Potential and optimisation of agriculture-based anaerobic digestion for environmental mitigation of agriculture-associated pollution

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Declaration of Authorship

I, Stephen Nolan, declare that this thesis entitled:

Potential and optimisation of agriculture-based anaerobic digestion for environmental mitigation of agriculture-associated pollution

and the work presented in it are my own and have been generated by me as a result of my own original research.

I confirm that:

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- With the exception of such quotations, this thesis is my own work
- I have acknowledged all main sources of help
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Signed: 

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Finally, to the One who set all things in motion, and holds all things together.
I will lift up my eyes to the hills.

Abstract

The current work has taken a holistic approach to understanding the potential for mitigation of pollution from agriculture using anaerobic digestion (AD), with a particular focus on reduction of pathogen load to the environment.

AD is a natural process whereby multi-species microbial communities operate synergistically to break down complex organic matter. This process produces biogas which can be used to generate electricity and/or heat, or upgraded to biomethane and injected into the gas grid or used as transport fuel. The residue from AD is called 'digestate' and can be used as an organic fertiliser/soil improver. Materials that fall under the scope of the EU Animal By-product (ABP) Regulations (EU Regulation 1069/2009 and EU Regulation 142/2011) are subject to rules aimed at protecting public and animal health. These Regulations require pasteurisation of AD raw materials or digestate at 70 °C for a minimum of 60 min with a maximum particle size of 12mm.

The EU legislation allows for derogation from the requirement for a pasteurisation treatment in AD plants transforming manure and non-ABP materials such as fats, oils and grease, "*provided the competent authority does not consider it to present a risk for the spread of any serious transmissible diseases.*" The Irish Department of Agriculture, Food and the Marine, the competent authority responsible for adherence to EU ABP legislation, established an alternative pasteurisation standard for digestate, known as the "National Transformation Parameter", 60 °C for 96 hours. The overall aim of this work was to determine the microbial sanitisation efficacy of the National Transformation Parameter when compared with the EU standard. Within this aim, the possibility for optimising the efficacy of AD as a tool for mitigation of the environmental impacts of agriculture was examined. Finally, a holistic analysis was undertaken of the potential for microbial, nutrient and metal transmission to watercourses, soil and grass, as well as gaseous emissions from landspreading of unprocessed slurry compared with slurry co-digested in AD.

Initial storage experiments demonstrated the efficacy of slurry co-digestion with fats, oils and grease as a means of reducing faecal indicator bacteria. Miniature-scale trials were validated as proxies for investigation of faecal indicator bacteria

(FIB) survival and biogas production where necessary for simultaneous examination of multiple variables. On that basis, 50 mL CSTR trials were established at different ratios of co-digestion feedstock, temperatures, retention times and loading rates. Response surface analysis was applied to model and optimise process parameters for different operational conditions. The model developed identified that with a combination of low organic loading and longer retention time, digestate sanitisation sufficient to satisfy EU standards is possible in AD at temperatures of 20 or 25°C, whilst also maintaining satisfactory methane production.

The Irish AD industry predominantly utilises mesophilic CSTR of slurry co-digested with food production waste. Hence, the aim of optimisation of sanitisation and biogas production under those conditions was addressed. By changing the feeding regime from daily to a three-day system, biogas yield per gram VS fed was increased by greater than 50% and coliform and *E.coli* numbers were reduced below the EU pasteurisation standard. An initial examination of the metagenomic datasets demonstrated the changing community dynamics, with increased abundance and diversity of key hydrolysers and methanogens, as well as some interesting shifts in bacteriophage concentrations.

Landspreading of unprocessed slurry presents risks of mobilisation during rainfall events thereby contributing to pathogen, nutrient and metal incidental losses. Field trials carried out as part of this work demonstrated the reduced microbial load from application of digestate from slurry co-digestion to grassland and consequent reduced runoff compared with unprocessed slurry. Pasteurisation at two conditions further reduced microbial contamination. These results have been used by project partners in a risk analysis to demonstrate reduced risk to human and animal health from landspreading of pasteurised and unpasteurised digestate, compared with slurry. Metal and nutrient analysis of soil, grass and runoff also demonstrated reduced pollution potential from digestate compared with slurry.

Finally, a comparative examination of greenhouse gas and ammonia emissions following landspreading found 72% and 50% lower methane and N_2O emissions respectively from plots treated with digestate compared with slurry. NH_3 emissions were not significantly different between treatments but were higher than untreated controls, while CO_2 emissions were not significantly different between treatments and controls.

Taken holistically, this work highlights the efficacy of AD with or without pasteurisation as a means of reducing agricultural pollution. Where the requirement for pasteurisation is a prohibiting factor for development of agriculture-based AD, this work demonstrates the potential for optimisation of sanitisation through adjustment of operational parameters. In that scenario, processing of slurry with food production waste is a multi-beneficial solution to reducing the environmental impacts of unmitigated landspreading of animal manure slurries.

Chapter 1

Introduction to Agriculture-based Anaerobic Digestion

Findings from this chapter and from a survey of Irish agriculture-based anaerobic digestion plants contributed to the review by Auer, A., Vande Burgt, N.H., Abram, F., Barry, G., Fenton, O., Markey, B.K., **Nolan, S**., Richards, K., Bolton, D., De Waal, T., Gordon, S. V, O'Flaherty, V., Whyte, P., Zintl, A., 2017. Agricultural anaerobic digestion power plants in Ireland and Germany: policy and practice, published in the Journal of the Science of Food and Agriculture. 97, 719–723. https://doi.org/10.1002/jsfa.8005

1.1 Agricultural Pollution

The European Green Deal aims to achieve carbon neutrality by 2050, through reduction of greenhouse gas (GHG) emissions, while also improving air and water quality and maintaining a sustainable level of agricultural production (COM, 2019). Air quality receives significant attention, particularly the contribution of ammonia to formation of particulate matter smaller then 2.5 microns (Behera and Sharma, 2010). Agriculture is responsible for 98% of ammonia and 10% of GHG emissions across the European Union (EU), whilst agricultural production in Ireland accounts for 34% of GHG emissions (EPA, 2019), 16.8% of which come from the 40 million tonnes of animal manure produced in Ireland each year (DAFM, 2019). This unprocessed manure is predominantly stored in a wet 'slurry' form over winter and landspread as an organic fertiliser (Pérez Domínguez et al., 2016), resulting in further fugitive gaseous carbon (C) and nitrogen (N) losses (Chantigny et al., 2009, 2001; Misselbrook et al., 2005), as well as potential contamination of water courses resulting from overland runoff.

Animal manures are also a potential reservoir for a range of bacterial, viral and parasitic pathogens and may present a significant risk of transmission of serious diseases to both humans and animals (Alam and Zurek, 2006; Bicudo and Goyal, 2003; Ferens and Hovde, 2011; Kearney et al., 1993). Diseased and clinically healthy animals, as well as those with latent infections can carry and excrete pathogens (Strauch, 1991), including *E. coli* O157, *Salmonella*, *Listeria*, *Campylobacter*, *Cryptosporidium, Ascaris*, *Mycobacterium avium* subspecies *paratuberculosis* and *Giardia* (Coklin et al., 2007; Grewal et al., 2006; Nicholson et al., 2005; Olson et al., 2004). Factors such as pathogen species and ability to survive storage, treatment and environmental exposure on grass until grazed contribute to the risk of infection associated with slurry spreading (Jones, 1980). Transmission may occur directly through ingestion of contaminated grassland post-application (Baloda et al., 2001; Braden and Tauxe, 2013), or indirectly through bioaerosols of infective material generated during landspreading, resulting in inhalation (Dungan, 2010; Millner, 2009). A further potential mechanism for transmission is through contamination of water sources by runoff, resulting from precipitation after land application (Douwes et al., 2003; Gerba and Smith, 2005; Venglovsky et al., 2009).

1.2 Anaerobic digestion of slurry as a mitigating measure

Anaerobic digestion (AD) has been identified at an EU-level as the most effective means of mitigating GHG emissions arising from agriculture, particularly manure management (EU RED II, 2018; Pérez Domínguez et al., 2016) as the GHGs that would otherwise be emitted from stored slurry are captured to produce renewable energy. On that basis, the EU's revised Renewable Energy Directive 2018/2001/EU (EU RED II, 2018) lists rules for calculating the GHG impact of biomass fuels, with AD of slurry resulting in a GHG emissions saving of 202% for biomethane used in transport and 246% for electricity generated from biogas, primarily through capture of methane (Giuntoli et al., 2017). Additional reported benefits of AD of slurry include odour and noxious gas control (Orzi et al., 2015, 2018), increased plant available N due to mineralization of complex organic N compounds (Möller and Müller, 2012), offset farm electricity and heating costs and diversification of farm income. AD of slurry has also been associated with significant reductions in pathogens (Kearney et al., 1993; Olsen and Larsen, 1987; Sahlström, 2003), reducing environmental load compared to landspreading of unprocessed slurry (Bicudo and Goyal, 2003).

1.3 The AD process

AD is a four stage sequential process that breaks down organic matter through the synergistic interactions of distinct microbial trophic groups in the absence of oxygen (Coates et al., 1996). This natural process can be harnessed as an organic waste management tool resulting in the production of biogas for electricity, heat and/or transport.. The four phases of AD; hydrolysis, acidogenesis, acetogenesis and methanogenesis (Batstone et al., 2002), occur naturally where high concentrations of wet organic matter accumulate in the absence of dissolved oxygen, such as in bogs, swamps, anaerobic interiors of landfill sites and the intestines of animals (Sawatdeenarunat et al., 2015), and have historically been harnessed as a simple means of producing renewable gas for heating and cooking.

The first stage, hydrolysis, involves the breakdown of complex carbohydrates, proteins and fats into sugars, amino acids and fatty acids (Gavala *et al.*, 1996). Hydrolytic enzymes, such as lipases, proteases, amylases and cellulases secreted by microbes, act on the insoluble, complex, polymeric matter to convert it into less

complex, soluble molecules. Hydrolysis of biopolymers into amino acids, sugars and fatty acids has regularly been found to be the rate-limiting step, especially when a feedstock with high levels of polymers is used (Eastman and Ferguson, 1981). The sugars and amino acids resulting from the hydrolytic stage are then degraded further in the acidogenesic step to form intermediates including acetate, propionate, lactate, formate, butyrate, H_2 and CO_2 (Gavala et al., 2003).

Unlike the prior stages, the latter two phases of AD are performed by obligate anaerobes, therefore the absence of oxygen is necessary for their operation. Propionate utilising acetogens, such as *Syntrophobacter wolinii* and butyrate decomposers such as *Sytrophomonos wolfei*, act on propionate and butyrate produced in the previous step to convert them to acetate, H_2 and CO_2, while fatty acid oxidising acetogens also convert the long-chain fatty acids from the hydrolysis stage to acetate, H_2 and CO_2 (Siegrist et al., 1993).

Finally, biogas with typical methane content of 50%-80% is produced from the activity of methanogenic archaea on the products of acetogenesis. Hydrogenotrophs such as *Methanobacterium*, *Methanobrevibacter*, *Methanomicrobium*, *Methanosarcina and Methanospirillum* have been identified in anaerobic digesters and chiefly reduce CO_2 to methane, with H_2 as the primary electron donor (Liu and Whitman, 2008). These hydrogenotrophs can also typically use formate. Acetate has been found to be utilised by only two genera; *Methanosaeta* and *Methanosarcina*. A third methanogenic pathway using methylated compounds is carried out by archaea from the Methanosarcinaceae family, but is relatively rare in AD systems (Liu and Whitman, 2008).

This final stage of AD is the most sensitive step, as the archaea are highly susceptible to changes in optimal conditions. Over-production of fatty acids in the preceding stages can lead to a decrease in pH, resulting in a deviation from the optimal range of pH 6.6 – 7.6 (Maspolim et al., 2015). This reduction in pH invariably leads to a number of problems, most significantly inactivation of methanogens and consequently, system failure (Appels et al., 2008). Instability, caused by the presence of inhibitors, feed overload, feed under-loading or inadequate temperature control, has often been a problem in AD systems due to their complex nature. This instability may be noted by a reduction in methane production, a drop in pH or a rise in volatile fatty acid (VFA) levels, while the level of hydrogen can also be used as an early indicator of instability (Lyberatos, 1998).

At the end of this process there is a portion of the original organic matter which remains due to being more recalcitrant and slower to digest, this is typically removed in order to allow for input of fresh, readily degradable matter in order to improve process rates. This recalcitrant matter, known as digestate, has been described as 'inocuous, stabilised and hygienised' (McKeown et al., 2012) and has significant onwards value as an organic fertiliser as it is typically rich in plant-available organic nitrogen (Ward et al., 2008).

The organic material utilised in AD is termed 'feedstock', with animal manure in slurry format forming the base feedstock in agriculture-based AD. A number of factors contribute to efficiency in the AD process, or lack thereof. Optimal process operation will vary depending on the particular mode of AD employed and the particular application, because the exact microbial communities vary depending on the feedstock in question (Hulshoff Pol et al., 1982; Brummeler et al., 1985; MacLeod, 1990; Bitton, 1994). The optimisation of certain factors, such as digester start-up conditions, degree of acclimatisation to the feedstock, pH, temperature and concentration of inhibiting compounds, is important if high quality biogas and product effluent are to be obtained (Lettinga et al., 1980; Hulshoff Pol et al., 1983; Wu et al., 1987). Sufficient substrate for the system, or 'organic loading rate', typically expressed as volatile solids (VS) or organic dry matter (oDM) per day is also important to ensure stable operation, and unless uncoupled in a two-phase system, determines the hydraulic retention time. Finally, the consistency and type of solids in the feedstock play an important role in maintaining balance and optimal performance.

1.4 Agriculture-based AD in Europe

Centralised agriculture-based anaerobic digestion of slurry has been employed in Denmark, whereby slurry from a number of farms is brought to a central location for digestion, whereas in Germany, Sweden and Austria, smaller farm-scale AD plants have been more typical (Holm-Nielsen et al., 2009). The deployment of these agriculture-based AD plants is however predicated on maintenance of a stable system and production of sufficient quantities of biogas for economic viability, neither of which are likely in systems mono-digesting slurry with typically low biogas yields of 15-30 m^3 per tonne (Weiland, 2010), as the carbon:nitrogen ratio is typically quite

low making the system vulnerable to failure (Nielsen and Angelidaki, 2008). Hence, in order to increase carbon these AD plants use agricultural residues and/or energy crops such as maize, thereby balancing the carbon:nitrogen ratio in the system and producing greater quantities of biogas (Rodriguez-Verde et al., 2014).

A proliferation of AD plants in Germany between the years 2000 and 2016 was primarily driven by governmental support, Erneuerbare Energien Gesetz (EEG; Renewable Energy Legislation) in the form of feed-in tariffs which guaranteed priority grid connection and a consistent income (Auer et al., 2017). These initiatives were supplemented with 'smart grid' technologies allowing convenient bi-directional power flow to small and medium-sized operators. In 2004, the EEG was modified to provide a bonus for using energy crops as feedstock and for developing combined heat and power (CHP). An unintended consequence ensued, namely conversion of more than 20% of food-producing land to growing energy crops, primarily maize, for co-digestion with slurry. Land rental prices rose due to competition, with negative effects on traditional agricultural practices, while mono-cropping vast swathes of land had negative impacts on biodiversity (Auer et al., 2017). As a result, German legislation was modified to encourage smaller scale farm-based AD plants primarily processing slurry with agricultural residues. The negative impact of the German approach has however, hampered efforts to implement support for agriculture-based AD in other EU Member States, particularly Ireland.

1.5 Agriculture-based AD in Ireland

Several factors have contributed to minimal uptake of AD in Ireland, including the lack of meaningful government support, long planning delays, difficulty in securing grid connection and an inappropriate climate for growing energy crops such as maize. Ireland is however known for growing one crop, grass, and there has been significant academic interest in the potential of grass silage for biogas production, with researchers pointing to the "hidden hectares" of underperforming grassland which, if optimised, could produce 30 % more grass (McEniry et al., 2013).

In this case the problems encountered in Germany with competition for food-production need not necessarily occur, as the extra grass could support an AD industry producing up to 28 % of fossil gas demand (SEAI, 2017), diversifying the rural economy and contributing to European Green Deal goals. In the absence of

clear government support however, grass silage for co-digestion is entirely dependent on market forces and hence considered too risky for the significant investment and operating costs required. Indeed, one such plant has been built by a semi-state research body but furloughed until such time as there are clear government supports available. Instead, in an effort to attain economic viability the fledgling Irish AD industry has turned to organic waste, primarily from food processing, greasetraps, paunch grass and unsold food waste as the co-digestion products to compliment slurry (Auer et al., 2017). These feedstocks attract gate fees ranging from €10-100 per tonne, and would otherwise be landspread without treatment, incinerated or exported. They do however present a potential problem not relevant to maize or grass silage as co-digestion material, namely the presence of animal by-products (ABP).

1.6 Animal by-products in AD

Animal by-products are materials of animal origin not intended for human consumption. The introduction of animal by-products to agriculture-based AD brings it under the scope of the EU Animal By-product (ABP) Regulations (EU Regulation 1069/2009 and EU Regulation 142/2011. The legislation classifies ABP into three categories according to risk to human and animal health, with Category 1 material presenting the highest risk and Category 3 being the lowest (EC, 2009). Common Category 3 material for AD plants include unsold food, catering waste or food processing waste arising from manufacturing or packaging problems, while typical Category 2 material includes digestive tract from slaughterhouses as well as manure (> 5,000 tonnes) from more than one farm.

As referred to earlier, unprocessed manure carries a significant human and animal health risk with regard to serious transmissible diseases when landspread, a risk typically reduced by AD processing. The addition of ABP to the feedstock mix however, introduces additional potential sources of pathogenic material and increases the risk of transfer between farms. AD digestate may also represent an additional risk compared with manure or slurry as the raw materials used in AD bioreactors are typically derived from multiple sources and spread on multiple farms.
These EU rules are therefore aimed at protecting public and animal health by requiring pasteurisation of AD raw materials or digestate at 70°C for a minimum of 60 minutes with a particle size of 12mm or less. The EU legislation allows for

derogation from the requirement for a pasteurisation treatment in AD plants transforming manure and non-ABP materials such as fats, oils and grease, "*provided the competent authority does not consider it to present a risk for the spread of any serious transmissible diseases.*" The legislation also allows for the deployment of alternative National Standards, provided that equivalent efficacy of those standards can be demonstrated by the competent authority.

The Irish Department of Food, Agriculture and the Marine, the competent authority responsible for adherence to EU ABP legislation, established an alternative pasteurisation standard for digestate at 60°C for 96 hours, known as the "National Transformation Parameter". The document detailing these parameters, and governing operation of anaerobic digestion plants in Ireland under EU or National legislation, is *CN11: Approval and operation of biogas plants transforming animal by-products and derived products in Ireland*. It details every conceivable aspect of the control of ABP to prevent transmission or cross-contamination of 'dirty' and 'clean' material post-pasteurisation, including storage, cleaning, vermin control and microbiological testing requirements. Faecal indicator bacteria (FIB) are used as proxies for pathogens to assess efficacy of the pasteurisation process. The preferred FIB is *E. coli*, with requirements that five samples are taken and tested to quantify *E. coli* as regularly as deemed necessary by the competent authority (typically once per week). Four of the five samples are allowed to have up to 1,000 colony forming units (cfu) per gram, while a fifth is allowed up to 5,000 cfu/gram. If these conditions are not met, the reason for failure must be identified, corrective action taken, and the batch must be repasteurised.

The overarching aim of this work was thus to determine the microbial sanitisation efficacy of the National Transformation Parameter when compared with the EU standard and the relative risk associated with subsequent landspreading of material subjected to either or none of the standards. The extent of that risk in general, or relative to the risk of spreading raw slurry, is not clearly understood. The underlying motivation for this project is to better understand that risk, with a view to informing future policy and practice for the AD industry in Ireland.

1.7 Thesis overview

In order to achieve these goals, a number of research outcomes needed to be achieved, thus creating the distinct work packages of this thesis, referred to as i) Miniature scale bioreactors ii) Modelling iii) Potential for optimisation: feeding regime iv) Comparative landspreading.

i) Miniature scale bioreactors

Although previous studies of farm-based AD have reported reductions in pathogen numbers (Dennehy et al., 2018; Jiang et al., 2018; Kearney et al., 1993; Sahlström, 2003), surveys of farm-based AD plants carried out as part of this project have detected the presence of pathogens in digestate, including *Cryptosporidium parvum, Mycobacterium avium* subsp. *paratuberculosis* and astroviruses. Hence the starting point of research in this area should be to determine the extent of survival of a broad range of the various potential human pathogens, or non-human pathogenic proxies thereof, which presents several difficulties.

The first of these is the risk of transmission of serious potential pathogens from laboratory or worse, full-scale AD to the environment. Physical containment of the pathogens can reduce this risk, however there exist significant difficulties using current containment strategies for examination of time-series survival. Porous containment vessels (tea-strainers) could be used but may release small viruses, whilst sentinel chambers capable of retaining viruses would inevitably prevent realistic interaction of the pathogen with the AD liquor. A third difficulty with examination of specific pathogens is cultivating sufficient quantities of pathogenic material with which to spike AD bioreactors. Miniature-scale bioreactors could potentially solve all of these difficulties, but have not been reported at length in the literature. Therefore it is first necessary to determine the adequacy of miniature-scale bioreactors as proxies for pathogen survival in larger volume AD plants.

ii) Modelling

Bioreactor performance in terms of biogas yield and pathogen sanitisation potential may be significantly impacted, positively or negatively, by a variety of factors. These include: pH, ammonia production, microbial competition, initial pathogen load, addition of co-digestion substrates such as food production waste with varying

pathogen risks and varying chemical compositions and operating conditions (Orzi *et al*., 2015; Smith *et al*., 2005). Some of the most important operating conditions in farm-based AD include temperature, organic loading rate, retention time and the ratio of slurry to organic waste. Typical AD temperatures in farm-based AD range from 35°C (mesophilic) to 55°C (thermophilic), whilst low- or ambient-temperature AD has garnered some attention (Alvarez et al., 2006; Kashyap et al., 2003; McKeown et al., 2012; Resende et al., 2014; Safley and Westerman, 1994).

Research examining the sanitisation potential of AD has tended to consider a single operational factor, and within the range of possibilities for that factor typically focuses on a narrow set of operational conditions. For example, temperature is commonly the single operational factor being considered, whereby a comparative performance analysis (biogas yield and/or pathogen removal) may be undertaken between mesophilic and thermophilic AD, with little consideration for the intermediate temperatures (Beneragama et al., 2013; Sahlström, 2003). This difficulty in biological studies arises because of the need for replication in triplicate at minimum in order to carry out reliable statistical analysis. Therefore, assessment of the impact of changes to multiple variables in a single trial would require multiple bioreactors, with each additional factor adding layers of complexity, as well as financial, human resource and space restrictions. For full-scale AD plants there is significant risk inherent in making changes to operational parameters, given the sensitivity of the microbial community to change and the financial implications of perturbation or failure of the system.

These constraints could be overcome through utilisation of design of experiment methods such as response surface methodology to reduce the number of experimental units required (Feng et al., 2017; Wang et al., 2013), combined with mathematical modeling, whereby results from a limited set of statistically significant data may be extrapolated out to better understand the potential interactions between multiple variables, and their impact on performance (Kainthola et al., 2019a). Modeling and optimisation studies for agriculture-based co-digestion have focused primarily on biogas production without addressing pathogen removal efficacy (Álvarez et al., 2010; Dennehy et al., 2016a; Lu et al., 2019). Indeed the most widely used model in the AD industry, ADM1 (Batstone et al., 2002), does not incorporate pathogen or faecal indicator bacteria (FIB) removal, and requires measurement of an

extensive list of physico-chemical parameters, putting it beyond the reach of most farm-based AD plants.

Hence, in order to assess the pathogen sanitation and biogas production implications of variation of a number of operational parameters, and the synergistic impact of their interactions, it is necessary to develop a decision support model accessible to biogas plant operators and or regulators.

iii) Potential for optimisation: feeding regime

A pasteurisation step of one hour at 70°C is required under EU regulations if ABP are utilised in an AD plant, or 2x48 hours at 60°C under the Irish National Transformation Parameter. These pasteurisation steps have been calculated to consume 57% and 4544% of AD energy output respectively if pre-digestion pasteurisation is carried out, or 30% and 1893% respectively for post-AD pasteurisation at EU and Irish standards respectively, assuming digestate is at 40°C entering the pasteuriser (Coultry et al., 2013).

The requirement for pasteurisation may therefore be prohibitive in terms of capital cost and energy consumption. Hence, optimisation of AD for sanitisation as well as improved biogas output is desirable, given that validation of an alternative standard is allowed if sanitisation standards are achieved. Furthermore, AD has demonstrated potential for demand-driven energy supply through flexible feeding of the system (Mauky et al., 2017), whereby the supremacy of the conventional hourly or daily drip-feed approach to AD, and possibility for alternative approaches to optimise utilisation of feedstocks, is beginning to be questioned. As the potential for optimised feeding regimes has focused primarily on biogas production it is necessary in the scope of the present work to also examine the potential for improved digestate sanitisation.

iv) Comparative landspreading

AD of slurry reduces agriculture associated greenhouse gas emissions by capturing biogas, producing a nutrient rich digestate by-product. Agriculture-based digestate is typically landspread as an organic fertiliser/soil improver, returning nutrients and remaining carbon to the soil and offsetting chemical fertiliser use. However, North Atlantic European grassland systems are known for low nutrient use efficiency and high rainfall and hence organic amendments can be mobilised during heavy rainfall

events thereby contributing to pathogen, nutrient and metal incidental losses. Co-digesting slurry with organic waste mitigates agriculture-associated environmental impacts but may alter microbial, nutrient and metal profiles and their transmission to watercourses, and/or soil persistence, grass yield and uptake, as well as gaseous emission profiles. The impact of EU and alternative pasteurisation regimes on transmission potential of these various pollutants is not clearly understood, particularly in pasture-based agricultural systems. Hence, holistic studies incorporating nutrient and metal runoff, soil persistence and uptake in grass, as well as grass yield, GHG and ammonia emissions from slurry compared with digestate are necessary to fully understand the mitigation potential of AD.

In an effort to further knowledge in the field of anaerobic co-digestion of slurry with organic waste, we aimed to tackle the following knowledge gaps as detailed above: i) the adequacy of miniature-scale bioreactors as proxies for pathogen survival in larger volume AD plants; ii) a decision support model to predict the pathogen sanitation and biogas production implications of variation of a number of operational parameters; iii) the possibility of process reconfiguration for improved sanitisation as well as biogas output; iv) the impact of EU and alternative pasteurisation regimes on transmission potential of various pollutants, particularly in pasture-based agricultural systems.

The knowledge gaps investigated in this work were chosen as they are relevant to the potential and optimisation of agriculture-based AD for environmental mitigation of agriculture-associated pollution.

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Chapter 2

Toward assessing farm-based anaerobic digestate public health risks: Comparative investigation with slurry, effect of pasteurisation treatments, and use of miniature bioreactors as proxies for pathogen spiking trials

Having reviewed the agricultural anaerobic digestion (AD) literature and the current legislation and practice regarding pathogen survival and transmission to the environment, it was necessary to establish comparative survival data, with or without AD and pasteurisation. Furthermore, project partners highlighted the complexity and risk of carrying out spiking trials in full-scale or laboratory-scale bioreactors, under multiple conditional combinations. Hence, the potential for using miniature bioreactors, and the accuracy thereof, was examined.

I wrote this paper with the help of my supervisors and collaborators, having carried out the laboratory work and data analysis. The Bayesian modeling was carried out with Nicholas Waters under the guidance of his supervisor, Leighton Pritchard. The paper presented here is the manuscript as published in *Frontiers in Sustainable Food Systems* using their typesetting template, in accordance with NUIG's requirements. It was published in July 2018 (Nolan et al. 2018).

2.1 Pasteurisation and miniature bioreactor Manuscript

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Toward Assessing Farm-Based Anaerobic Digestate Public Health Risks: Comparative Investigation With Slurry, Effect of Pasteurization Treatments, and Use of Miniature Bioreactors as Proxies for Pathogen Spiking Trials

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Manure and slurry may contain a range of bacterial, viral, and parasitic pathogens and land application of these organic fertilizers typically occurs without prior treatment. *In-situ* treatment through farm-based anaerobic digestion (AD) of such organic fertilizers co-digested with food-production wastes is multi-beneficial due to energy recovery, increased farm incomes and noxious gas reduction. Before risk assessment can be carried out at field scale an investigation of the fate of relevant target pathogens during the actual AD process must be undertaken, requiring the development of practical test systems for evaluation of pathogen survival. The present study examines miniature (50 mL) and laboratory (10 L) scale AD systems. Treatments included slurry co-digested with fats, oils, and grease (FOG) under typical operating and pasteurization conditions used in farm-based AD, in batch-fed miniature and laboratory mesophilic (37°C) continuously stirred tank reactors. Biogas production, pH, chemical oxygen demand, volatile solids, and ammonia concentration were measured throughout the trial, as were fecal indicator bacteria (FIB) i.e., total coliforms, *Escherichia coli,* and *Enterococcus* species. The miniature and laboratory bioreactors performed similarly in terms of physicochemical parameters and FIB die-off. In the absence of pasteurization, after 28 days, enterococci numbers were below the $<1{,}000$ cfu g^{-1} threshold required for land application, while *E. coli* was no longer detectable in the digestate. For comparison, FIB survival in slurry was examined and after 60 days of storage, none of the FIB tested was $<1{,}000$ cfu g^{-1}, suggesting that slurry would not be considered safe for land application if FIB thresholds required for AD digestate were to be applied. Taken together

we demonstrate that (i) miniature-scale bioreactors are valid proxies of farm-based AD to carry out targeted pathogen survival studies and (ii) *in situ* AD treatment of slurry prior to land application reduces the level of FIB, independently of pasteurization, which in turn might be indicative of a decreased potential pathogen load to the environment and associated public health risks.

Keywords: anaerobic digestion, fats, oils and grease, fecal indicator bacteria survival, miniature bioreactors, slurry

INTRODUCTION

Approximately 1.4 billion tons of manure are produced in Europe each year, 80% of which is in the form of slurry, predominantly from cattle (Crowe et al., 2000; Foged et al., 2011). Manure and slurry represents valuable organic fertilizers, but typically contain a broad range of bacterial, viral, and parasitic pathogens (Bicudo and Goyal, 2003; Alam and Zurek, 2006; Ferens and Hovde, 2011). Human and animal pathogens commonly isolated from manure include *E. coli* O157, *Salmonella*, *Listeria*, *Campylobacter*, *Cryptosporidium, Ascaris*, *Mycobacterium avium* subspecies *paratuberculosis,* and *Giardia* (Nicholson et al., 2004; Olson et al., 2004; Grewal et al., 2006). These pathogens can be transferred to the environment as bioaerosols during landspreading (Millner, 2009; Dungan, 2010), ingested directly from grass or vegetables (Baloda et al., 2001; Braden and Tauxe, 2013), or may be washed off into connected water bodies, posing a significant threat to human and animal health (Douwes et al., 2003; Gerba and Smith, 2005; Venglovsky et al., 2009). Furthermore, manure is a potent source of noxious and greenhouse gases (GHG), which are released to the atmosphere during storage in slatted tanks and subsequent landspreading (Chadwick et al., 2011). A number of methods for limiting the impact of manure storage and landspreading, both in terms of GHG capture or mitigation and pathogen reduction have been examined, including aeration, and acidification during storage, animal diet manipulation, or alternative landspreading techniques (Nicholson et al., 2004; Franz et al., 2005; Webb et al., 2010). Typically these proposed solutions, however, consider either pathogens or GHG in isolation. Composting, for example, is suggested as an effective solution to reduce pathogens in manure (Ros et al., 2006; Vinnerås, 2007; Mc Carthy et al., 2011; Millner et al., 2014), with scant reference to gaseous N or CH_4 loss to the environment (Rao et al., 2007). Conversely, methods for reduction of ammonia or other GHG losses from manure, such as acidification, rarely consider the fate of pathogens during such treatments (Kai et al., 2008; Petersen et al., 2012). In fact, in this context, the recommended direct incorporation of slurry into soil might lead to increased pathogen survival, as it inevitably reduces UV exposure (Avery et al., 2004; Hutchison et al., 2004).

Rather than tackling pathogen survival or GHG emissions from manure in isolation, a technological solution that addresses both would clearly be preferable. To that end, biogas production as a treatment for manure holds great promise (Monteny et al., 2006). In addition to the obvious benefits of energy recovery, noxious gas and GHG mitigation, farm-based AD could potentially reduce pathogen loads in the environment and the associated public health risks (Olsen and Larsen, 1987; Kearney et al., 1993; Sahlström, 2003; Jiang et al., 2018). Pathogen survival may be significantly impacted, positively or negatively, by a variety of factors. These include: pH, ammonia production, microbial competition, initial pathogen load, operating conditions of farm-based AD plants and addition of co-digestion substrates such as food production waste with varying pathogen risks (Smith et al., 2005; Orzi et al., 2015). Indeed, the AD of slurry alone is hindered by an imbalanced C:N ratio resulting in low potential methane yields of 25–30 m^3 ton^{-1} (Weiland, 2010). To overcome this limitation, co-digestion of slurry with locally sourced organic waste is typically implemented. This in turn helps to balance the C:N ratio and thus improves the relatively low methane yield of slurry alone, whilst taking advantage of its inherent buffering capacity, microbial populations, nutrients, and moisture content (Hamelin et al., 2014; Moset et al., 2017; Neshat et al., 2017).

Congealed fats, oils, and grease (FOG) are a significant problematic food production waste internationally, causing environmental and human health issues when allowed to form "fatbergs" in municipal sewage systems (Wallace et al., 2017). Grease-traps required for licensing in the food-processing industry as well as those in restaurants mitigate the problem, but create large quantities of organic waste, which requires further treatment. The typical biogas yield of FOG (4–8 m^3 kg VS $^{-1}$) dwarfs that of slurry alone (0.148 m^3 kg VS $^{-1}$), making co-digestion of FOG with slurry in farm-based AD plants a sustainable treatment option, cheaply increasing methane output (Møller et al., 2004; Weiland, 2010; Long et al., 2012). In Ireland, successful implementation of grease-trap legislation provides a steady supply of organic waste in the form of FOG, which is used as a feedstock in the majority of Irish farm-based AD plants. The co-digestion of slurry with organic waste, however, typically requires some pasteurization treatment to be carried out as stipulated by the legislation. In that context, two pasteurization processes are available in Ireland, set out by (i) the European Union Commission (Directive No. 142/2011) as 60 continuous minutes at 70°C and (ii) the Irish Department of Agriculture, Food and the Marine as a total of 96 h at 60°C (DAFM, 2014). Pasteurization can be applied either pre- or post-AD processing with the corresponding digestate quality being assessed using fecal indicator bacteria, typically *E. coli* and/or enterococci. According to Regulation (EC) No.1069/2009 and Regulation (EU) No. 142/2011, for AD digestate to be deemed safe for landspreading FIB levels must <1,000 cfu g^{-1}. As highlighted by Dennehy et al. (2018), further investigations into the effect of AD processing on pathogen loads must be carried out to

determine the need for pasteurization. In addition, in order to meaningfully and accurately carry out risk assessment of digestate landspreading, the determination of the fate of relevant target pathogens during AD processing is necessary. Although previous studies of farm-based AD have reported reductions in target pathogen numbers (Olsen and Larsen, 1987; Kearney et al., 1993; Sahlström, 2003; Dennehy et al., 2018; Jiang et al., 2018), investigations into pathogen survival are typically hampered by difficulties in cultivating sufficient pathogen quantities and the public health concerns associated with spiking large volume bioreactors. Some solutions have been deployed in an effort to overcome this, including the containment of pathogens using sentinel chambers or filters held in steel baskets and submerged into digesters (Gray and Hake, 2004; Wagner et al., 2008). While this may successfully contain the pathogens and thus reduce the associated public health risks, such an experimental set-up greatly limits the interactions of the target pathogens with the surrounding matrix. There is, therefore a crucial need to develop an alternative solution closely mimicking real-life scenarios whereby interactions between pathogens and the AD liquor are not hindered.

Thus the aims of this study were to: (i) propose and validate the use of miniature-scale (50 mL) bioreactors as proxies for 10 L bioreactors; (ii) determine FIB survival under typical operating and pasteurization conditions used in farm-based AD systems; and (iii) assess the suitability of AD as a means of reducing the environmental impact of slurry management.

MATERIALS AND METHODS

Feedstock Selection, Collection, and Storage

In order to determine feedstock composition and operating conditions, a characterization of current Irish AD facilities was carried out. All Irish farm-based AD plants currently operate at mesophilic temperatures and process slurry co-digested with food production waste, including FOG (Auer et al., 2016). By visiting these AD facilities and utilizing knowledge gained in Auer et al., the following operation conditions and feedstock composition were determined.

First, a cattle slurry:FOG ratio of 2:1 was used, with a view to replicating full-scale farm-based AD. The FOG was sourced from the Bioenergy and Organic Fertilizer Services (BEOFS) AD plant in Camphill, County Kilkenny, Ireland, collected in a 25 L drum, stored at 4°C, and mixed thoroughly before use. Cattle slurry for feeding the bioreactors was collected from a dairy farm in County Galway, Ireland in October, 2016. The slatted housing storage tanks were agitated to homogenize the slurry before collection of the sample using a bucket attached to a pole, in accordance with Brennan et al. (2011) and Peyton et al. (2016). Slurry was stored in a 25 L sealed container at 4°C for 2 days prior to use as feedstock, at which time it was mixed thoroughly. In order to establish levels of farm to farm variation, dairy cattle slurry was collected from two additional farms in County Galway during October 2016. For comparison between digestate and stored slurry, triplicate slurry samples for each farm were stored in a shed at ambient Irish environmental temperatures during October–December, to mimic on-farm storage.

Inoculum Development

Digestate from the BEOFS full-scale mesophilic continuously-stirred tank reactors (CSTR) co-digesting FOG with slurry was used as the starting inoculum, as it was adapted to the chosen substrate. This inoculum was found, through biomethane potential assays (BMP, data not shown), to be sub-optimal for biogas production. Therefore, augmentation with a mixture of slurry and methanogenic anaerobic granular sludge was deemed necessary to bolster both hydrolysis and methanogenesis. A series of specific methanogenic assays (SMA) were carried out using non-gaseous (acetate, ethanol, propionate, butyrate) and gaseous substrates (H_2/CO_2) as described by Coates et al. (1996). Based on the SMA results, a 2:1:1 ratio of granular sludge:BEOFS:slurry was selected as the optimum inoculum mixture (**Figure S1**).

Miniature- and Laboratory-Scale Bioreactors Operation

Three 10-L CSTRs (R1–R3) were operated at 37°C in batch with a 28-day solid retention time. Prior to operation, the inoculum and starting liquor were adjusted to pH 7 by adding $NaHCO_3$. The organic loading rate for each bioreactor was 30 g VS L^{-1} in a 2:1 inoculum to feedstock ratio with a 7 L working volume. Submerged, motor-propelled axial stirrers with large scale paddles were centrally installed in the bioreactor ceilings, with an externally positioned motor, as is typical of agricultural biogas plants (Weiland, 2010). Miniature batch tests (33 mL in 50 mL glass bottles) using identical inoculum and feedstock ratios to the 10 L bioreactors were run simultaneously at 37°C under shaking conditions in a New Brunswick Scientific Innova°44 incubator and destructively sampled in triplicate at regular intervals (days 0, 7, 14, 21, 28), for comparison. Their contents, as well as samples collected from the 10 L bioreactors were analyzed as described below.

Analytical Methods

Biogas volume from the 10 L and 50 mL bioreactors was determined using the water displacement method and 10 mL syringes attached with a stopcock, respectively. Methane content of the biogas was analyzed using a Varian gas chromatograph equipped with a flame ionization detector. The carrier gas was nitrogen and the flow rate was 25 mL min^{-1}. Analysis of TS and VS was performed gravimetrically according to standard methods (APHA., 2005). Soluble chemical oxygen demand (sCOD) was determined by analyzing the supernatant of centrifuged samples. Total chemical oxygen demand (tCOD) and sCOD analyses were performed according to the Standing Committee of Analysts. (1985). NH_3 concentrations (mg L^{-1}) were determined using the HACH AmVer High-Range Ammonia test, available from HACH.

Pasteurization

In addition to the unpasteurized 50 mL bioreactors used for comparison with the 10 L CSTRs, four pasteurization conditions were examined at the miniature scale to determine the impact on

bioreactor performance and FIB survival. At each time point, two pre-AD pasteurization conditions (P1: 60°C for 96 h; P2: 70°C for 1 h) were used on the food production waste, and two post-AD pasteurization conditions (P3: 60°C for 96 h; P4: 70°C for 1 h) were applied to the digestate. These assays were carried out in triplicate for each time point, totaling 75 miniature-scale assays. Water baths set to the appropriate temperatures were used for pasteurization, and temperature probes were employed to ensure the designated temperature was achieved.

Fecal Indicator Bacteria Monitoring

In line with the EU Regulation, total coliforms, *E. coli,* and enterococci numbers were monitored throughout the trial. Most probable numbers (MPN) of total coliforms and *Escherichia coli* were quantified using IDEXX Colisure with Quanti-Tray/2000 incubated at 35°C for 24 h. MPN of enterococci were determined using IDEXX Enterolert kit with Quanti-Tray/2000 incubated at 41°C for 24 h. Slurry and digestate samples were diluted as necessary to fall within the detection range (1 - 2419.6 cfu 100 mL^{-1}) in sterilized phosphate buffered saline (Colisure) and sterilized distilled water (Enterolert).

Assessing Treatment Effects on FIB Die-Off With Bayesian Hierarchical Modeling

Bayesian hierarchical modeling was used to compare the effects of vessel volume and pasteurization conditions on FIB die-off. Weak Cauchy-distributed priors were used for the pooled parameter estimates of the regression, to allow for outliers (Gelman and Hill, 2006). Stan version 2.17.0 (Carpenter et al., 2017) was used to generate samples from the model using the Rstan interface (Stan Development Team, 2017). The data, model, analysis scripts, and interpretation of the results can be found at https://github.com/nickp60/SI_Nolan_etal_2018. A difference in parameter estimates was considered significant if the 95% confidence intervals were exclusive.

RESULTS

Slurry Characterization

The slurry collected from the three farms was tested prior to AD, for initial FIB levels as well as total solids and volatile solids (**Table 1**). TS and VS were consistent across the samples tested, whilst coliforms and *E. coli* numbers were highest in samples from Farm C. In all cases, enterococci numbers were lower than coliforms and *E. coli*.

Miniature- (50 ml) and Laboratory-Scale (10 L) Bioreactor Performance Is Similar

The recorded performance data in the comparative trial displayed similar trends for miniature- and laboratory-scale bioreactors. The pH for both bioreactor scales remained between 7.6 and 8.1 throughout the experiment (**Figure S2**). Volatile solids (VS) degradation was comparable for the 50 mL and 10 L bioreactors with 64 and 61% VS removal, respectively within the first 7 days (**Figure 1A**). Similar trends in ammonia concentration (**Figure 1B**) were also observed across the two scales, with an increase over the first 2 weeks of the trial from 937 to 1,233 mg L^{-1} in the 10 L bioreactors and from 865 to 1,038 mg L^{-1} in the 50 mL bioreactors. This increase likely results from the breakdown of organic compounds. As ammonia concentration has been identified as an important factor in pathogen reduction (Watcharasukarn et al., 2009, the similarity between the scaled bioreactors is of particular relevance.

Soluble and total chemical oxygen demand (sCOD and tCOD) concentrations were also consistent across the two bioreactor scales (**Figures 1C,D**). Soluble COD and tCOD removal primarily occurred within the first 7 days, reaching a maximum of 87–88% by Day 28 for both bioreactor scales (**Figures 1C,D**). The majority of methane production occurred within 14 days, reaching 77.5 and 82% of the total recorded in the 10 L and 50 mL bioreactors within that time frame (**Figure S3A**). Although similar methane production trends were observed at both bioreactor scales, the larger scale bioreactors approached the theoretical yield proposed by Batstone et al. (2002) of 350 mL CH_4 g^{-1} of COD at Day 21 compared to Day 28 for the 50 mL bioreactors (**Figure 1E**). This could partly be attributed to the more thorough mixing occurring in the larger bioreactors.

Bayesian Hierarchical Modeling

A Bayesian hierarchical model was developed to compare the effects of vessel volume and pasteurization conditions on FIB survival. In short, both the initial effect (from day 0 to day 7) and the latter effect (from day 7 to day 28) of the conditions were considered in relation to the underlying behavior of the data. This piece-wise approach was able to accurately model both the initial perturbation (the addition of feedstock to the inoculum) and the recovery of the system (https://github.com/nickp60/SI_Nolan_etal_2018).

Fecal Indicator Bacteria Survival Is Comparable in 50 ml and 10 L Bioreactors

Fecal indicator bacteria levels should be reduced to <1,000 cfu g^{-1} for the safe landspreading of digestate (Regulation (EC) No.1069/2009 and Regulation (EU) No. 142/2011). Total coliforms survival showed similar trends in both 50 mL and 10 L bioreactors, with a 3.7 and 4.3 log_{10} reduction after 7 days (**Figure 2A**). A similar trend in *E coli* die-off was also observed in both bioreactor scales (**Figure 2B**). The initial 3.5–4.3 log_{10} reductions of both coliforms and *E. coli* occurring within 7 days (**Figures 2A,B**), followed by relatively stable survival until 21 days suggests the presence of resilient cells with increased ability to survive under mesophilic AD conditions. Although enterococci numbers were slightly above 1,000 cfu g^{-1} after 21 days, greater than 3.0 log_{10} reduction was observed after 28 days in both bioreactor scales (**Figure 2C**). The parameter estimates obtained from piece-wise modeling of the FIB die-off data showed well-overlapping confidence intervals, indicating no significant difference between the two bioreactor volumes.

Pre-pasteurization Impacts Scod Removal and Methane Yield

Two pre-AD (P1: 60°C for 96 h and P2: 70°C for 60 min) and two post-AD pasteurization regimes (P3: 60°C for 96 h

TABLE 1 | Slurry characterization.

Farm	Coliforms	*E. coli*	Enterococci	TS	VS
A	6.90 ± 0.09	6.48 ± 0.08	5.31 ± 0.11	7.47 ± 0.36	5.83 ± 0.52
B	7.13 ± 0.13	6.95 ± 0.39	6.12 ± 0.38	7.59 ± 0.1	5.80 ± 0.27
C	7.48 ± 0.21	7.43 ± 0.19	5.76 ± 0.48	7.27 ± 0.45	5.65 ± 0.83

Mean ($n = 3$) slurry pathogen indicator numbers (log_{10} cfu g^{-1}) and TS/VS% from 3 cattle farms.

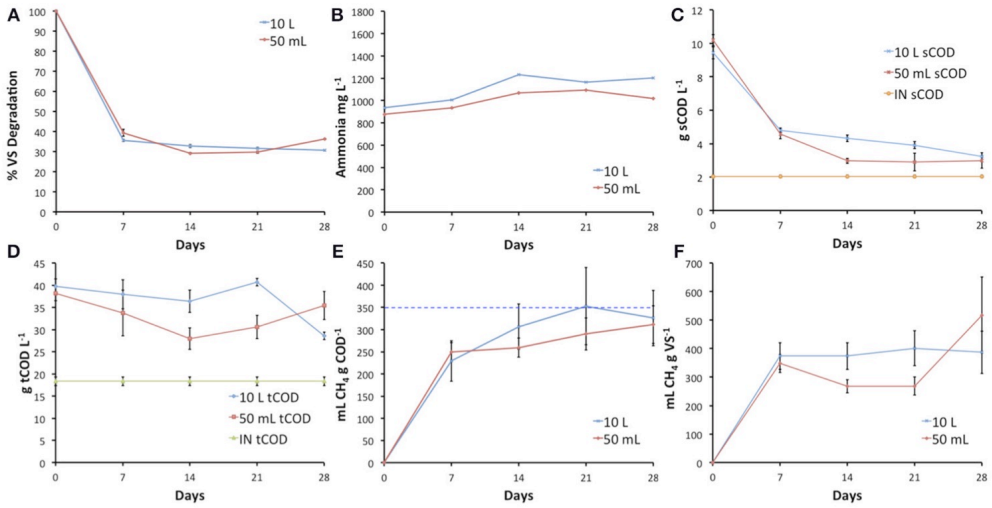

FIGURE 1 | Comparison of means ($n = 3$) of key process performance indicators in 10 L and 50 mL mesophilic continuously stirred bioreactors, with standard deviation error bars. **(A)** Volatile solids degradation %. **(B)** Ammonia concentration (mg L^{-1}). **(C)** Soluble COD removal (g L^{-1}). **(D)** Total COD removal (g L^{-1}). **(E)** Volume of methane produced per gram of COD removed, with dashed line denoting theoretical methane yield (Batstone et al., 2002). **(F)** Volume of methane produced per gram VS.

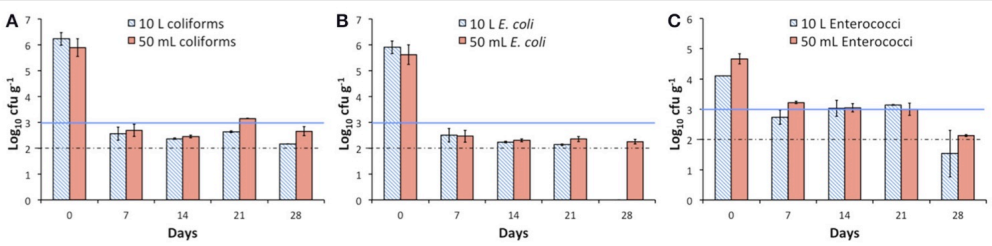

FIGURE 2 | Comparison of average fecal indicator bacteria survival in 10 L and 50 mL bioreactors ($n = 3$), with standard deviation error bars. **(A)** Total coliforms; **(B)** *E. coli*; **(C)** Enterococci. Blue line denotes EU digestate standard requirement, dashed line represents the limit of detection.

and P4: 70°C for 60 min) were tested at the miniature scale. Volatile solids degradation was relatively consistent across all conditions, as was ammonia concentration (**Figures 3A,B**). For both total COD and soluble COD, the rate of removal within the first 7 days was notably higher for AD of feedstock that had been pre-pasteurized at 60°C for 96 h (89 vs. 74–80% for sCOD, **Figure 3**; and 93 vs. 82–85% for tCOD; data not shown). The impact of P1 on COD removal was observed at the first time point only, as by Day 14, the other conditions displayed similar results (**Figure 3C**). Although the total volume of methane produced for P1 was similar to the other conditions, high levels of COD removal combined with low biogas quality (22–40% CH_4 Day 2, 54–68% CH_4 Day 5; **Figure S3B**) resulted in lower yields of 146 mL CH_4 g COD^{-1} by Day 7 (**Figure 3D**),

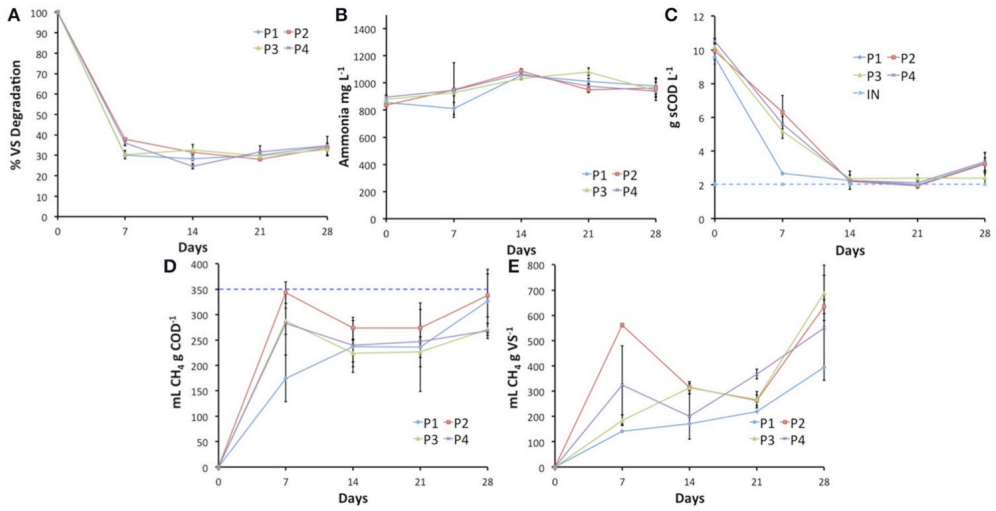

FIGURE 3 | Comparison of means ($n = 3$) of key process performance indicators following four pasteurization treatments, with standard deviation error bars. **(A)** Volatile solids degradation %; **(B)** Ammonia concentration (mg L^{-1}); **(C)** Soluble COD removal (g L^{-1}); **(D)** Volume of methane produced per gram of COD removed, with dashed line denoting theoretical methane yield (Batstone et al., 2002); **(E)** Volume of methane produced per gram VS.

compared with 227 mL CH_4 g COD^{-1} for no pasteurization. Pre- pasteurization at the EU standard (P2) improved methane yield, approaching the maximum theoretical methane yield of 350 mL CH_4 g COD^{-1} within 7 days (**Figure 3D**; Batstone et al., 2002). As expected, the two post-AD conditions had no impact on the AD process itself and the results for key performance indicator data recorded for P3 and P4 (**Figure 3**) were comparable to those of the unpasteurized condition presented in **Figure 1**.

Post-AD Pasteurization Decreases Fecal Indicator Bacteria Survival

Pre-AD pasteurization (P1 and P2) was carried out on the food production waste prior to mixing with slurry and feeding into bioreactors, as is standard practice. This resulted in a reduction in *E. coli* (1.19–1.33 $\log_{10}$) numbers on Day 0, particularly for P1, but had minimal impact on total coliform numbers compared with no pasteurization (**Figures 2**, **4**). Overall, the effect of pre-pasteurization treatments (P1 and P2) on FIB survival was not statistically significant. Post-AD treatment under Irish and EU transformation parameters (P3 and P4) resulted in lower coliform and *E. coli* numbers in the digestate, when compared with unpasteurized (**Figures 2**, **4**). When comparing pre-pasteurization with post pasteurization, the post-pasteurized treatments showed significantly lower coliform counts; whilst the other indicators shared similar trends (P1 and P2; **Figure 4**). At all post-AD pasteurization time-points, coliforms and *E. coli* were below the limit of detection in the majority of replicates, while enterococci numbers were below 1,000 cfu g^{-1} within 7 days (**Figure 4**).

AD Treatment Effectively Reduces Fecal Indicator Bacteria Levels Compared to Stored Slurry

Cattle slurry from three dairy farms was stored in a shed at ambient environmental temperature for 56 days (between 4 and 13°C in Galway, Ireland). Over the first 7 days of storage there was a 0.32 and 0.36 $\log_{10}$ reduction in coliforms and *E. coli* numbers respectively, and a slight increase in enterococci numbers. Hence, within 7 days of AD treatment, the resulting digestate was superior to stored slurry in terms of FIB inactivation (**Figure 5**). It is worth noting that an initial dilution factor of 1–1.5 $\log_{10}$ is evident in the digestate when compared with unprocessed slurry. This is due to the mixing of slurry with FOG and microbial inoculum prior to AD processing. After 2 months of storage, none of the FIB tested in slurry had dropped below the EU minimum digestate quality standards of 1,000 cfu g^{-1} (**Figure 5**).

DISCUSSION

Systematic examination of the fate of key viral, bacterial and protozoan pathogens in farm-based anaerobic co-digestion of various wastes is hampered by availability of sufficient pathogenic biomass as well as health and safety concerns associated with spiking large-volume bioreactors. This makes the use of larger scale bioreactors for pathogen survival studies impractical. Here, we carried out a comparative trial across two bioreactor scales, of 50 mL and 10 L, in order to assess the potential use of miniature-scale AD bioreactors as proxies for larger scales. Across all the major physicochemical parameters recorded, both bioreactor

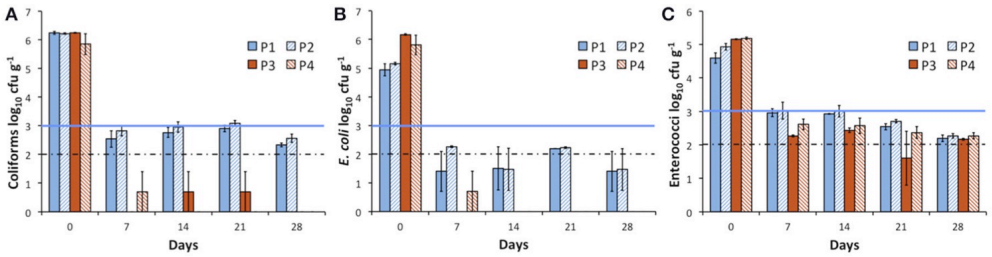

FIGURE 4 | Comparison of means ($n = 3$) of fecal indicator bacteria survival, presented as $\log_{10}$ colony forming units per gram following four pasteurization treatments, with standard deviation error bars. P1: Pre-AD, 60°C for 96 h; P2: Pre-AD, 70°C for 1 h; P3: Post-AD, 60°C for 96 h; P4: Post-AD, 70°C for 1 h. **(A)** Total coliforms; **(B)** *E. coli*; **(C)** Enterococci. Blue line denotes EU digestate standard requirement, dashed line represents the limit of detection.

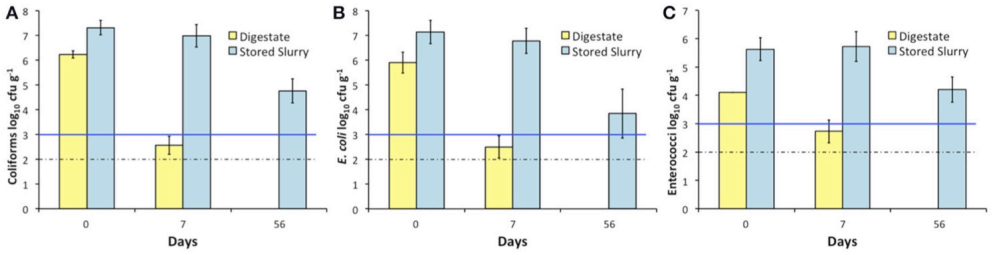

FIGURE 5 | Comparison of FIB removal in 10 L mesophilic continuously stirred tank bioreactors ($n = 3$) and stored slurry ($n = 9$), with standard deviation error bars. All FIB survived 56 days storage at ambient temperature at levels exceeding EU limits; 1 week of AD processing was sufficient to bring FIB counts beneath the EU limits, and eventually beneath the limit of detection. Blue line denotes EU digestate standard requirement, dashed line represents the limit of detection. **(A)** Total coliforms ($\log_{10}$ cfu g^{-1}); **(B)** *E. coli* ($\log_{10}$ cfu g^{-1}); **(C)** Enterococci ($\log_{10}$ cfu g^{-1}).

scales displayed similar trends. The volatile solids removals obtained in the present study were in line with those reported in the literature (64–67%—Neves et al., 2009; Luste et al., 2012). The majority (61–64%) of the volatile solids degradation at both scales occurred within 7 days (**Figure 1A**), demonstrating the potential for reduced retention time of the substrate in the bioreactors. Reported methane yields vary significantly, depending on feedstock mixtures and ratios, retention time, and temperatures, but a range between 200 and 489 mL g VS^{-1} is typical of co-digestion containing manure as the primary constituent with food production waste (200–350 mL g VS^{-1}–Neves et al., 2009; 470 mL g VS^{-1}–Creamer et al., 2010; 260 mL g VS^{-1}–Luste et al., 2012; 320–489 mL g VS^{-1} Dennehy et al., 2016). The range of 220–488 mL CH_4 g VS^{-1} recorded in the present work falls within those previously reported. Here we demonstrate, at 50 mL and 10 L bioreactor scales, that mesophilic AD of slurry co-digested with FOG effectively reduces coliforms and *E. coli* numbers within 7 days (**Figures 2A,B**). Similarly, whilst examining the effect of varying ratios of pig slurry co-digested with food waste in dry-AD, Jiang et al. (2018) recently reported coliform and *E. coli* inactivation within 7 days, identifying free VFA concentration as a primary factor in inactivation. Dennehy et al. (2018) found similarly reduced levels of *E. coli* (1.2–2.2 $\log_{10}$ cfu g^{-1}) in mesophilic CSTR co-digesting pig manure with food waste, although higher total coliform values were reported (4–6 $\log_{10}$). The higher total coliforms reported by Dennehy et al. (2018) may be due to reduced mixing (1 h per day), decreased hydraulic retention time and feeding regime employed (daily feeding vs. batch) when compared to the present study.

Using Bayesian hierarchical modeling provided a flexible framework for assessing the statistical significance of the indicator die-off rates. As the vast majority of change in FIB numbers occurred within the initial 7 days, taking a piecewise approach allowed assessment of both the initial effect of the feedstock addition under the different pasteurization schemes, and also the long-term effect on FIB counts in the system as it stabilized over time. We hope that by releasing both the data and models used to assess the data, such an approach will become a regular tool in assessing bioreactor performance, particularly in relation to pathogen survival.

The results obtained for both bioreactor scales indicate higher enterococci survival in mesophilic anaerobic co-digestion of slurry with FOG, compared with coliforms or *E. coli*. This observation is in agreement with the previously reported examination of four full-scale Swedish biogas plants, one thermophilic and three mesophilic, co-digesting manure with kitchen, and food-processing waste, where higher numbers

of enterococci than coliforms were consistently found in the digestate, despite the use of pre-AD pasteurization in all four plants (Bagge et al., 2005). Furthermore, the enterococci survival results of Bagge et al. (2005) mirror closely those of Dennehy et al. (2018), whereby ∼3 $\log_{10}$ cfu g^{-1} were consistently recorded, using a continuously fed system and three different ratios of pig manure to food waste. Based on these observations, enterococci are recommended as a better indicator for pathogen survival during AD processes (Larsen et al., 1994; Sahlström, 2003).

Numerous studies have examined the impact of pre-AD pasteurization on process performance, typically anticipating improved methane yield caused by preliminary hydrolysis of the feedstock (Luste and Luostarinen, 2010). The corresponding results have however varied widely, ranging from a methane production reduction of up to 34% during the co-digestion of slaughterhouse waste (SHW) with the organic fraction of municipal solid waste (Cuetos et al., 2010) to no significant effect during the AD of SHW (Hejnfelt and Angelidaki, 2009; Ware and Power, 2016), through 14–25% improvements during the co-digestion of SHW and slurry (Paavola et al., 2006; Luste and Luostarinen, 2010). Edström et al. (2003) initially reported a 400% increase in BMPs of pasteurized vs. unpasteurized SHW, although this yield was not achieved in laboratory or pilot-scale trials. The variability of these results is likely due to differences in biochemical properties of the feedstocks used, as demonstrated in a study examining the effects of pre-treatment on five different components of SHW (Luste et al., 2009). In the present study, the methane output when FOG was pre-pasteurized at 70°C for 1 h was statistically higher than the other conditions in the first 7 days of this trial (**Figure 3D,E**), although Carrere et al. (2016) advise against extrapolating such results to full-scale plants without complex modeling. Although methanogenesis appears to have been impacted differentially by the two pre-AD pasteurization conditions tested, FIB survival was similar for both conditions. Slightly higher FIB numbers were recorded after 28 days in systems processing pre-pasteurized feedstock (P1 and P2; **Figure 4**). This may be indicative of reduced competition for resources, whereby pre-pasteurization reduced the microbial populations in the feedstock, enabling increased FIB survival and/or regrowth of resilient strains or cells.

A number of pasteurization conditions were examined by Coultry et al. (2013) to determine the energy consumption and consequent economic impact on viability of AD plants. Pre-AD pasteurization was demonstrated to be prohibitively expensive; most notably, the energy required to meet the Irish national transformation standard (P1) equates to 4,544% of the digester's output, which is an 80-fold increase in energy consumption when compared with the already prohibitive EU requirement (P2). These numbers are likely to be lower in practice however, as only the imported materials are pasteurized before mixing in with indigenous slurry, reducing the pasteurization treatment efficacy as seen in the FIB survival results for P1 and P2. The energy cost of post-AD pasteurization is mitigated by the mesophilic digestate, but was still found by Coultry et al. (2013) to be substantial, at 30 and 1,893% of the digester's annual energy output for EU (P4) and national standards (P3) respectively. Although some measures could be taken to reduce these costs, such as separation of liquid and solids, they are clearly a substantial burden to the economic viability of bioreactor operation. This burden hinders adoption of farm-based AD and is worth reconsideration in light of the reduction in FIB numbers in unpasteurized trials and the absence of hygienization requirements for unprocessed slurry. The FIB survival rates monitored in the stored slurry are in line with previous studies such as that of Nicholson et al. (2005), who found that *E. coli* O157, *Salmonella* and *Campylobacter* survived for up to 3 months during dairy slurry storage. Similarly, *Mycobacterium avium* subspecies *paratuberculosis* has been found to survive beyond 56 days in stored slurry at ambient temperatures (Grewal et al., 2006). Furthermore, survival of pathogens in stored slurry increases with temperatures below 10°C, such as those typical of winter storage months in north-western European climates (Kudva et al., 1998). In terms of potential pathogen load to the environment, as assessed via the monitoring of FIB levels, we have demonstrated that mesophilic anaerobic co-digestion of slurry with food production waste is superior to simple slurry storage without treatment. Moreover, the slight increase in numbers of enterococci over the first 7 day period of slurry storage (**Figure 5**) highlights the potential risk of pathogens thriving in this environment. Based on these findings, if the EU standard for digestate was applied to slurry (<1,000 cfu g^{-1}), all livestock farms would be required to adopt some form of treatment.

Previous studies have examined the agronomic benefits of anaerobic digestion (AD) of slurry. Benefits include increased homogeneity and decreased viscosity, due to the reduction in volatile solids, resulting in more uniform landspreading (Massé et al., 1997). As detailed by Massé et al. (2011), other studies have demonstrated the added fertilizer value of digestate compared with slurry and mineral fertilizer, resulting from improved plant N uptake and increases in N and P mineralization (Massé et al., 2007; Chantigny et al., 2009). When these agronomic improvements, energy production, waste reduction and mitigation of GHG emissions are considered together with reduced pathogen load to the environment, widespread adoption of AD as a means of slurry amendment prior to landspreading should be encouraged (Clemens et al., 2006). The enterococci survival observed in this study highlights however the scope for future work to improve pathogen inactivation during farm-based AD. Optimization of operational conditions for FIB reduction is currently underway. Future work focusing on landspreading field trials will be necessary to assess further comparative risk from digestate and unprocessed slurry.

CONCLUSION

In this study we demonstrate that (i) miniature 50 mL bioreactors are valid proxies of farm-based AD to carry out targeted pathogen survival investigations and (ii) *in situ* AD treatment of slurry prior to land application reduces the level of FIB compared to slurry storage alone, independently of pasteurization, which in

turn might be indicative of a decreased potential pathogen load to the environment and associated public health risks. While pathogen indicator die-off was observed, enterococci survival highlights the opportunity for process optimization with a focus on hygienization.

AUTHOR CONTRIBUTIONS

FA and SN designed the research. SN and AA ran the bioreactors. NW and LP carried out the modeling. SN and FA analyzed the data. SN, FA, NW, and LP wrote the paper with input from VO, FB, OF, KR, and DB.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2018.00041/full#supplementary-material

Figure S1 | Comparison of methanogenic activity performance of various ratios of granular sludge:BEOFS digestate:slurry for development of inoculum ($n = 3$). FOG: Fats, oils and grease; PRO: Propionate; BUT: Butyrate; ETH: Ethanol; ACE: Acetate.

Figure S2 | Recorded pH for **(A)** 10 L and 50 mL bioreactors processing unpasteurized slurry and FOG; **(B)** 50 mL bioreactors testing pasteurization conditions.

Figure S3 | Mean methane percentages for 10 L and 50 mL **(A)** and P1-P4 **(B)** at all timepoints ($n = 3$), with standard deviation error bars. Detailed information about the models used, in addition to the data and analysis script, can be found at http://nickp60.github.io/SI_Nolan_etal_2018.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.2 Future work

Having established that miniature bioreactors (50 mL) are appropriate proxies from which to glean information relevant to larger-scale AD, and that some survival of faecal indicator bacteria may occur in AD, it is necessary to test multiple combinations of variables to establish an optimum set of conditions for biogas production and/or digestate sanitisation.

Given the infinite possible combination of variables, it is not feasible to attempt to find the optimal operational conditions by testing each possible combination, particularly in a full-scale set up with major economic implications. Hence, it is necessary to carry out designed experiments to glean baseline data and employ modeling tools to establish the optimum set points for conditions such as temperature, loading rate and feedstock ratio.

2.3 Supplementary Figures

Figure S1

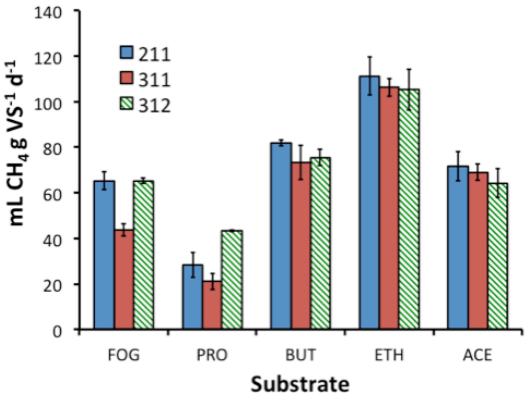


Figure S2

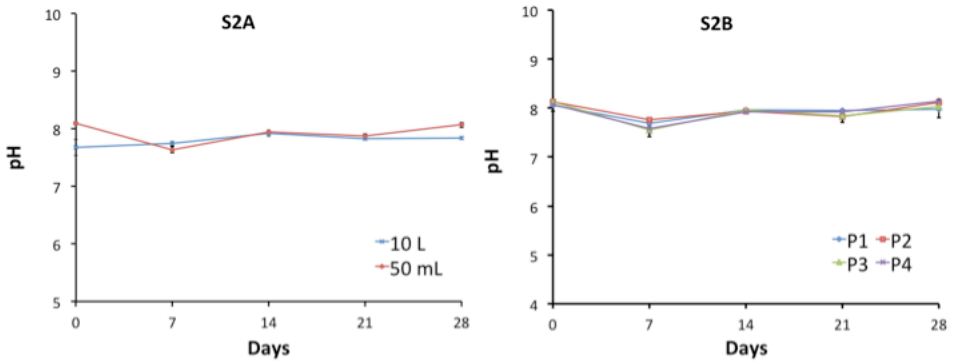
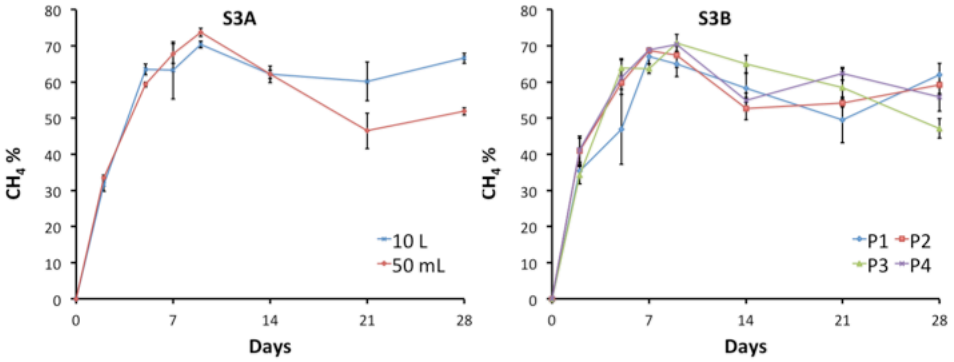


Figure S3



Chapter 3

Towards the development of an anaerobic digestion plant operation support tool for optimising methane production and digestate sanitisation in farm-based applications

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I wrote this paper with the help of my supervisors and collaborators, having carried out the laboratory work. The data analysis and visualisation was undertaken with Camilla Thorn and Nicholas Waters. The modeling was carried out with Peyman Sadrimajd under the guidance of his supervisor, Piet Lens. The paper presented here is the manuscript as submitted to *Applied Energy*. The reviewers requested validation experiments to be carried out, and those experiments were interrupted by COVID-19 restrictions. They will be re-established with a view to resubmitting to *Applied Energy*.

Highlights

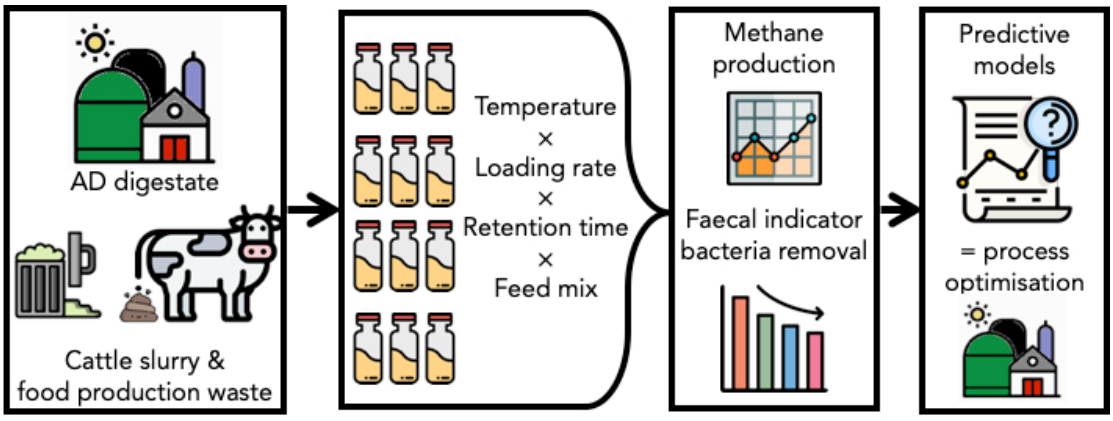
- Modeling methane production optimisation can streamline bioreactor start-up and operation
- Modeling should be used prior to operational or feedstock changes to predict impact on bioreactor performance
- Modeling can be used to predict insufficient sanitisation or increased pathogen load in specific feedstock mixture and loading rate combinations
- Farm-based anaerobic digestion (AD) at sub-mesophilic temperatures is an attractive process option

Abstract

Agriculture-based anaerobic digestion (AD) of slurry with various organic secondary raw materials represents a multi-beneficial solution to waste and energy management. Operational parameters, including co-digestion feedstock availability, can vary significantly within and between farm-AD plants. Although AD digestate typically has a lower pathogen load than untreated slurry, the impact of this variability on pathogen survival in the bioreactor is not clear. The impact of operational parameters on bioreactor performance and digestate sanitisation (mixing ratio of co-digestion feedstock, temperature, retention time and loading rate) during slurry and food production waste anaerobic co-digestion in miniature-scale (50 mL) continuously stirred tanks was investigated. Faecal indicator bacteria (FIB) – faecal coliforms, *Escherichia coli* and *Enterococcus* species – were monitored throughout the trials.

Results showed that FIB reduction was highest at 55°C, achieving sanitisation of all feedstocks at all loading rates within six days, whilst methane output was more consistent across operational conditions at 37°C. Response surface analysis was applied to model and optimise process parameters for different operational conditions. The model developed identified that with a combination of low organic loading and longer retention time, digestate sanitisation sufficient to satisfy EU standards is possible in AD at temperatures of 20 or 25°C, whilst also maintaining satisfactory methane production. The outcomes of the present study should inform farm-AD plant operations and underpin the on-going development of a comprehensive risk prediction tool for digestate land application.

Graphical Abstract



Keywords: Anaerobic co-digestion; slurry; food production waste; faecal indicator survival; digestate; predictive modeling

Abbreviations:

AD - Anaerobic digestion
EU - European Union
S - Dairy cattle slurry
FIB - Faecal indicator bacteria
FOG - Fats, oils and grease
FW - Food production waste

3.1 Introduction

The EU Circular Economy Action Plan aims to "close the loop" of product life cycles, by re-envisioning waste as 'secondary raw materials' from which maximal value is to be derived. The plan identifies waste from food (production, distribution and storage) and bio-based materials as two priority areas, which must be addressed (COM, 2015). Agriculture-based anaerobic co-digestion of animal slurries with various organic wastes directly addresses these two priority areas and is an effective means of increasing farm profitability whilst reducing landfill related greenhouse gas emissions, leachate generation and fossil-fuel dependence. Traditionally farm-based AD produces biogas for electricity and/or heat generation with a high fertiliser value by-product, the digestate, which is landspread. Unprocessed slurry may contain a

range of pathogens, which present a risk to human and animal health during storage and landspreading (Nicholson et al., 2005; Venglovsky et al., 2009; Watabe et al., 2003). Slurry AD with organic waste typically results in reduced pathogen levels in the digestate when compared with unprocessed slurry, in turn reducing the risk to human and animal health (Nolan et al., 2018; Sahlström, 2003). However, the EU's stated aim of maintaining quality standards for waste-based fertilisers necessitates an efficient and accurate prediction of hygienisation and methane potential of co-digestion feedstocks (COM, 2015).

Cattle and pig slurry are the primary feedstocks for agriculture-based anaerobic co-digestion. Across Europe, 1.4 billion tonnes of manure are produced annually, the majority of which is landspread (Foged, 2011). Of the 1.4 billion tonnes of manure, approximately 92% comes from cattle and pig production (79% and 13.4% respectively). When utilised as an AD feedstock, the slurry fraction of cattle and pig manure provides active hydrolytic and methanogenic microbial populations, which help to maintain bioreactor activity. The biomethane potential of slurries vary between 25 - 30 m^3 t^{-1}, depending on the animal source (Weiland, 2010), much of which is released to the atmosphere during storage and spreading. Although capturing this methane using AD could significantly mitigate greenhouse gas emissions (Massé et al., 2011), this biomethane potential is typically insufficient to justify mono-digestion, given the large capital costs required (Pantaleo et al., 2013). Furthermore, mono-digestion is susceptible to underperformance or process failure due to an imbalanced C:N ratio, ammonia inhibition (Nielsen and Angelidaki, 2008), and micronutrient or trace element deficiency (Mata-Alvarez et al., 2014).

Co-digestion of slurry with secondary raw materials is therefore preferable, whereby another feedstock such as food processing waste, is mixed in, to produce a stabilising buffering effect (Rodriguez-Verde et al., 2014), thereby increasing the methane yield and farm profitability (Álvarez et al., 2010). The resulting digestate typically has an improved fertiliser nutrient concentration and bioavailability as well as lower pathogen load when compared to untreated slurry, with the added non-negligible environmental and economic benefits of offsetting reliance on chemical fertilisers (Nolan et al., 2018; Sahlström, 2003; Ward et al., 2008).

The availability of co-digestion feedstocks can vary significantly, as can their methane potential (Álvarez et al., 2010; Rodriguez-Verde et al., 2014). These may

introduce additional pathogens to the bioreactor and resulting digestate. Modeling and optimisation studies for agriculture-based co-digestion have focused primarily on the synergistic effects of feedstocks on the biomethane potential without addressing digestate sanitisation (Álvarez et al., 2010; Dennehy et al., 2016a). However, EU legislation (Regulation (EC) No.1069/2009) and Regulation (EU) No. 142/2011), stipulates that digestate faecal indicator bacteria (FIB) levels must be below 1,000 cfu g^{-1} in order to be considered safe for landspreading.

A review of 30 countries found that many outside the EU do not have specific AD regulatory policies regarding digestate sanitisation, potentially increasing risk of pathogen spread to the environment in countries with no regulations (Global Methane Initative, 2014). To date, pathogen and FIB survival/reduction has primarily been reported for mono-digestion of slurry, with sparse consideration for co-digestion. Furthermore, the impact of the interaction between varying feedstock mixture, loading rate or retention time on digestate sanitisation has scarcely been considered (Beneragama et al., 2013). One such study on co-digestion of pig manure with domestic food waste examined the impact of feedstock mixtures and retention time on FIB survival, and found a slight increase in *E. coli* survival as retention time decreased, but only examined at one temperature (39°C) (Dennehy et al., 2018).

Process temperature is considered to be the dominant controlled factor in pathogen survival during AD, with increased reduction in pathogen load at thermophilic temperatures (Beneragama et al., 2013; Gerba and Smith, 2005; Olsen & Larsen, 1987; Sahlström, 2003). Historically, agriculture-based AD bioreactors have been operated at mesophilic or thermophilic temperatures and have used the biogas on site in a combined heat and power (CHP) plant to produce electricity. CHP plants are however, typically an inefficient means of producing electricity (35% conversion efficiency), with 40% of the energy potential being converted to heat (Murphy et al., 2004), which is primarily used to maintain bioreactor temperatures and for pasteurisation (Auer et al., 2017; Ward et al., 2008). Developments in gas pipeline connectivity and centralised injection points, as well as improved gas-scrubbing technology have facilitated a move away from CHP towards direct grid injection. One consequence of this move is the loss of the waste heat previously used to maintain meso- or thermophilic temperatures, requiring electrical heating instead. In this new scenario, low or ambient temperature AD becomes an attractive

alternative, particularly in regions where mesophilic ambient temperatures dominate (Resende et al., 2014). The potential of low-temperature AD for wastewater treatment is well established (McKeown et al., 2012), but not for agriculture-based co-digestion, with studies on mono-digestion of slurry typically demonstrating decreased methane yields at lower temperatures (Alvarez et al., 2006; Kashyap et al., 2003; Safley & Westerman, 1994). Even though the reduced energy requirement should offset lower methane yields, the impact on FIB survival needs to be investigated to properly assess the feasibility of ambient temperature AD for farm-based applications.

Determining the effect of multiple parameters on pathogen and FIB survival in an AD setting is complicated by the sheer number of potential variables. Some of these factors are under the control of the AD plant operator, including: variance in organic loading rate, feedstock ratios with diverse pathogen load, retention time or temperature (Smith et al., 2005). Statistical analysis and mathematical modeling make process parameter optimisation and scenario investigation possible. Additionally, design of experiment (DoE) methods can be used to reduce the number of experimental units required to study specific factors and increase information gain to allow for qualitative optimisation. The financial and time constraints inhibiting extensive experimentation of multiple variables can be overcome through modeling, so that lab-scale experimental data can be utilised more efficiently to determine optimal settings at industrial scale, mitigating the risk inherent in making operational changes (Kainthola et al., 2019b; Lu et al., 2019). Response Surface Methodology (RSM) is a DoE that incorporates optimisation and statistical analyses, which has previously been applied to AD processes (Feng et al., 2017; Han et al., 2012; Kainthola et al., 2019b; Riaño et al., 2011; Wang et al., 2013).

Anaerobic Digestion Model No. 1 (ADM1) (Batstone et al., 2002), a first principle mathematical model, has been widely employed, with over 2,400 citations currently, and primary application in the field of AD of sewage sludge under various process conditions (batch, mixed batch, fluidised bed, fixed bed) (Batstone et al., 2006). It is formulated as a set of ordinary differential equations (ODE) or alternatively as a set of differential algebraic equations (DAE). These sets of equations describe the rates of change and interaction of biochemical and physicochemical components of AD systems. However, as information related to

FIB removal (such as *E. coli*, enterococci, coliforms) is not incorporated in the ADM1 system of equations, ADM1 was not a viable option for this study. In addition, optimising operational conditions (biogas production or pathogen die off) would require the development of a separate optimisation algorithm to be used alongside ADM1. Furthermore, an extensive number of measurements (e.g. pH, NH_3, TKN, tCOD, P, TS, VS, VFA, alkalinity, CH_4) are required for ADM1 calibration and validation (Batstone et al., 2002) and the expertise, time and facilities necessary to acquire them are not commonly available. Hence a modeling approach that requires the monitoring of fewer physico-chemical parameters would be more favourable for widespread use.

Thus the aims of this study were to (i) examine the impact of various co-digestion operational parameter variables on FIB survival and reactor performance, and (ii) develop a decision support model for plant operators, and responsible governmental bodies, capable of (a) predicting biogas production and FIB die-off for a given secondary raw material under various operational conditions, and (b) optimising operational parameters to maximise biogas production while maintaining EU regulatory digestate standards.

3.2 Material and Methods

3.2.1 Experimental Setup

To investigate the effect of three continuous factors (temperature, organic loading (OL), and retention time (RT)) and one categorical factor (feedstock recipe) on biogas production and faecal indicator die-off, experiments with a custom response surface design were performed (Table 3.1). The range of variation for each factor was chosen based on potential configurations of full-scale AD plants, with the centre level for each variable corresponding to typical operational parameters used in AD plants. A schematic representation of the experimental set-up is presented in Figure 3.1.

The volatile solids (VS) fed and the retention times (RT) used in the experimental microcosms were both calculated based on a semi-continuously fed, full-scale AD plant. Thus, microcosms were set up with sufficient VS to provide an equivalent organic loading rate (OLR) of either 0.5 g, 2 g or 3.5 g VS per litre per day at either a 21, 42 or 63 day RT. In such a semi-continuously fed AD plant, a 21 day RT would be achieved by feeding every 3 days; likewise a 42 day cycle by feeding every 6 days and a 63 day cycle by feeding every 9 days. Destructive sampling was therefore performed at days 3, 6 and 9 to represent these three RTs.

Table 3.1 presents the range and levels of independent variables employed. For clarity, these were batch-type experiments, whereas "continuous" refers to the variable type, as distinct from "categorical".

Table 3.1: Experimental range and levels of independent variables.

Variables	Temperature (°C)	Loading Rate (g VS l^{-1} d^{-1})	Time (days)	Recipes (S:FW ratio)
Low level	19	0.5	3	1:3
Medium level	37	2	6	2:1
High level	55	3.5	9	3:1
Type	Continuous	Continuous	Continuous	Categorical

S: Dairy cattle slurry; FW: Food production waste.

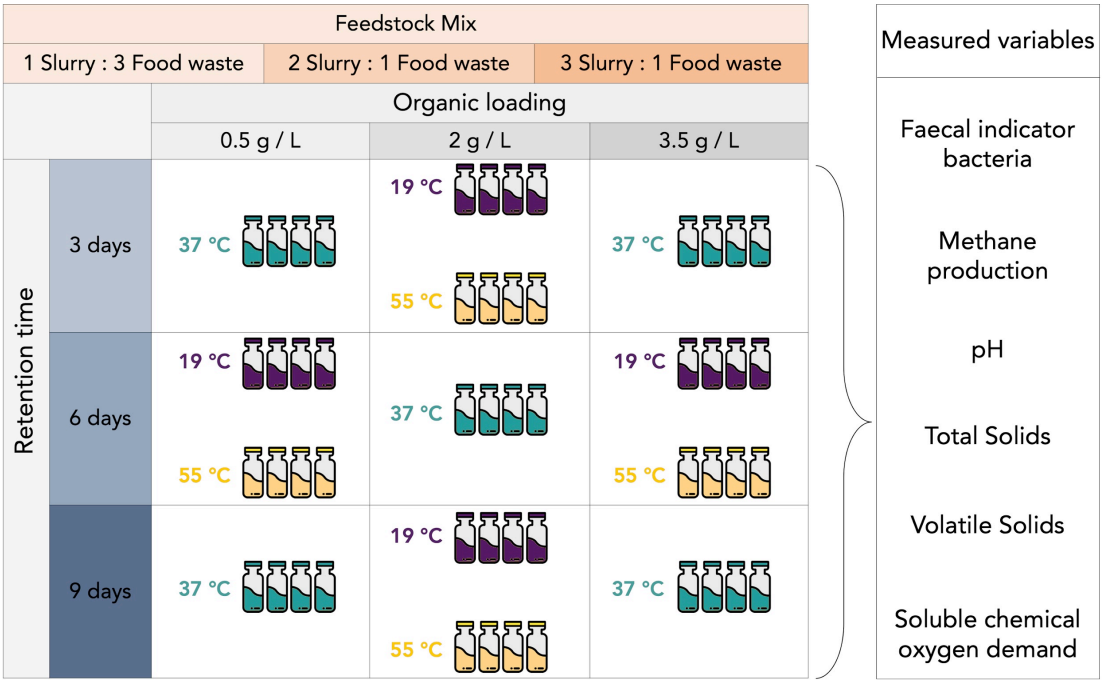


Figure 3.1: Graphical representation of the experimental set up employed in this study, where three feedstock recipes were tested at each of the 13 combinations of conditions represented; three temperatures, low, medium and high loading rates and short, medium and long retention time. A minimum of four biological replicates were investigated per condition, with 12 replicates for each recipe in the central condition (2 g VS/L, 6 days, 37°C).

Inoculum from 10 L mesophilic laboratory-scale bioreactors co-digesting dairy cattle slurry (S) with food production waste was pre-incubated at each experimental temperature for three days prior to feeding. Experiments were performed utilising miniature batch tests (36 mL in 50 mL glass bottles), as previously described by Nolan *et al*. (2018), with an inoculum to feedstock ratio of 2:1 on a VS basis, so that final total volume depended on substrate VS concentration. These were incubated at the three selected temperatures (Table 3.1) shaking at 80 rpm (New Brunswick Scientific Innova°44 incubator) and destructively sampled on Day 3, 6 and 9.

Two types of food production waste were used in these experiments; fats, oils and grease (FOG) from industrial grease traps and bakery waste (BW). FOG was sourced from the Bioenergy and Organic Fertiliser Services (BEOFS) AD plant in Camphill, Co. Kilkenny, Ireland, collected in a 25 L drum, stored at 4°C, and mixed thoroughly before use. BW was collected from an industrial dough-production bakery, homogenised and stored at -20°C until used. Dairy cattle slurry (S) for the trial was collected from a dairy farm in County Galway, Ireland. The slatted storage

tanks were agitated to homogenise the slurry before collection using a bucket attached to a pole, in accordance with Brennan *et al*. (2011) and Peyton *et al*. (2016). Slurry was stored at 4°C for one week prior to use as feedstock, at which time it was mixed thoroughly on a volume basis with the food production waste described above to create three distinct feedstocks: i) 1S:3FW (1 part S to 3 parts FW); ii) 2S:1FW (2 parts S to 1 part FW) and iii) 3S:1FW (3 parts S to 1 part FW). The food production waste component of the 2S:1FW feedstock recipe was FOG, to provide a reference recipe for which FIB survival data has already been reported (Nolan et al., 2018). Bakery waste was used as the food production waste component in the 3S:1FW and 1S:3FW recipes.

3.2.2 Analytical methods

Biogas volume was determined by capturing the gas in graduated 50 mL syringes. Methane content of the biogas was analysed using a gas chromatograph (GC) equipped with a flame ionisation detector. The carrier gas was nitrogen and the flow rate was 25 mL min^{-1}. Analysis of total and volatile solids (TS/VS) was performed gravimetrically according to standard methods (APHA, 2005) and total and soluble chemical oxygen demand (tCOD/sCOD) analysis was performed according to the Standing Committee of Analysts (1986). NH_3 concentrations (mg L^{-1}) were determined using the HACH AmVer High-Range Ammonia test, while pH was also monitored throughout the trial.

3.2.3 Faecal Indicator Bacteria

Faecal coliform and *Escherichia coli* numbers were quantified using IDEXX Colisure with Quanti-Tray/2000 after incubation at 35°C for 24 hours. Enterococci numbers were quantified using IDEXX Enterolert kit with Quanti-Tray/2000 after incubation at 41°C for 24 hours. FIB numbers were determined for Day 0 and at each time point.

3.2.4 Data Analysis

Raw and formatted (cleaned) data are available at https://github.com/nickp60/SN_minitrials. Data were pre-processed and visualised with R (R Core Team, 2019) and ggplot2 (Wickham, 2009). Error bars are the standard deviation of at least 4 replicates. Details of the data analysis can be found in

Supplementary Material. Minitab (Minitab® 17 version) was used for implementation of the custom response surface design, analysis and optimisation.

For response surface analysis full quadratic models were fitted to experimental data in order to examine the effect of all possible combinations of the factors investigated, namely organic load, temperature, retention time, and feedstock recipe, on the prediction of FIB survival, and methane production, as follows:

$$Y_i = \beta_0 + \sum_i \beta_i x_i + \sum_{ii} \beta_{ii} x_i^2 + \sum_{ij} \beta_{ij} x_i x_j$$

with Y_i as predicted response, β_0 as a constant, β_i as linear coefficients, β_{ii} as quadratic coefficients, βij as interaction coefficients and x_i and x_j as independent variables.

3.3 Results and Discussion

3.3.1 Faecal indicator bacteria survival

Faecal indicator bacteria are used as a proxy for pathogen hygienisation in AD digestate. EU legislation (Regulation (EC) No.1069/2009 and Regulation (EU) No. 142/2011) requires FIB in digestate to be below a limit of 1,000 cfu g^{-1} to be considered safe for landspreading. The removal rate required to achieve this maximum threshold depends on the starting FIB load, which in turn depends on the feedstock recipe (Table 3.2).

Table 3.2. Mean (n=3) feedstock FIB numbers and total and volatile solids (TS/VS) before AD treatment. S stands for slurry and FW for food processing waste. 1S:3FW, 2S:1FW and 3S:1FW correspond each to a ratio of 1:3, 2:1 and 3:1 slurry to food processing waste.

Feedstock	Coliforms log_{10} cfu g^{-1}	*E. coli* log_{10} cfu g^{-1}	Enterococci log_{10} cfu g^{-1}	TS %	VS %
1S:3FW	6.60±0.04	6.51±0.13	4.97±0.12	28.1±0.22	24.5±0.25
2S:1FW	6.94±0.13	6.68±0.11	5.69±0.22	8.3±0.02	6.5±0.03
3S:1FW	6.96±0.21	6.88±0.19	6.30±0.19	16.4±0.08	13.2±0.30

Coliforms and *E. coli* were removed more efficiently than enterococci numbers under all conditions investigated, suggesting that enterococci may be a more conservative indicator of pathogen removal (Figure 3.2; Nolan et al., 2018; Sahlström, 2003). The results displayed in Figure 3.2 are aggregated by loading rates for ease of interpretation, as similar trends were observed for all loading rates (Figures 3.S1 to 3.S3). A table of recorded FIB absolute log numbers is included in the supplementary material (Table 3.G.1).

AD operational temperature had the most significant impact on FIB survival, with 55°C resulting in reduction below the limit of detection (100 cfu g^{-1}) for coliforms and *E. coli* within three days for all feedstock recipes (Figure 3.2), in line with previous research (Smith et al., 2005). Enterococci survival was higher in

thermophilic AD, with insufficient removal in 1S:3FW and 3S:1FW (3.43 $\log_{10}$ ± 0.06 and 3.63 $\log_{10}$ ± 0.07 respectively) after 3 days corresponding to a 21 day retention time (Figure 3.S3). The 1,000 cfu g^{-1} limit required by EU legislation was however achieved at 55°C for all FIB at all conditions at the 42 and 63 day retention time (Figure 3.S3, Day 6 and Day 9). At 37°C feedstock recipe 2 (2S:1FW) displayed better FIB removal than the other two recipes, particularly for coliforms and *E. coli*, achieving satisfactory removal (shaded in green) for those two indicators at both low and high loading rates (0.5 g and 3.5 g VS L^{-1} day^{-1}) after 9 days (Figure 3.S2). Enterococci removal was however insufficient to satisfy EU requirements in any recipe, loading rate or retention time at either 19°C or 37°C (Figure 3.2, Figure 3.S1 & 3.S2).

At 19 and 37°C, RT had a more significant impact on coliform and *E. coli* survival than loading rate (Figures 3.S1 and 3.S2). For enterococci however, the combination of low loading rate and high-slurry feedstock recipe was most significant, resulting in a 0.72 - 2.13 $\log_{10}$ cfu reduction for recipe 3 (3S:1FW) at 19 and 37°C respectively (Figure 3.2). However, given the higher initial enterococci load in recipe 3 (Table 3.2), even the >2 $\log_{10}$ reduction at 37°C was insufficient to achieve the EU digestate standard. Enterococci numbers in the digestate from feedstock recipe 1 (1S:3FW) were between 0.82 and 1.77 $\log_{10}$ higher than in the initial feedstock across all retention times and loading rates at 19°C and up to 1.35 $\log_{10}$ higher in the 3.5 g VS L^{-1} day^{-1} loading rate at 37°C (Table 3.2; Figures 3.S1 and 3.S2). This is an important result to consider when examining potential new feedstocks for agriculture based anaerobic co-digestion, as inadvertently increasing pathogen load to the environment could have detrimental consequences. In this scenario, post-AD pasteurisation of digestate would need to be incorporated to satisfy EU standards.

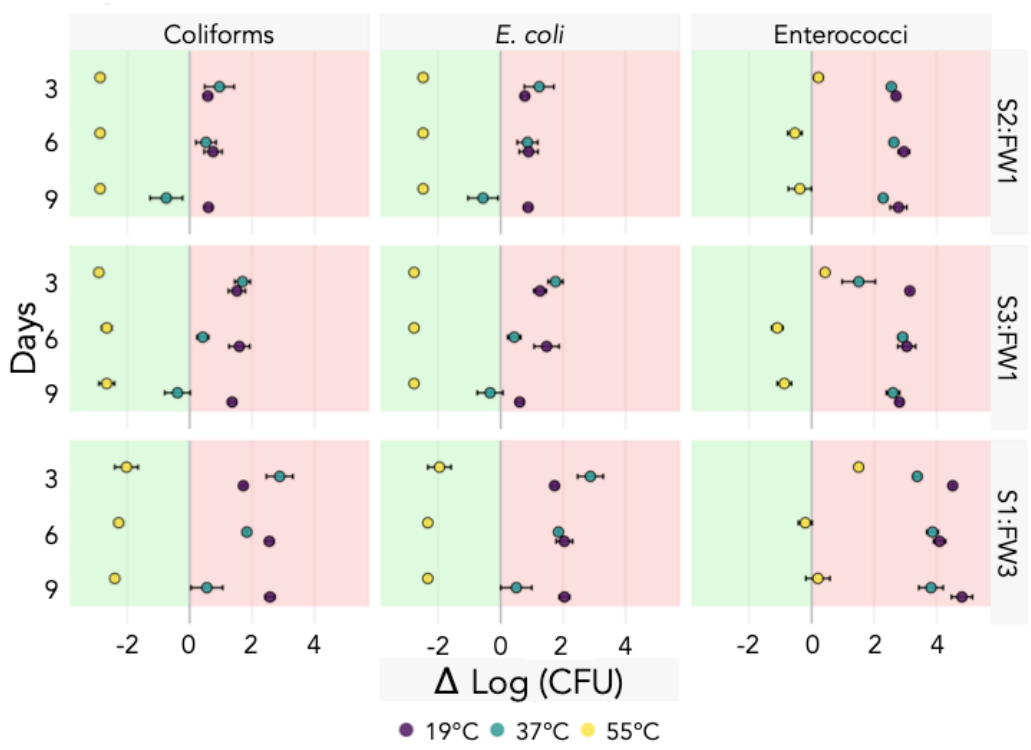


Figure 3.2: FIB removal as a function of retention time and feedstock recipe calculated as the difference between the required removal (per feedstock, according to EU digestate standard of 1,000 CFU g-1) and the actual FIB counts. Negative values indicate FIB removal greater than that required to meet EU standards, and are therefore shaded in green, while positive values represent insufficient FIB removal and are shaded in red. Temperature is indicated by marker colour where purple =19°C, green = 37°C and yellow = 55°C. Error bars indicate standard error of the mean (n≥4).

3.3.2 Physico-chemical performance

The AD performance is primarily considered in terms of methane production, with ancillary objectives including soluble COD reduction and solid degradation, reducing the digestate pollution potential. Methane production, sCOD consumption and solid degradation were all more efficient at 37°C across all conditions (Supplementary Material, Tables 3.F1 and 3.F2). In the medium retention time however, methane output at 19°C for one of the feedstock recipes (1S:3FW) at high organic loading (3.5 g VS L^{-1} day^{-1}), was close to that achieved at mesophilic temperature and low organic loading (0.5 g VS L^{-1} day^{-1}) with the short retention time (32.1±1.22 mL CH_4 vs 32.5 ± 1.75 mL CH_4; Supplementary Material, Table 3.F1). Thus methane production at lower temperatures and a longer retention time is comparable to low loading rate in 37°C (Supplementary Material, Table 3.F1). This finding was also observed in a longer time-scale semi-continuously-fed study (data not shown), in which methane output at 19°C approached that of the 35°C within 40 days, similar to Sutter & Wellinger (1988). Mesophilic inoculum was used, and it is likely that if inoculum had longer to acclimatise to the low and high temperatures, better methane production at both could be observed.

The pH did not vary significantly between conditions or over time, ranging between 6.84 and 7.92 with an average of 7.36 and standard error of 0.01 (Supplementary Material, Table 3.F1). The lower temperature and high loading rate resulted in lower pH, particularly for feedstock recipe 1, likely resulting from lower buffering capacity in low slurry ratio feed, and initial overloading of acidogenesis products (1S:3FW, pH 6.86 ± 0.01) (Supplementary Material, Table 3.F1). Although pH below 6.8 is not optimal for methane production (Ward et al., 2008), the combination of high ratio of food production waste in the feedstock and high loading rate gave the highest methane output at lower temperature, despite the lower pH (Supplementary Material, Table 3.F1).

Typically in AD systems methane production should correlate with volatile solid and/or soluble COD degradation. In this study methane production was highest at 37°C, as were sCOD and solid reduction (Supplementary Material, Table 3.F1). Feedstock recipe 3 (3S:1FW) displayed lowest sCOD removal at 19 and 37°C (37.5 and 36.0% respectively), in line with the lower methane production observed for that recipe in all but one of the 37°C conditions (Supplementary Material, Table 3.F2).

This is as expected, given that cattle slurry contains recalcitrant lignocellulosic biofibers with high levels of lignin which cannot be easily broken down by anaerobic digestion, thus resulting in low biomethane potential (Bruni et al., 2010; Møller et al., 2004; Triolo et al., 2011). This is borne out in our data as a relatively high VS content (13%) but lower initial sCOD load (the easily digestible fraction) compared with the other feedstock recipes (20g L vs 145g and 28g for recipes 1 and 2 respectively; Supplementary Material, Table 3.F2).

Concentrations of NH_3-N ranged between 865 and 2305 mg L^{-1}, with a median of 1570 ± 15.5 (Supplementary Material, Table 3.F1). Free ammonia has been identified as a key methanogenic inhibitor, particularly at thermophilic temperatures (Angelidaki and Ahring, 1994; Chen et al., 2008). The higher NH_3-N concentrations observed at 55°C (1712 ± 28 mg L^{-1}), with 1563 ± 30 and 1528 ± 20 mg L^{-1} for 19 and 37°C respectively, might partly explain the lower methane production observed at 55°C (Maximum cumulative CH_4 of 21.5 ± 2.27 mL for 55°C vs 168 ± 7.21 mL for 37°C; Supplementary Material. Table 3.F1).

3.3.3 Determination of optimal operational parameters

Quadratic models for each feedstock recipe and FIB numbers together with the related statistical analysis are provided in Supplementary Material. Most of the model coefficients were statistically significant and the prediction power of all models was good (R^2-c. 80%). We assessed different scenarios for bioreactor control parameter optimisation to meet EU standards for FIB die-off to below 2.5 log (to provide certainty of achieving the 1,000 cfu g^{-1} limit) and/or maximising methane production (confidence level for all intervals = 0.95) (Table 3.3).

The first priority constraint applied was efficient sanitisation (EU FIB limit), followed by methane production. The third constraint explored in the models was the impact of temperature control, with a view to predict the optimum loading rate, retention time and recipe required to achieve sanitisation.

Table 3.3: Optimum operational variables at different temperatures with EU FIB limit as primary constraint, with or without methane production as secondary constraint and temperature as third optimisation constraint. Table should be read from left to right.

Optimisation Constraints			**Parameter Estimates**			
EU FIB limit	**Maximise methane production**	**Temp control**	**Temp ^{o}C**	**OL g VS L^{-1}**	**Retention Time (Days)**	**Recipe**
✓	-	-	49.54	0.5	5.45	1S:3FW
✓	✓	-	45.89	3.5	9.00	2S:1FW
✓	✓	20	20	3.5	9.00	2S:1FW
✓	-	20	20	0.5	4.18	2S:1FW
✓	-	20	25	0.5	5.55	2S:1FW
✓	-	30	30	0.5	7.00	2S:1FW
✓	-	35	35	0.5	9.00	2S:1FW
✓	-	45	45	0.5	8.96	2S:1FW
✓	-	48	48	0.5	7.50	1S:3FW
✓	-	50	50	0.5	4.27	1S:3FW

The optimal conditions depend on the desired outcome. For example, if sanitisation is the primary aim, then the conditions displayed in the first row of Table 3.3 were found to be optimal, whereas the optimal conditions for achieving the FIB limit whilst maximising biogas output are displayed in the second row. Although temperature and retention time are critical factors in pathogen destruction, regardless of the temperature applied, if achieving the EU digestate standard is the only focus, then low organic loading (0.5 g VS L^{-1} day^{-1}) is optimal. As temperature increases

from 20°C, the time required for satisfactory sanitisation also increases to a long retention time at 35°C (Table 3.3). Whilst the retention time required to meet EU standards decreases above 45°C, the impact of the 2°C change from 48 to 50°C is notable, leading to a reduction in the corresponding required retention time from 7.5 days to 4.27 days, indicating an exponential relationship. Although these higher temperatures are conducive to faster digestate sanitisation, they also tend to correlate with reduced microbial diversity and consequent system instability (Kim et al., 2002; Labatut et al., 2014; Lee et al., 2017). Furthermore, the excessive energy required to maintain this higher temperature, limits the usefulness of thermophilic AD as a practical full-scale AD system, particularly in a temperate or cold climate.

The feedstock recipe with a higher slurry ratio (3S:1FW) was not optimal under any of the constraints applied, consistent with the reported relatively low methane potential of slurry (Bruni et al., 2010; Møller et al., 2004; Triolo et al., 2011; Weiland, 2010), and the high initial FIB numbers (Table 3.2). Above 48°C, the recipe which demonstrated most efficient digestate sanitisation is 1S:3FW, This recipe has a lower initial FIB load than the other recipes (Table 3.2), and at higher temperatures was not prone to the increase in enterococci numbers observed at ambient and mesophilic temperatures (Figure 3.2). For all temperature conditions below 48°C, the optimal recipe for efficient digestate sanitisation was 2S:1FW.

When maximisation of methane production was applied, in addition to digestate EU standards, feeding a higher rate and leaving it in the system for longer, were deemed optimal, particularly with the 2S:1FW recipe), with or without temperature control (Table 3.3). The parameter estimates indicate that ambient temperature is a feasible option for both methane production and efficient digestate sanitisation, and that a longer retention time, should be combined with a high loading rate to achieve both goals (Table 3.3). This is longer than the 40-day retention time previously deemed necessary for optimal methane production at ambient temperature in a continuous flow system (Sutter & Wellinger, 1988), but accomplishes the goal of efficient digestate sanitisation, which had not been previously taken into consideration. In light of the recent development toward direct biogas injection and the associated reduction in waste heat availability, ambient-temperature AD becomes an attractive alternative for farm-based applications, capable of producing relatively high biogas yield, particularly with longer retention times.

3.4 Conclusions

Digestate FIB numbers were observed to increase when using one specific feedstock recipe at 19 and 37°C, depending on the operating time. This highlights the importance of preliminary screening and modeling for proposed operational conditions, as well as the need for post-AD pasteurisation for some feedstock combinations. The model developed identified that a combination of low organic loading and longer retention time, digestate sanitisation sufficient to satisfy EU standards is possible in AD at temperatures of 20 or 25°C, whilst also maintaining satisfactory methane production. This may open up increased opportunity to upgrade biogas to biomethane rather than using it in relatively inefficient CHP units, as there would be reduced need for heat input to maintain temperature. Models, such as the one presented here, can be used to optimise operational conditions for methane and/or FIB removal prior to start-up or feedstock recipe changes.

3.5 Data Availability

Raw and cleaned data are available at https://github.com/nickp60/SN_minitrials.

3.6 References

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3.7 Supplementary Material

3.7.1 Supplementary Tables

A. Custom response surface design of experiment

Responses studied by the custom response surface analysis were *E. coli*, coliforms, enterococci and methane. Factors are presented in Table 3.A1 and Table 3.A2. Models incorporated full quadratic terms. The experiment design had no blocks and the total number of runs was 180.

Table 3.A.1. Continuous factors and their uncoded levels for the custom response surface design. Temp, OL and time stands for temperature, organic loading and retention time.

name	**low**	**high**
temp	19	55
OL	0.5	3.5
time	3	9

Table 3.A.2. Categorical factor and related uncoded levels for the custom response surface design. i) 1S:3FW (1 part S to 3 parts FW); ii) 2S:1FW (2 parts S to 1 part FW) and iii) 3S:1FW (3 parts S to 1 part FW)

name	**levels**
recipe	"1S:3FW" "2S:1FW" "3S:1FW"

B. Response Surface Regression: Coliforms versus Temp, OL, Time, Recipe

Table 3.B.1. Analysis of variance

Source	**DF**	**P-Value**
Model	17	0
Linear	5	0
Temp	1	0
OL	1	0
Time	1	0
Recipe	2	0
Square	3	0
Temp*Temp	1	0
OL*OL	1	0.904
Time*Time	1	0.352
2-Way Interaction	9	0.121
Temp*OL	1	0.07
Temp*Time	1	0.558
Temp*Recipe	2	0.038
OL*Time	1	0.065
OL*Recipe	2	0.872
Time*Recipe	2	0.874
Error	162	
Lack-of-Fit	21	0
Pure Error	141	
Total	179	

Table 3.B.2. Model summary

S	**R-sq**	**R-sq(adj)**	**R-sq(pred)**
0.827033	83.87%	82.17%	79.9%

Table 3.B.3. Coded coefficients

Term	**Coef**	**P-Value**
Constant	4.65	0
Temp	-2.0607	0
OL	0.4601	0
Time	-0.4842	0
Recipe		
2S:1FW	-0.4217	0
3S:1FW	0.0082	0.925
Temp*Temp	-1.424	0
OL*OL	0.015	0.904
Time*Time	-0.116	0.352
Temp*OL	-0.217	0.07
Temp*Time	-0.07	0.558
Temp*Recipe		
2S:1FW	0.291	0.016
3S:1FW	-0.056	0.638
OL*Time	-0.222	0.065
OL*Recipe		
2S:1FW	-0.061	0.608
3S:1FW	0.021	0.862
Time*Recipe		
2S:1FW	0.061	0.607
3S:1FW	-0.023	0.846

Table 3.B.4. Regression equation for each feedstock recipe in uncoded units

Recipe	Equation
2S:1FW	Coliforms = 0.25 + 0.2509 Temp + 0.833 OL + 0.160 Time - 0.004396 Temp*Temp + 0.0067 OL*OL - 0.0129 Time*Time - 0.00805 Temp*OL - 0.00130 Temp*Time - 0.0494 OL*Time
3S:1FW	Coliforms = 1.45 + 0.2316 Temp + 0.888 OL + 0.132 Time - 0.004396 Temp*Temp + 0.0067 OL*OL - 0.0129 Time*Time - 0.00805 Temp*OL - 0.00130 Temp*Time - 0.0494 OL*Time
1S:3FW	Coliforms = 2.23 + 0.2217 Temp + 0.901 OL + 0.127 Time - 0.004396 Temp*Temp + 0.0067 OL*OL - 0.0129 Time*Time - 0.00805 Temp*OL - 0.00130 Temp*Time - 0.0494 OL*Time

C. Response Surface Regression: *E. coli* versus Temp, OL, Time, Recipe

Table 3.C.1. Analysis of variance

Source	**DF**	**P-Value**
Model	17	0
Linear	5	0
Temp	1	0
OL	1	0
Time	1	0
Recipe	2	0
Square	3	0
Temp*Temp	1	0
OL*OL	1	0.861
Time*Time	1	0.333
2-Way-Interaction	9	0.037
Temp*OL	1	0.002
Temp*Time	1	0.918
Temp*Recipe	2	0.087
OL*Time	1	0.131
OL*Recipe	2	0.752
Time*Recipe	2	0.63
Error	162	
Lack-of-Fit	21	0
Pure-Error	141	
Total	179	

Table 3.C.2. Model summary

S	**R-sq**	**R-sq(adj)**	**R-sq(pred)**
0.798127	84.0%	82.3%	80.2%

Table 3.C.3. Coded coefficients

Term	**Coef**	**P-Value**
Constant	4.574	0
Temp	-1.918	0
OL	0.4816	0
Time	-0.5467	0
Recipe		
2S:1FW	-0.3619	0
3S:1FW	-0.0432	0.609
Temp*Temp	-1.556	0
OL*OL	-0.021	0.861
Time*Time	-0.117	0.333
Temp*OL	-0.359	0.002
Temp*Time	-0.012	0.918
Temp*Recipe		
2S:1FW	0.248	0.033
3S:1FW	-0.068	0.556
OL*Time	-0.175	0.131
OL*Recipe		
2S:1FW	-0.058	0.618
3S:1FW	-0.028	0.811
Time*Recipe		
2S:1FW	0.111	0.337
3S:1FW	-0.058	0.615

Table 3.C.4. Regression equation for each feedstock recipe in uncoded units

Recipe	Equation
2S:1FW	Ecoli = -0.63 + 0.2904 Temp + 1.045 OL + 0.096 Time - 0.004801 Temp*Temp - 0.0094 OL*OL - 0.0129 Time*Time - 0.01330 Temp*OL - 0.00022 Temp*Time - 0.0388 OL*Time
3S:1FW	Ecoli = 0.64 + 0.2729 Temp + 1.065 OL + 0.040 Time - 0.004801 Temp*Temp - 0.0094 OL*OL - 0.0129 Time*Time - 0.01330 Temp*OL - 0.00022 Temp*Time - 0.0388 OL*Time
1S:3FW	Ecoli = 1.16 + 0.2666 Temp + 1.140 OL + 0.041 Time - 0.004801 Temp*Temp - 0.0094 OL*OL - 0.0129 Time*Time - 0.01330 Temp*OL - 0.00022 Temp*Time - 0.0388 OL*Time

D. Response Surface Regression: Enterococci versus Temp, OL, Time, Recipe

Table 3.D.1. Analysis of variance

Source	DF	P-Value
Model	17	0
Linear	5	0
Temp	1	0
OL	1	0
Time	1	0.695
Recipe	2	0
Square	3	0
Temp*Temp	1	0
OL*OL	1	0
Time*Time	1	0.994
2-Way-Interaction	9	0.011
Temp*OL	1	0.716
Temp*Time	1	0.004
Temp*Recipe	2	0.019
OL*Time	1	0.41
OL*Recipe	2	0.203
Time*Recipe	2	0.455
Error	162	
Lack-of-Fit	21	0
Pure-Error	141	
Total	179	

Table 3.D.2. Model summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.635807	86.8%	85.4%	83.5%

Table 3.D.3. Coded coefficients

Term	**Coef**	**P-Value**
Constant	5.676	0
Temp	-1.8134	0
OL	0.296	0
Time	-0.0255	0.695
Recipe		
2S:1FW	-0.3993	0
3S:1FW	0.2194	0.001
Temp*Temp	-1.3168	0
OL*OL	-0.4455	0
Time*Time	0.0007	0.994
Temp*OL	0.0335	0.716
Temp*Time	-0.2702	0.004
Temp*Recipe		
2S:1FW	0.2363	0.011
3S:1FW	-0.0225	0.806
OL*Time	0.0758	0.41
OL*Recipe		
2S:1FW	-0.1439	0.119
3S:1FW	0.1414	0.125
Time*Recipe		
2S:1FW	-0.1039	0.259
3S:1FW	0.0953	0.3

Table 3.D.4. Regression equation for each feedstock recipe in uncoded units

Recipe	**Equation**
2S:1FW	Enterococci = 1.405 + 0.2407 Temp + 0.747 OL + 0.107 Time - 0.004064 Temp*Temp - 0.1980 OL*OL + 0.0001 Time*Time + 0.00124 Temp*OL - 0.00500 Temp*Time + 0.0168 OL*Time
3S:1FW	Enterococci = 1.776 + 0.2263 Temp + 0.937 OL + 0.174 Time - 0.004064 Temp*Temp - 0.1980 OL*OL + 0.0001 Time*Time + 0.00124 Temp*OL - 0.00500 Temp*Time + 0.0168 OL*Time
1S:3FW	Enterococci = 2.489 + 0.2157 Temp + 0.844 OL + 0.145 Time - 0.004064 Temp*Temp - 0.1980 OL*OL + 0.0001 Time*Time + 0.00124 Temp*OL - 0.00500 Temp*Time + 0.0168 OL*Time

E. Response Surface Regression: Methane versus Temp, OL, Time, Recipe

Table 3.E.1. Analysis of variance

Source	DF	P-Value
Model	17	0
Linear	5	0
Temp	1	0.1
OL	1	0
Time	1	0
Recipe	2	0.099
Square	3	0
Temp*Temp	1	0
OL*OL	1	0.026
Time*Time	1	0.038
2-Way-Interaction	9	0
Temp*OL	1	0.894
Temp*Time	1	0.639
Temp*Recipe	2	0.771
OL*Time	1	0
OL*Recipe	2	0.07
Time*Recipe	2	0.212
Error	162	
Lack-of-Fit	21	0
Pure-Error	141	
Total	179	

Table 3.E.2. Model summary

S	R-sq	R-sq(adj)	R-sq(pred)
16.8947	87.8%	86.6%	84.7%

Table 3.E.3. Coded coefficients

Term	**Coef**	**P-Value**
Constant	92.38	0
Temp	-2.85	0.1
OL	16.19	0
Time	23.06	0
Recipe		
2S:1FW	-2.46	0.17
3S:1FW	-1.35	0.45
Temp*Temp	-74.04	0
OL*OL	-5.72	0.026
Time*Time	-5.3	0.038
Temp*OL	-0.32	0.894
Temp*Time	-1.15	0.639
Temp*Recipe		
2S:1FW	1.75	0.473
3S:1FW	-0.99	0.685
OL*Time	14.58	0
OL*Recipe		
2S:1FW	-5.67	0.021
3S:1FW	2.98	0.224
Time*Recipe		
2S:1FW	4.23	0.085
3S:1FW	-2.86	0.243

Table 3.E.4. Regression equation for each feedstock recipe in uncoded units

Recipe	Equation
2S:1FW	Methane = -287.3 + 17.000 Temp - 1.82 OL + 10.47 Time - 0.22851 Temp*Temp - 2.54 OL*OL - 0.589 Time*Time - 0.0120 Temp*OL - 0.0212 Temp*Time + 3.240 OL*Time
3S:1FW	Methane = -277.9 + 16.847 Temp + 3.95 OL + 8.11 Time - 0.22851 Temp*Temp - 2.54 OL*OL - 0.589 Time*Time - 0.0120 Temp*OL - 0.0212 Temp*Time + 3.240 OL*Time
1S:3FW	Methane = -275.8 + 16.860 Temp + 3.76 OL + 8.60 Time - 0.22851 Temp*Temp - 2.54 OL*OL - 0.589 Time*Time - 0.0120 Temp*OL - 0.0212 Temp*Time + 3.240 OL*Time

F. Physicochemical data for trials, feedstock recipes and inoculum

Table 3.F1 Physicochemical data for all conditions at all data-points. Error bars indicate standard error ($n \geq 4$).

Temp	Day	OL	Recipe	NH_3 mg/L	pH	sCOD g/L	TS %	VS %	mL CH_4
19°C	3	2	1	1788±76	6.99±0.01	11.93±0.44	5.75±0.06	3.59±0.04	9.55±0.33
			2	1695±12	7.52±0.05	10.50±0.07	6.08±0.07	3.97±0.04	2.20±0.21
			3	1779±208	7.06±0.00	12.59±0.44	5.76±0.07	3.57±0.03	8.75±0.69
	6	0.5	1	1546±48	7.43±0.02	10.36±0.13	5.47±0.10	3.37±0.03	11.50±2.02
			2	1503±32	7.54±0.03	9.63±0.65	5.70±0.02	3.48±0.00	9.08±0.57
			3	1384±23	7.49±0.00	9.71±0.36	5.62±0.04	3.36±0.02	11.68±0.59
		3.5	1	1533±31	6.86±0.01	9.36±1.58	6.01±0.02	3.81±0.02	32.08±1.23
			2	1410±124	7.23±0.01	9.91±0.39	6.58±0.05	4.44±0.03	7.80±0.39
			3	1319±22	6.91±0.01	13.08±0.06	5.99±0.02	3.83±0.01	23.50±0.84
	9	2	1	1629±57	7.07±0.01	11.11±0.22	5.70±0.02	3.53±0.02	27.93±1.93
			2	1556±122	7.30±0.02	10.38±0.27	5.99±0.08	3.89±0.04	16.15±0.60
			3	1615±55	7.17±0.01	10.99±0.36	5.68±0.02	3.52±0.02	28.10±0.94
37°C	3	0.5	1	1530±128	7.40±0.00	8.91±0.10	5.55±0.05	3.42±0.02	32.53±1.74
			2	1355±245	7.50±0.01	8.48±0.47	5.49±0.21	3.48±0.09	30.90±2.53
			3	1668±186	7.47±0.02	10.51±0.25	5.46±0.20	3.41±0.09	25.88±3.63
		3.5	1	1586±52	6.98±0.02	12.08±0.60	5.98±0.03	3.90±0.03	65.23±1.73
			2	1660±49	7.12±0.01	9.84±0.21	6.37±0.08	4.41±0.08	34.68±5.46
			3	1660±64	7.05±0.01	8.51±0.49	5.88±0.07	4.04±0.18	67.05±1.15
	6	2	1	1498±21	7.42±0.01	8.27±0.08	5.57±0.02	3.42±0.02	97.89±1.84
			2	1446±33	7.45±0.01	7.33±0.18	5.81±0.03	3.67±0.05	94.61±2.07
			3	1442±18	7.45±0.01	8.27±0.16	5.53±0.04	3.43±0.03	84.64±5.03
	9	0.5	1	1479±163	7.51±0.05	7.64±0.19	5.18±0.08	3.19±0.04	76.45±3.83
			2	1523±63	7.46±0.00	7.70±0.12	5.27±0.06	3.25±0.02	89.00±3.41
			3	1496±78	7.48±0.02	7.74±0.18	5.16±0.04	3.22±0.01	68.33±2.67
		3.5	1	1423±90	7.44±0.02	8.21±0.32	5.37±0.05	3.46±0.02	161.35±5.41
			2	1606±94	7.29±0.01	6.94±0.16	5.62±0.05	3.85±0.05	168.43±7.21
			3	1549±34	7.38±0.02	8.00±0.08	5.15±0.12	3.35±0.05	156.60±4.33
55°C	3	2	1	1804±56	7.16±0.01	13.62±0.43	5.88±0.01	3.61±0.01	6.43±2.45
			2	1805±87	7.86±0.03	9.90±0.16	6.18±0.07	3.99±0.07	1.43±0.39
			3	1620±76	7.21±0.01	11.31±0.19	6.06±0.03	3.77±0.02	5.13±1.14
	6	0.5	1	1809±124	7.47±0.01	11.02±0.28	5.58±0.04	3.43±0.11	5.63±0.27
			2	1648±1	7.79±0.09	10.42±0.24	5.54±0.07	3.32±0.04	4.40±0.43
			3	1749±189	7.73±0.01	9.82±0.23	5.54±0.02	3.27±0.02	4.33±0.29
		3.5	1	1809±62	7.18±0.02	8.51±0.11	6.13±0.07	3.85±0.11	18.55±2.40
			2	1726±58	7.50±0.02	9.36±0.17	7.39±0.02	4.83±0.02	6.63±1.01
			3	1685±63	7.23±0.01	13.81±0.14	6.06±0.02	3.73±0.01	16.40±3.46
	9	2	1	1668±96	7.44±0.01	12.60±0.18	5.68±0.02	3.43±0.01	21.50±2.27
			2	1637±109	7.40±0.00	13.18±0.14	5.95±0.10	3.77±0.06	13.98±1.21
			3	1583±84	7.47±0.01	12.19±0.30	5.76±0.09	3.51±0.05	15.43±1.14

Table 3.F2 Physicochemical data for all feedstock recipes and inoculum. Error bars indicate standard error (n≥4).

Recipe	NH_3 mg L^{-1}	pH	sCOD g L^{-1}
1S:3FW	370	4.9	145 ± 18
2S:1FW	1900	6.28	28 ± 0.7
3S:1FW	1200	6.72	20 ± 1.2
Inoculum	2000	7.97	11 ± 0.2

Table 3.G.1. Absolute log numbers recorded during trial by temperature and feedstock mixture for coliforms, *E. coli* and enterococci

	Coliforms	FS 1	FS 2	FS 3
19°C	OLR 2*HRT 3	1.35E+05	3.26E+04	4.49E+05
	OLR 0.5*HRT 6	7.77E+05	1.41E+04	6.85E+04
	OLR 3.5*HRT 6	1.24E+06	2.11E+05	2.18E+06
	OLR 2*HRT 9	1.89E+06	3.23E+04	2.00E+05
37°C	OLR 0.5*HRT 3	4.14E+05	1.25E+04	2.82E+05
	OLR 3.5*HRT 3	1.60E+06	8.01E+05	7.50E+05
	OLR 2*HRT 6	2.22E+05	1.19E+05	3.87E+04
	OLR 0.5*HRT 9	4.19E+04	3.35E+04	4.44E+03
	OLR 3.5*HRT 9	7.83E+04	2.69E+03	1.89E+04
55°C	OLR 2*HRT 3	7.75E+01	0.00E+00	0.00E+00
	OLR 0.5*HRT 6	0.00E+00	0.00E+00	0.00E+00
	OLR 3.5*HRT 6	2.50E+01	0.00E+00	5.00E+01
	OLR 2*HRT 9	0.00E+00	0.00E+00	2.50E+01
	E. coli	FS 1	FS 2	FS 3
19°C	OLR 2*HRT 3	1.17E+05	1.93E+04	1.50E+05
	OLR 0.5*HRT 6	1.52E+05	9.60E+03	3.75E+04
	OLR 3.5*HRT 6	7.76E+05	1.20E+05	1.64E+06
	OLR 2*HRT 9	3.87E+05	2.55E+04	2.65E+04
37°C	OLR 0.5*HRT 3	3.99E+05	1.09E+04	2.59E+05
	OLR 3.5*HRT 3	1.49E+06	7.20E+05	7.45E+05
	OLR 2*HRT 6	2.02E+05	1.16E+05	3.22E+04
	OLR 0.5*HRT 9	9.98E+03	1.27E+04	3.74E+03
	OLR 3.5*HRT 9	7.95E+04	1.97E+03	1.74E+04
55°C	OLR 2*HRT 3	7.75E+01	0.00E+00	0.00E+00
	OLR 0.5*HRT 6	0.00E+00	0.00E+00	0.00E+00
	OLR 3.5*HRT 6	0.00E+00	0.00E+00	0.00E+00
	OLR 2*HRT 9	0.00E+00	0.00E+00	0.00E+00
	Enterococci	FS 1	FS 2	FS 3
19°C	OLR 2*HRT 3	2.31E+06	1.58E+05	2.61E+06
	OLR 0.5*HRT 6	1.33E+06	4.77E+05	7.83E+05
	OLR 3.5*HRT 6	1.92E+06	4.89E+05	3.16E+06
	OLR 2*HRT 9	6.57E+06	3.59E+05	1.08E+06
37°C	OLR 0.5*HRT 3	1.95E+05	9.26E+04	1.35E+05
	OLR 3.5*HRT 3	1.88E+05	1.33E+05	1.84E+05
	OLR 2*HRT 6	3.18E+05	1.50E+05	1.63E+06
	OLR 0.5*HRT 9	3.69E+05	7.07E+04	7.79E+05
	OLR 3.5*HRT 9	4.09E+06	7.93E+04	1.48E+06
55°C	OLR 2*HRT 3	2.80E+03	5.78E+02	4.40E+03
	OLR 0.5*HRT 6	2.50E+01	5.00E+01	1.25E+02
	OLR 3.5*HRT 6	1.75E+02	3.00E+02	2.50E+02
	OLR 2*HRT 9	2.58E+02	2.28E+02	3.40E+02

3.7.2 Supplementary Figures

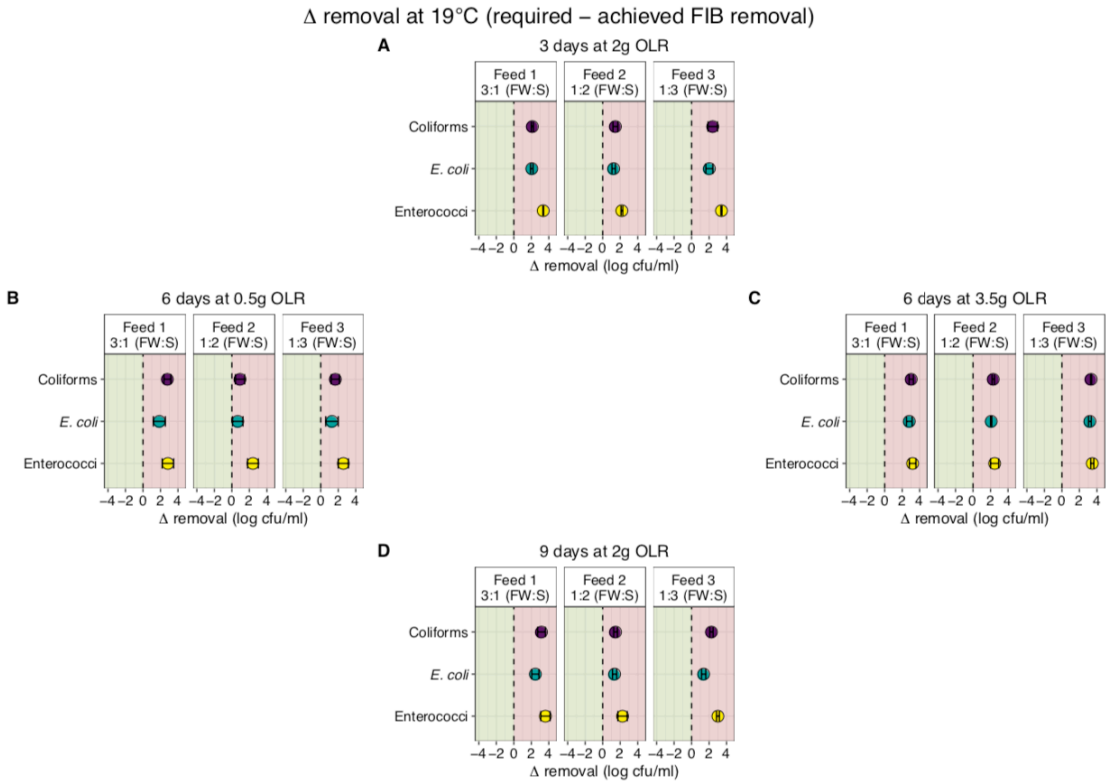

Figure 3.S1: FIB removal by feedstock recipe at 19°C on days 3, 6 and 9, where Δ removal is calculated by subtracting the log cfu of achieved FIB removal from the log cfu of removal required for each feedstock to achieve EU digestate standards (1,000 cfu g^{-1}). The green and red shading indicates sufficient and insufficient FIB removal respectively (where sufficient removal ≤ 0 Δ). Different operating conditions are separated by panels where A = 3 days RT at 2 g OL; B = 6 days RT at 0.5 g OL; C = 6 days RT at 3.5 g OL; D = 9 days RT at 2 g OL. Error bars indicate standard deviation (n ≥ 4).

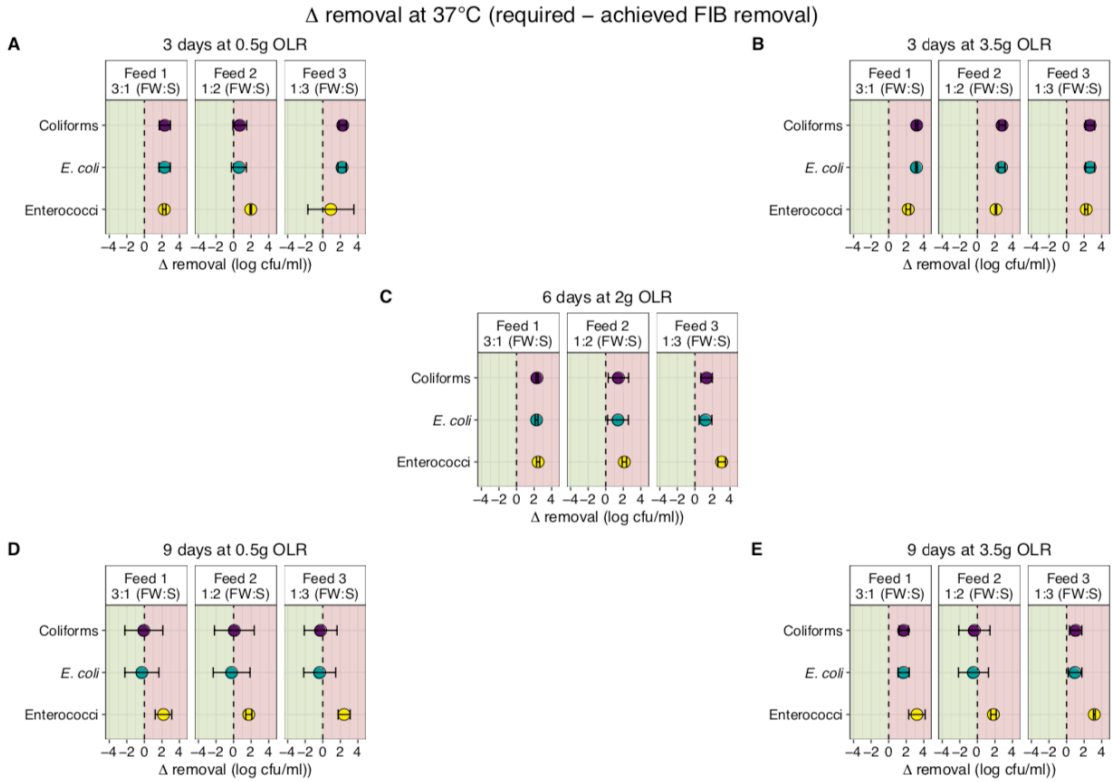

Figure 3.S2: FIB removal by feedstock recipe at 37°C on days 3, 6 and 9, where Δ removal is calculated by subtracting the log cfu of achieved FIB removal from the log cfu of removal required for each feedstock to achieve EU digestate standards (1,000 cfu g^{-1}). The green and red shading indicates sufficient and insufficient FIB removal respectively (where sufficient removal ≤ 0 Δ). Different operating conditions are separated by panels where A = 3 days RT at 2 g OL; B = 6 days RT at 0.5 g OL; C = 6 days RT at 3.5 g OL; D = 9 days RT at 2 g OL. Error bars indicate standard deviation ($n \geq 4$).

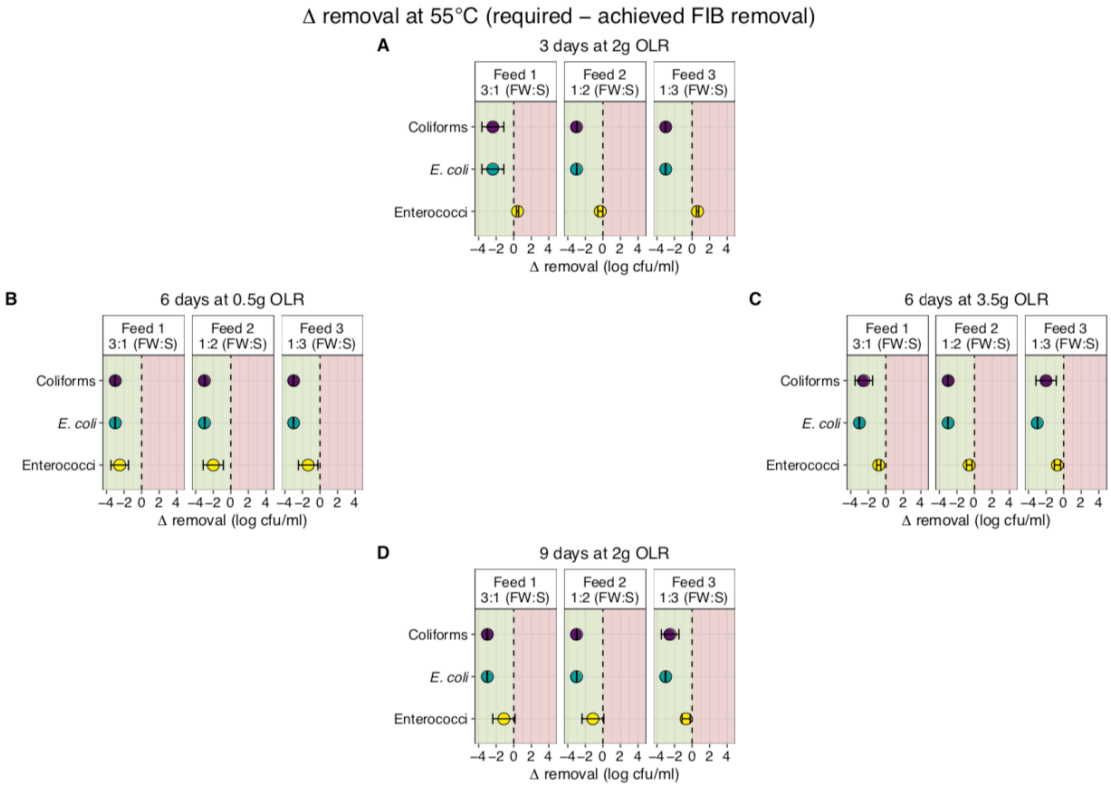

Figure 3.S3: FIB removal by feedstock recipe at 55°C on days 3, 6 and 9, where Δ removal is calculated by subtracting the log cfu of achieved FIB removal from the log cfu of removal required for each feedstock to achieve EU digestate standards (1,000 cfu g^{-1}). The green and red shading indicates sufficient and insufficient FIB removal respectively (where sufficient removal ≤ 0 Δ). Different operating conditions are separated by panels where A = 3 days RT at 2 g OL; B = 6 days RT at 0.5 g OL; C = 6 days RT at 3.5 g OL; D = 9 days RT at 2 g OL. Error bars indicate standard deviation (n ≥ 4).

Chapter 4

Anaerobic digestion process optimisation for biogas output and sanitisation

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I wrote this chapter with the help of my supervisors, having carried out the laboratory work and process data analysis. The metagenomic data analysis and visualisation was undertaken with Nicholas Waters. A version of this chapter is being prepared for submission to *Applied Energy*.

Abstract

Anaerobic digestion (AD) of slurry with food production waste has been demonstrated to reduce pathogen load and greenhouse gas emissions while providing a useful renewable energy source. While the resulting by-product, digestate, can be a valuable organic fertiliser, regulatory requirements for its pasteurisation may be prohibitive in terms of capital cost and energy consumption. Efforts toward optimisation of AD to date have focused on biogas yield, yet improvements in pathogen load reduction may potentially negate the need for a costly pasteurisation step. Hence, optimisation of AD for sanitisation as well as improved biogas output is desirable. To this end, triplicate mesophilic 10 L CSTR bioreactors were fed with slurry and FOG for 216 days, initially, five days per week, with a two-day break over weekends (FR1). Indicator die-off during this period did not meet EU digestate standard requirements of less than 1,000 CFU g^{-1}. However a decline in coliform and *E. coli* numbers was observed over the weekend, with a subsequent increase in numbers during weekdays. Based on these results, a change to three-day interval feeding was implemented (FR2), with a view to improving FIB removal. All other conditions were maintained at this stage so that the two feeding regimes could be compared directly.

Changing the feeding regime to FR2 resulted in an initial build up of COD with sCOD in the digestate increasing to an average of 13 ± 0.4 g L^{-1} between Day 135 and 150 and tCOD in the digestate averaging 81 ± 2.4 g L^{-1} during the same period. Beyond Day 150 methane production in all bioreactors increased significantly as the excess COD that built up during the perturbation period was rapidly consumed, generating a peak of 690 ± 35 mL CH_4 g VS^{-1} fed between Day 166 and 168. When the excess COD was consumed, a stable period followed from Day 180 onwards, where an average of 430 ± 7.4 mL CH_4 g VS^{-1} fed was sustained (i.e. FR2 had 58 % higher methane yield per gram VS fed than FR1). As no operational parameters (OLR, temperature, RT, mixing rate etc.) were altered apart from the feeding regime, the COD and CH_4 phenomena recorded during the transition and stable period of FR2 may be indicative of improved microbial efficiencies. This necessitated a closer examination of community dynamics during the three main phases, namely FR1, FR2 transition phase and FR2 stable phase.

Furthermore, after FR2 transition phase the required reduction in coliform and *E. coli* numbers to satisfy EU sanitisation standards was consistently achieved. Whether the reduction in *E. coli* numbers was due to competition, inhibition for example from fungal toxins, or predation by bacteriophage or protozoan grazing is not clear. Hence, DNA was extracted from samples from the three phases for shotgun metagenomics. MG-RAST was used to carry out phylogenetic and functional analysis of the metagenomes. The results of this work indicate the possibility for manipulation of AD for improved sanitisation and biogas production and highlight the need to better understand the important role of microbial dynamics in AD systems.

Abbreviations

AD - Anaerobic digestion

EU - European Union

DCS - Dairy cattle slurry

FIB - Faecal indicator bacteria

FOG - Fats, oils and grease

FW - Food processing waste

4.1 Introduction

Animal manures represent a valuable source of fertilisers at costs far reduced from those associated with synthetic fertilisers. However, land application of manures is associated with GHG emissions, as well as significant pathogen risk to the environment and the animals themselves. Agriculture-based anaerobic digestion (AD) offers an opportunity to reduce manure-associated pathogen load to the environment (Nag et al., 2019; Nolan et al., 2018; Sahlström, 2003). However, due to its low methane yield, it is often co-digested with other organic wastes including food wastes. While these bolster methane production, they can include animal by-products (ABP) and are consequently deemed to carry an increased disease transmission risk to humans and animals. ABP waste is categorised into three groups, with Category 1 assigned to higher risk, Category 2 medium risk and Category 3 considered lower risk. ABPs are strictly regulated within the EU, and when utilised in AD, typically necessitate a pasteurisation step pre- or post-AD, either to EU Standard or validated national equivalent (DAFM, 2014; EC, 2009).

Regular faecal indicator bacteria (FIB; *E. coli* or *Enterococci*) testing is required to ensure satisfactory reduction below 1,000 colony forming units (cfu), in four of five samples, and up to 5,000 cfu in one of five samples (DAFM, 2014; EC, 2009). Within certain specific intake limits a pasteurisation step is not mandated, such as non-ABP (e.g. greasetrap waste); processed animal protein; ABP generated on-site; up to 5,000 tonnes per annum of imported Category 2 manure, digestive tract, milk or milk based products; processed Category 3 material; Category 3 milk and milk based products (DAFM, 2014). Yet, despite the typical reduction in pathogen load observed in agriculture-based AD, survival of FIB above the prescribed limits has frequently been reported in the absence of a pasteurisation step.

Several factors contribute to the survival or satisfactory removal of FIB, and theoretically by extension, pathogenic agents. Foremost of these factors is temperature, with thermophilic AD consistently demonstrating superior FIB removal compared with mesophilic or ambient temperature AD (Avery et al., 2014; De Leon and Jenkins, 2002; Olsen and Larsen, 1987; Jiang et al., 2020; Moset et al., 2015; Nag et al., 2019; Scaglia et al., 2014; Smith et al., 2005). Of the eight

currently operational agriculture-based AD plants in Ireland, none are thermophilic, primarily due to operational difficulties associated with instability in the limited thermophilic microbial community (Auer et al., 2017; Hejnfelt and Angelidaki, 2009). Retention time has an effect on FIB survival (Kearney et al., 1993; Nag et al., 2019; Nolan et al., 2018; Olsen and Larsen, 1987; Sahlström, 2003), but beyond twenty days this effect is minimal (Dennehy et al., 2018; Smith et al., 2005), and most agriculture-based AD plants operate with retention times in excess of fifty days. Organic loading rate (OLR) is a factor in pathogen survival given that theoretically higher concentrations of pathogenic material may be added under a higher OLR (Strauch, 1991), while conversely, higher OLR potentially increase concentrations of volatile fatty acids and free ammonia, both of which are associated with pathogen reduction (Dennehy et al., 2016b; Jiang et al., 2018; Orzi et al., 2015b), particularly at acidic pH (Kunte et al., 1998; Sahlström, 2003). Other factors affecting pathogen survival include mixing efficacy and bypass flow (Smith et al., 2005), as well as microbial competition in limited resource conditions (Kearney et al., 1994; Orzi et al., 2015b; Ward et al., 1999).

The effect of feeding regime on sanitisation is not typically considered in the literature. Sahlström (2003) identifies ‘economic and practical reasons’ as the justification for unwavering adherence to a continuous feeding (typically once per hour, at least once per day) in full-scale systems. This approach may have developed with the proliferation of AD systems, primarily in Germany, feeding energy crops such as maize silage or grain, whereby there is minimal feedstock variance, consistent biogas output and no consideration necessary for the control of pathogens. Therefore, feed inputs are determined based on standard reported biomethane potentials to target biogas production matching combined heat and power (CHP) fuel demand. Operators are typically generalists, requiring mechanical, electrical, plumbing and clerical skills, as well as contributing to farm operations. These AD plants are typically controlled via supervisory control and data acquisition (SCADA) systems, which add a layer of dissociation between operator and system. Minimal understanding of microbial community dynamics is required to operate an AD system under such conditions, and given the consistent nature of the inputs and outputs this ‘black box’ approach suffices, albeit with limited potential for optimisation.

Introduction of organic waste to the system complicates operation by adding layers of administrative control and inconsistencies to the feedstock, which if not monitored or controlled could cause under or over-feeding with potentially detrimental effects, such as VFA or ammonia-induced inhibition (Cuetos et al., 2010; Hejnfelt and Angelidaki, 2009; Nielsen and Angelidaki, 2008). One such organic waste is greasetrap waste, commonly available because of requirements for food processing facilities and restaurants to prevent escape of fats, oils and grease (FOG) to wastewater systems (Long et al., 2012). Greasetrap waste can vary considerably, typically containing 0-15% FOG, and high concentrations of long chain fatty acids, which may be inhibitory to AD (Hwu and Lettinga, 1997; Rinzema et al., 1994). Even so, the recommended feeding regime has been continuous feeding (Angelidaki and Ahring, 1992; Cavaleiro et al., 2009), although intermittent feeding was suggested, at least in upflow anaerobic sludge blanket (UASB) reactors (Coelho et al., 2007).

As agriculture-based AD technology has matured, the desire to optimise the system and maximise output has led to some developments in approach to feeding regime (Willeghems and Buysse, 2016). Simultaneously, the requirement for flexibility and demand-driven output in renewable energy production has risen, in line with increased utilisation of fluctuating sources such as wind and solar energy (Bonk et al., 2018; Hahn et al., 2014; Mulat et al., 2016; Szarka et al., 2013). In particular some scope has emerged for improving functional stability in AD systems generally (Bonk et al., 2018; De Vrieze et al., 2013), and more specifically, biogas output from AD of fats, oils and grease (Ziels et al., 2018), via ‘pulse’ (i.e. semi-continuous) feeding regime, in place of continuous daily feeding. Perhaps understandably, the effect of this feeding regime manipulation on microbial community dynamics have focused primarily on biogas output with little, if any, consideration for the effect on digestate sanitisation.

Objectives

The operational parameters used in Irish AD systems may not reduce pathogen load in digestate sufficiently, necessitating a costly mitigation step, such as pasteurisation. All Irish AD systems currently feed continuously (at least once per day), and most co-digested slurry with (amongst other organic wastes) some form of FOG. Published work has demonstrated the possibility of manipulating feeding regime to improve AD of FOG, but the effect on biogas output from co-digestion of slurry with FOG is unclear. Furthermore, the potential for improved sanitisation via feeding manipulation is not known. Hence, the objectives of this work were to determine the impact of feeding regime adjustment on digestate sanitisation and methane production in AD of slurry co-digested with FOG, and to utilise metagenomics to develop a better understanding of the effect on community dynamics.

The main hypotheses of this chapter are as follows:

1. AD operational parameters can be optimised to improve sanitisation in systems outside the scope of pasteurisation legislation.
2. Biogas output from AD of slurry co-digested with FOG can be optimised by altering feeding regime.
3. Microbial dynamics underpin the anticipated sanitisation and biogas yield improvements

4.2 Materials and methods

4.2.1 Replicated AD system

Based on characterisation of Irish on-farm AD plants, triplicate 10 L laboratory-scale continuously stirred tank reactors (CSTRs) were operated at 37 ^{o}C and fed slurry co-digested with fats, oils and grease. The digestate produced was monitored throughout using enterococci and coliforms as pathogen indicators. Triplicate 10 L CSTR bioreactors were fed with slurry and FOG for 216 days, initially, five days per week, with a two-day break over weekends (FR1). Indicator die-off during this period did not meet EU digestate standard requirements of less than 1,000 CFU g^{-1}. However a decline in coliform and *E. coli* numbers was observed over the weekend, with a subsequent increase in numbers during weekdays. Based on these results, a change to three-day interval feeding was implemented (FR2), with a view to improving FIB removal. All other conditions, including OLR, HRT, temperature, and mixing were maintained at this stage so that the two feeding regimes could be compared directly.

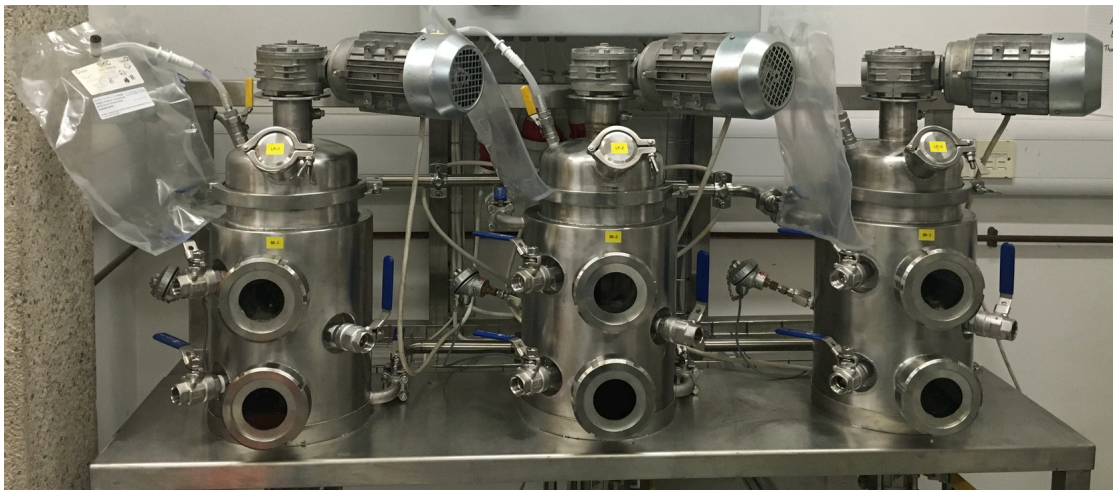


Figure 4.1 – Triplicate 10 L continuously stirred tank reactors (CSTRs)

4.2.2 Feedstock

The FOG was sourced from an AD plant in Co. Kilkenny, Ireland, collected in a 25 L drum, stored at 4°C and mixed thoroughly before use. Dairy cattle slurry (DCS) for the trial was collected from a dairy farm in County Galway, Ireland. The underground slatted storage tanks were mechanically agitated to homogenise the slurry before collection using a bucket attached to a pole, in accordance with

Brennan *et al*. (2011) and Peyton *et al*. (2016). Slurry was stored at 4°C prior to use as feedstock and mixed thoroughly before use. To prepare feedstock, DCS was mixed with FOG at a 2:1 DCS:FOG ratio, based on the mixing ratio used in practice at the source farm-based AD plant. This bioreactor trial ran for 240 days in total. After 30 days lower FIB numbers were observed in slurry stored at 4°C. Upon determination that storage for a period of time longer than two weeks affected FIB numbers, slurry was collected weekly. The mixed feedstock was tested at each time point for total and volatile solids, pH, total and soluble COD, NH_3 (Table 4.1) and FIB (Figure 4.3) before being fed through the feeding port on top of each bioreactor.

Table 4.1 Average physicochemical data of mixed feedstock throughout trial

TS %	VS %	tCOD g/L	sCOD g/L	NH_3 Mg/L
10.02±0.06	7.78±0.04	165.78±8.38	20.40±0.56	1013.2±65.7

4.2.3 Feeding regime

An organic loading rate (OLR) of 2 g VS L^{-1} d^{-1} was used to establish the trial, with a target retention time of 28 days. Volatile solids of the input feedstock were determined regularly to account for any fluctuations in solids content between DCS collections. Initially (first 99 days) bioreactors were fed and sampled each weekday, with 3x feedstock each Friday to maintain the 2 g VS L^{-1} d^{-1} OLR over the weekend. After 90 days of operation the feeding regime was changed to feeding every three days, whilst maintaining the 2 g VS L^{-1} d^{-1} OLR. Hence, in a 21-day cycle, bioreactors received an average of 42 g VS L^{-1} (8 L working volume), regardless of feeding regime employed. The three-day semi-continuous feeding regime was maintained until the end of the trial.

4.3.4 Physicochemical analysis

Biogas produced by the 10 L bioreactors was collected in 25 L Tedlar SCV gas bags and volume was determined at each sampling point using water displacement. Methane content of the biogas was analysed using a Varian 450 gas chromatograph (GC) equipped with a flame ionisation detector. The carrier gas was nitrogen and the flow rate was 25 mL min^{-1}. Analysis of total and volatile solids (TS/VS) was

performed gravimetrically according to standard methods (APHA, 2005) and total and soluble chemical oxygen demand (tCOD/sCOD) analysis was performed according to the Standing Committee of Analysts (1985). NH_3 concentrations (mg L^{-1}) were determined using the HACH AmVer High-Range Ammonia test, following the manufacturer's instructions.

4.2.5 Faecal indicator bacteria (FIB)

As the Irish Department of Agriculture, Food and the Marine require *E. coli* numbers of less than 1000 cfu per gram in digestate samples to be considered safe for landspreading, this work focused primarily on *E. coli* survival. To gain a more comprehensive understanding of FIB dynamics however, numbers of three FIB were assessed throughout. Faecal coliform and *Escherichia coli* most probable numbers (MPN) were determined using IDEXX Colisure with Quanti-Tray/2000 incubated at 35 °C for 24 hours. MPN of enterococci were quantified using IDEXX Enterolert kit with Quanti-Tray/2000 incubated at 41 °C for 24 hours. Initial FIB numbers in the slurry, prior to AD, and associated TS and VS are presented in Table 4.1. FOG was tested regularly but was not found to have FIB above the limit of detection (100 cfu g^{-1}).

4.2.6 DNA extraction

At each time point, triplicate 2 mL aliquots of sample from each bioreactor were flash frozen in liquid nitrogen and stored at -80°C for further analysis. Upon completion of the trial and determination of significant trends in coliform and *E. coli* removal, eight time points were selected for metagenomic analysis. The time points chosen are denoted by red circles in Figure 4.3, constituting eight time points from the triplicate bioreactors (24 samples total), representative of the two feeding regimes (FR1 and FR2) and the interim perturbation period. During FR1 *E. coli* numbers tended to rise during daily feeding to a high point towards the end of the working week, with spikes in removal observed over the two-day break, hence for FR1 the four sampling points reflected before and after weekend feeding (Thursday and Monday) x2 (Day 78/82 & Day 92/96). The perturbation period demonstrated a steady increase in *E. coli* removal, hence two sampling points were chosen, near the

beginning and end of that phase (Day 102 & Day 123). During stable three-day semi-continuous feeding (FR2) phase *E. coli* was consistently satisfactorily removed, hence two sampling points were chosen from that phase (Day 153 & Day 213).

Nucleic acid and protein were co-extracted from the 24 samples using the method developed by Thorn et al. (2019), based on Griffiths et al. (2000) and Benndorf et al. (2007). Briefly, phase separation was used to isolate nucleic acids while proteins were recovered and washed from the phenol phase. Extracted nucleic acid samples were stored at -80°C prior to metagenomic sequencing. Protein was stored in 0.1 M ammonium acetate in MeOH at -20°C. The RNA and protein are available for further research, but were outside the scope of the present work.

4.2.7 Metagenomics

Metagenomic sequencing, assembly and annotation

Shotgun sequencing was carried out by Teagasc Sequencing Platform at Moorepark, with two runs of 12 samples each in Illumina NextSeq High Output 300 cycles. Sequencing reads from this study have been deposited in Metagenome Rapid Annotation using Subsystem Technology (MG-RAST[1]).

4.2.8 Taxonomic and Functional Annotation of Metagenomes

Initial analysis was carried out via the online metagenome analysis tool MG-RAST webserver due to computation limitations. This comes with some limitations, the biggest of which was a read pairing issue. Hence the analysis herein presents only the taxonomic content of the samples, not the functional capacity suggested by the sequencing data. Reads were neither assembled or filtered prior to submission for taxonomic and functional analysis, as recommended by MG-RAST version 3.0 (Mason et al., 2014; Wilbanks et al., 2014). Read paired ends were merged prior to analysis according to the instructions provided by MG-RAST. Low-quality sequences were filtered out using MG-RAST default settings and artificial replicate and irrelevant sequences (human or mouse) were removed automatically. The pairing of the reads is done incorrectly on MG-RAST. It appears that their approach

is more geared towards 16S amplicon sequencing, where it is advantageous to have overlaps between your read pairs. In the case of shotgun metagenomics, the reads do not necessarily overlap, as this can aid later assembly efforts by covering larger stretches of the genome. In practice, selecting "Join read pairs" on MG-RAST resulted in a file with only about 1/4 of the input reads, presumably only those fragments that happened to have an overlap (whether it was a real overlap from sequencing both ends of a DNA fragment less than 2x the read length, or whether an overlap occurred by chance). After several attempts to upload the reads to the server and to fix this "merging" error to no avail, correspondence with the MG-RAST team indicated that MG-RAST does not treat paired reads properly, it can only *merge* reads that overlap. Hence for this analysis, only the forward reads were considered, as considering both the forward and reverse reads adds complexity to the statistical analysis without greater insight.

4.2.9 Metagenomic Analysis

After uploading the data, the forward reads were added to a "Project" entitled "AD Policy F" with all 96 (24 samples run on 4 sequencer lanes) forward read files at https://www.mg-rast.org/mgmain.html?mgpage=project&project=82a225b6116d67703935393037),

On the "analysis" page, each metagenome was loaded and analysed with the RefSeq taxonomic database, after which it was determined that normalisation and filtering needed to be applied as some of the datasets failed to yield many annotated sequences, possibly due to known issues with annotation in MG-RAST's backend. Outliers were identified using the common formula of selecting values outside of $ 1.5 * IQR$, where IQR is the interquartile range between first and third quartile.

The following total abundance quartiles were attained:

0 %	25 %	50 %	75 %	100 %
17645	3356635	3690654	3971614	4455714

In this case, any value less than the 25% quartile was marked as an outlier - 1.5∗(3971614−3356635)1.5∗(3971614−3356635); excluding 14 datasets on the basis of low abundances (Figure 4.2).

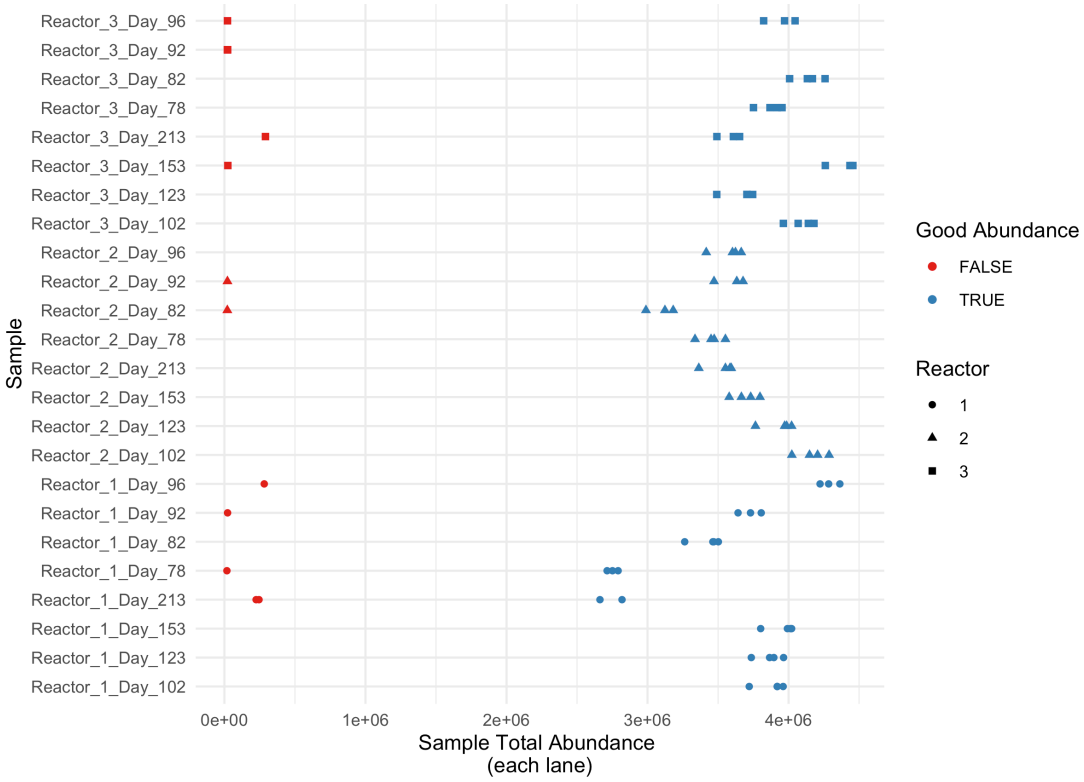


Figure 4.2: Identifying and removing low-abundance outliers on MG-RAST

The dataset was then normalised according to the total abundance of the dataset. As such. the normalised abundances for future analysis were written out, as well as the mean-aggregated per-genus by-reactor abundances and the per-day ones, rounded. Thereafter, an “overall taxonomic profile” was attained, per reactor, per day (Figure 4.5), before “specific taxonomic analyses” were carried out to determine the abundances over time of certain relevant genera (Figures 4.6-4.15), particularly some potentially relevant archaea and bacteriophage.

4.3 Results and discussion

4.3.1 Optimisation of sanitisation and methane production

The EU standard requires coliforms and *E. coli* in digestate to be less than 1,000 cfu g^{-1}. Figure 4.3 displays the required *E. coli* removal to achieve the EU digestate standard (blue) and the actual *E. coli* removal achieved at each time point. After the initial stabilisation period, a phenomenon was observed wherein strong FIB reduction was seen in periodic "spikes" (seen as green spikes in Figure 4.3). This was attributed to the nature off the FR1 conditions (feeding 1x volume and sampling every day for 4 days per week, followed on day 5 by feeding 3x volume to last the remaining 3 days of the week), with the spikes being observed on the Monday sampling following the 3x feed.

The feedstock slurry had been stored for the first six weeks of the trial, causing a reduction in initial FIB levels (Figure 4.3). Hence, from Day 67, fresh slurry was collected every two weeks and used in the feedstock. This change highlighted that although spikes in achieved *E. coli* removal were observed after weekends, the required standard was not achieved during FR1. The observed spikes did, however, indicate the potential for achieving improved FIB removal using a longer gap between semi-continuously fed batches. Hence on Day 99 (indicated by red line in Figure 4.3), the feeding regime was changed to three-day batches, whilst maintaining all other operational parameters (OLR: 2g VS L^{-1} day^{-1}; retention time: 21 days; temperature 37°C). Following a transition phase, coliform and *E. coli* removal steadily improved until the EU digestate standard (less than 1000 CFU g^{-1}) was consistently achieved (Figure 4.3).

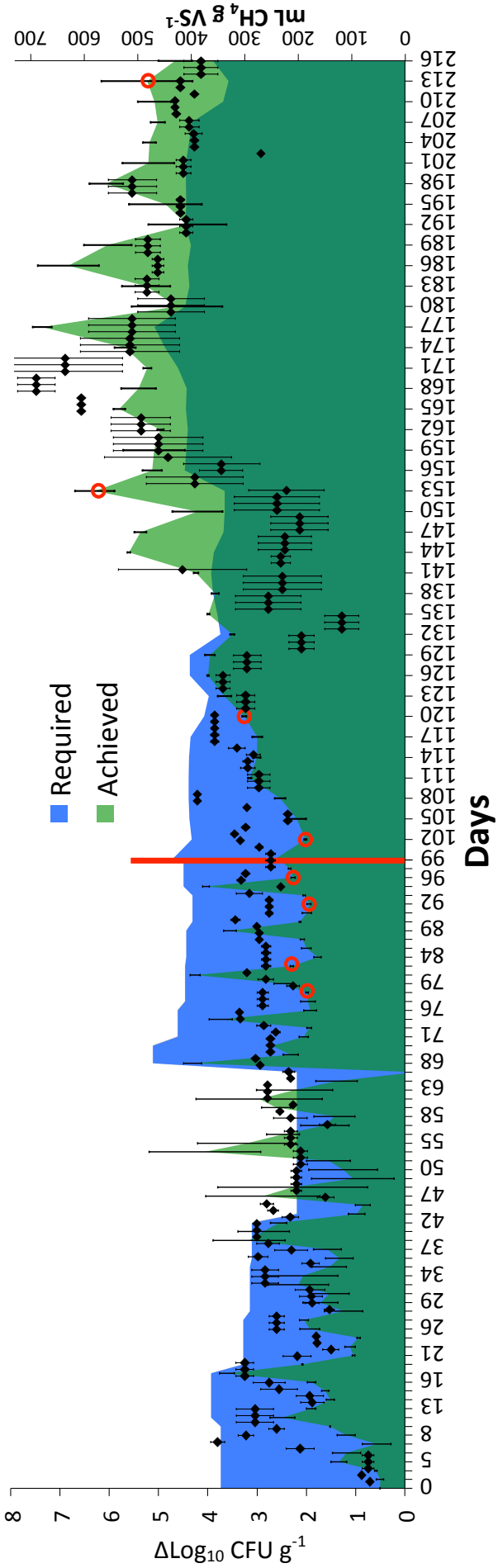
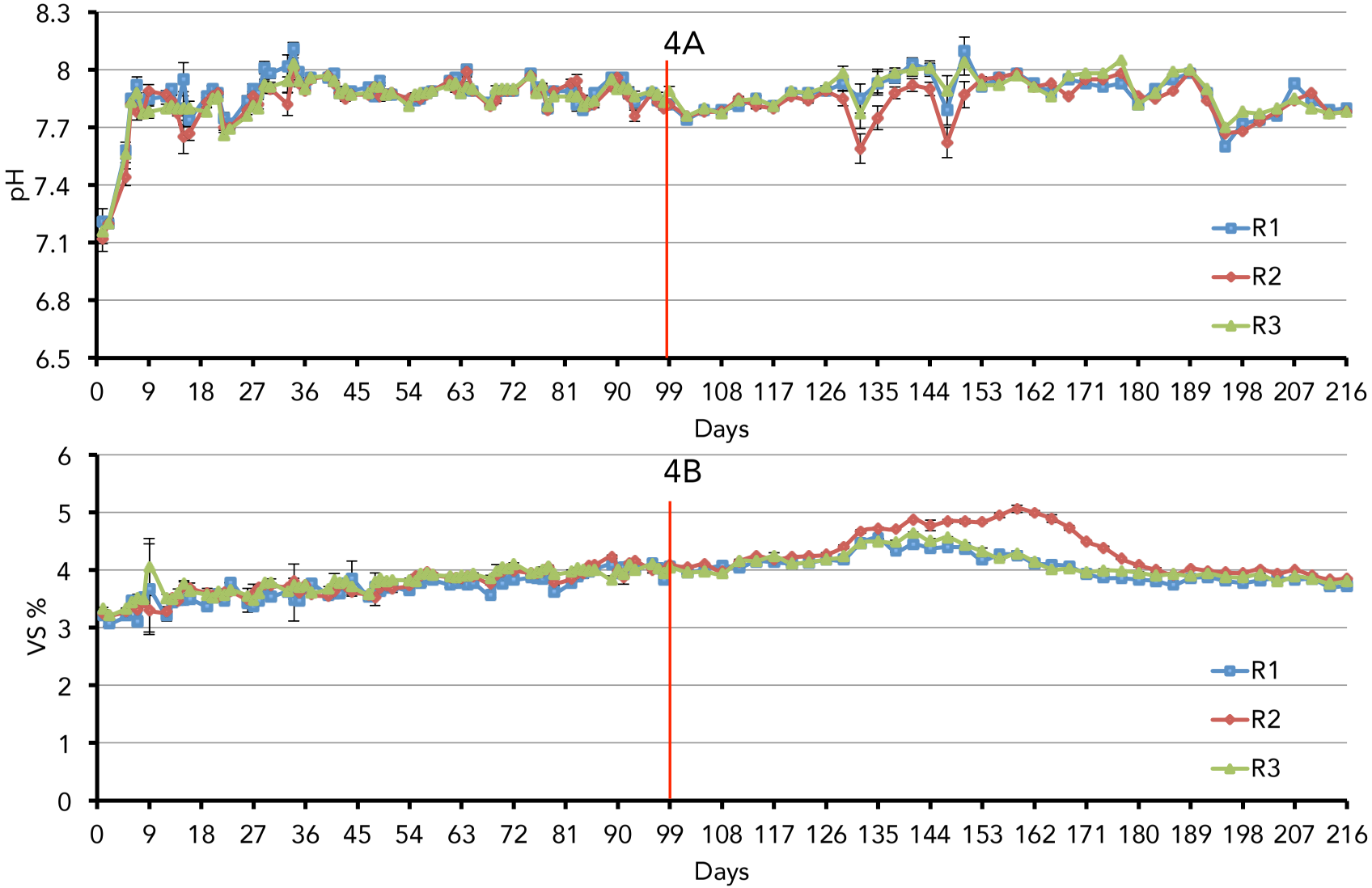


Figure 4.3: Displaying data from 216 day trial in CSTRs processing dairy slurry with FOG (n = 3) with blue shading denoting required and green shading denoting achieved *E. coli* **Δlog** removal, with satisfaction of the EU digestate standard <1,000 cfu g^{-1} as the set removal target. Red line indicates change of feeding regime from FR1 to FR2. Red circles indicate sample points for metagenomic analysis.

Remarkably, concomitant with improved FIB removal, we observed increased biomethane production as a result of the altered feeding regime. FIB reduction slightly pre-empted the altered trend observed for methane production. During the perturbation period following transition to FR2, methane production fell from a stable average (Day 31 - Day 99) of 249 ± 5 mL CH_4 g VS^{-1} to a low of 119 ± 32 mL CH_4 g VS^{-1} between Day 130 and Day 133, likely due to an overloading effect, requiring adaptation of the microbial community. This phenomenon negates the possibility that increased methane yield later in the trial was an artifact of reduced feeding interval disturbances. Methane production in Bioreactor 1 and 2 recovered more slowly than that of B3, beginning from Day 150.

Physicochemical analysis including pH, COD, solids and NH_3 was undertaken throughout to determine potential correlations with biogas production and sanitisation efficacy.

Results for pH, VS, sCOD and NH_3 analysis are presented in Figure 4.4 and are discussed below.


4A
pH
8.3
8
7.7
7.4
7.1
6.8
6.5
0 9 18 27 36 45 54 63 72 81 90 99 108 117 126 135 144 153 162 171 180 189 198 207 216
Days
R1
R2
R3
4B
VS %
6
5
4
3
2
1
0
0 9 18 27 36 45 54 63 72 81 90 99 108 117 126 135 144 153 162 171 180 189 198 207 216
Days
R1
R2
R3

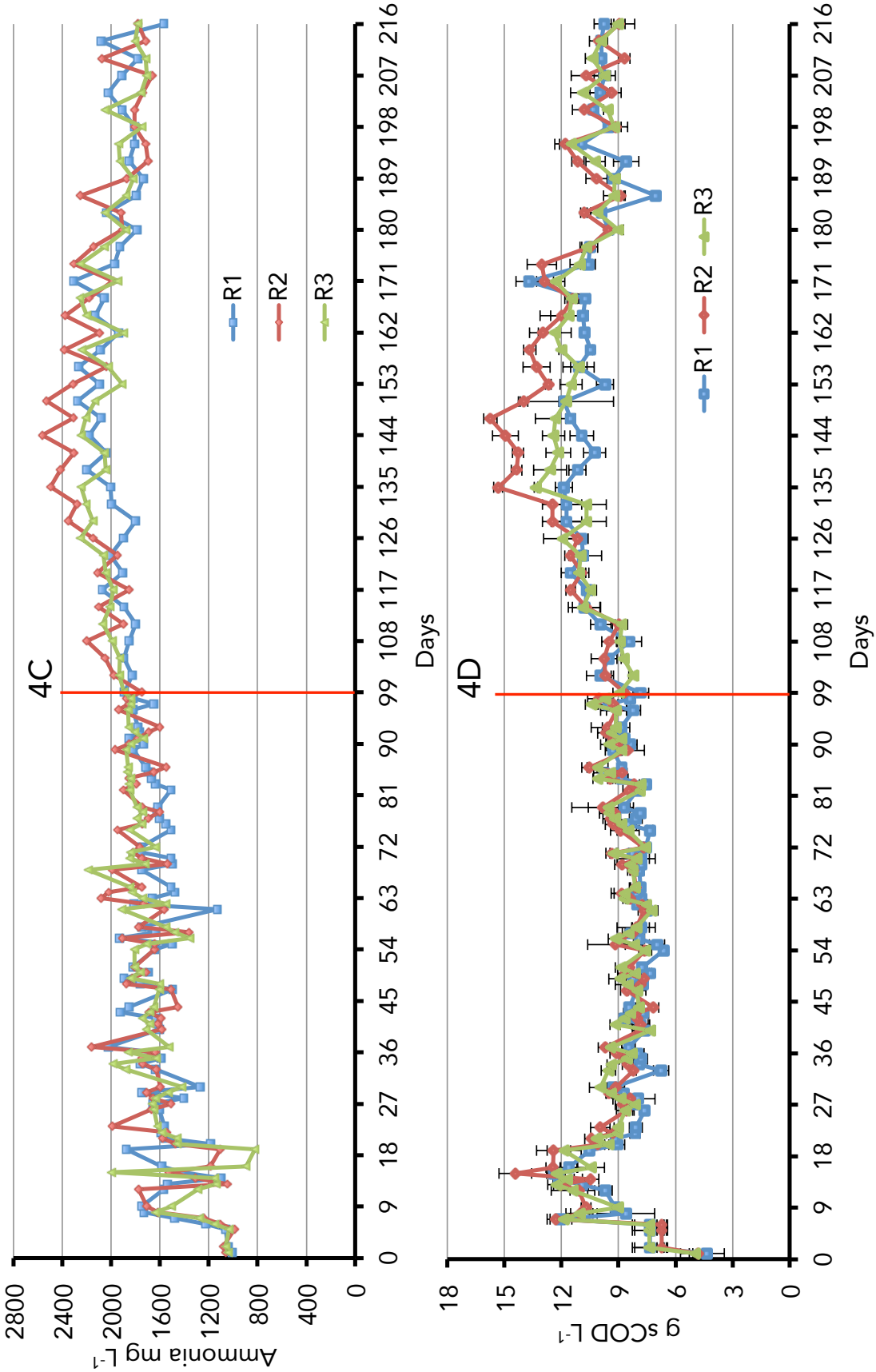

4C
Ammonia mg L-1
2800
2400
2000
1600
1200
800
400
0
R1
R2
R3
0 9 18 27 36 45 54 63 72 81 90 99 108 117 126 135 144 153 162 171 180 189 198 207 216
Days
4D
g sCOD L-1
18
15
12
9
6
3
0
R1
R2
R3
0 9 18 27 36 45 54 63 72 81 90 99 108 117 126 135 144 153 162 171 180 189 198 207 216
Days

Figure 4.4: Physicochemical analysis of samples from triplicate CSTRs processing DCS and FOG for 216 days initially fed daily (FR1: first 99 days) and then fed every three days (FR2), including pH (4A), volatile solids concentration (%) (4B), NH_3 (4C) and sCOD (4D), with error bars indicating standard error of technical replicates (n = 3). A vertical red line indicates the change from FR1 to FR2 on Day 99.

4.3.2 pH

pH is an important factor in methane production in AD systems, becoming an inhibiting factor below 6.8 and above 8.0 (Ward et al., 2008). Within six days of start-up, pH in all bioreactors stabilised at an average of 7.87 ± 0.07, which was maintained for the remainder of FR1 (Figure 4.4A). Extending the time between feeding while maintaining the same OLR could potentially result in an accumulation of hydrolysis and fermentation products, leading to a consequent drop in pH, which could inhibit methanogens (Mulat et al., 2016).

Upon changing feeding regime at day 99, pH fluctuated, initially dropping slightly (7.75 ± 0.01), then increasing above 8 in bioreactors 1 and 3 concurrently with a peak in NH_3 (between Day 141 and 150). In line with the bioreactors reaching stable methane production in FR2, pH returned to an average of 7.82 ± 0.09 between Day 180 and the end of the trial (Figure 4.4A). In a study examining the impact of shock loading on bioreactor performance, Kim & Lee (2015) observed far greater capacity for survival in AD systems with a mechanism for buffering to a constant pH then those without pH buffering capacity, surviving shock loads of 8.0g and 3.0g sCOD L^{-1} d^{-1} respectively. In that study pH was controlled near 7 by automatic addition of 3 N NaOH, whereas in the present work the significant buffering capacity of slurry is likely achieving the same effect naturally. While comparing once-per-day with twice-per-day feeding, Lv et al (2014) observed a greater biogas increase within 80 minutes of the once-per-day feeding, attributed to a spike in acetic acid and reduced pH. However, here it is unlikely that pH was the primary factor in either the increased methane production or FIB removal observed during FR2 because the pH range recorded was consistently suitable for methane production (well within the 6.8-8.0 range established by Ward et al., 2008), and was not acidic enough to significantly affect FIB.

4.3.3 NH_3 – impact on FIB and methane production

Free ammonia has been identified as a key factor in FIB removal in AD (Jiang et al., 2020) and can also be inhibitory to the anaerobic digestion process (Capson-Tojo et al., 2020). Average NH_3 concentration was significantly higher during the second feeding regime (FR2: 2004 ± 24 mg L^{-1}) when compared with FR1 (1638 ± 12 mg L^{-1}). During the perturbation/adaptation period between feeding regimes, NH_3 concentrations peaked (Day 150) at 2330 ± 119 mg L^{-1}, which could have become toxic to the methanogenic population if sustained (Angelidaki & Ahring, 1993; Capson-Tojo et al., 2020; Kayhanian, 1999; Nielsen & Angelidaki, 2008; Regueiro et al., 2015).

By Day 150 however, NH_3 concentration had begun to decline, averaging 1849±22 mg L^{-1} between Day 180 and the end of the trial (Figure 4.4B). Sustained NH_3 concentrations above 2,000 mg L^{-1} have been found to cause ammonia toxicity in anaerobic digestion (Rajagopal et al., 2013) and has also been attributed to improved sanitisation. However, it is not likely that free-ammonia inhibition was the primary factor in the improved sanitisation observed following the adaption period in FR2, given that: 1. ammonia concentration declined to a stable level in line with bioreactor stability whilst maintaining improved coliform removal, and 2. the average NH_3 concentration for the "post-weekend/Monday" time points on which satisfactory coliform and *E. coli* removal spikes were observed during FR1 was not significantly different to the daily-fed samples (1625 ± 26 vs 1641 ± 15 mg L^{-1} respectively). The perturbations in biogas production observed following transition to FR2 may be associated with NH_3 inhibition (NH_3 > 2,000 mg L^{-1}) as the slow decline (30 days) observed is indicative of the effects of a steady buildup of NH_3 as opposed to a shock event.

The elevated NH_3 levels in Bioreactor 2 compared with Bioreactor 3 (2530 vs 2130 mg L^{-1} respectively) correlated closely with slower recovery of methane production in B2 after the perturbation period, possibly due to biological differences amongst the three replicates. Methanogenic populations exhibit differential sensitivity to elevated free ammonia nitrogen concentrations, with *Methanosaeta* giving way to mixotrophic archaea such as *Methanosarcina* as concentrations increase, until ultimately more resilient hydrogenotrophic

methanogens such as *Methanoculleus* become dominant (Capson-Tojo et al., 2020).

4.3.4 Solids

Total and volatile solids (TS & VS) in the digestate were recorded throughout the trial. Similar to the other physicochemical parameters, consistent (although slightly increasing) averages of 5.54 ± 0.1 and 3.85 ± 0.16 for TS % (data not shown) and VS % (Figure 4.4C) respectively were maintained during FR1 after the initial 30-day stabilisation period. Upon modification of the feeding regime, TS % and VS % increased steadily, reaching averages of 6.56 ± 0.4 and 4.53 ± 0.33 respectively (19 % and 18 % increases over stable operation). This build up of solids in the bioreactors is indicative of unstable operation and aligns closely with the reduced methane production during this perturbation period (Figure 4.3). Once stable operation in FR2 was achieved, total and volatile solids in the digestate returned to almost identical levels as during FR1, 5.67 ± 0.14 and 3.88 ± 0.09% respectively (Figure 4.4C).

One of the benefits of agricultural AD is that it breaks down solids so that they can be more easily assimilated into the soil and used as fertiliser by plants, further reducing likelihood of ingestion by animals. As reduction of pollution potential from agriculture is a key goal of this work and the agricultural-AD sector broadly, precautions should be taken with digestate generated during transition phases between operational conditions as these can lead to incomplete breakdown of organic matter and therefore reducing AD pollution mitigation potential. Additional processing prior to landspreading may then be required; such as recycling digestate from the unstable phase back into the AD system for more complete degradation once stable operation has been achieved.

4.3.5 Chemical oxygen demand

Chemical oxygen demand can be used as an indicator of the biomethane potential of a feedstock. In AD, total and soluble COD should be lower in digestate than in the feedstock, hence reducing the pollution potential of the raw material (in this case, DCS and FOG). Soluble COD is the fraction that is most

readily available for methane production, whilst total COD (tCOD) is an indicator of the total methane potential of a feedstock, if given enough time to break down. Theoretical methane yield is 0.35 L per gram COD (0.35 mL/mg), although there are many factors that may contribute to actual yield, including mixing intensity and duration (Singh et al., 2020) and retention time (Mao et al., 2015) and microbial community dynamics.

Feedstock sCOD averaged 20.52 ± 0.37 g sCOD L^{-1}, while total COD in the feedstock averaged 166 ± 2.87 g tCOD L^{-1} over the course of the trial. Following a 30-day start-up period, sCOD in the digestate averaged 8 ± 0.1 g L^{-1}, between day 33 and 99, while tCOD averaged 56 ± 0.1 g L^{-1} during the same period. Changing the feeding regime to every three days resulted in an initial build up of COD with sCOD in the digestate increasing to an average of 13 ± 0.4 g L^{-1} between Day 135 and 150 and tCOD averaging 81 ± 2.4 g L^{-1} during the same period, peaking on Day 147 at 87 ± 6 g L^{-1}. Beyond Day 150 methane production in all bioreactors increased significantly as the excess COD that built up during the perturbation period was rapidly consumed, generating a peak of 690 ± 35 mL CH_4 g VS^{-1} fed between day 166 and 168. When the excess COD was consumed, a stable period followed from Day 180 onwards, where an average of 430 ± 7.4 mL CH_4 g VS^{-1} fed was sustained (i.e. FR2 had 58% higher methane yield per gram VS fed than FR1).

As no operational parameters (OLR, temperature, RT, mixing rate etc.) were altered apart from the feeding regime, the COD and CH_4 phenomena recorded during the transition and stable period of FR2 may be indicative of improved microbial efficiencies, necessitating an examination of community dynamics during the three main phases, namely FR1, FR2 transition phase, FR2 stable phase.

4.3.6 Metagenomics: Biogas yield

The metagenomics dataset was analysed using MG-RAST to examine the effect of feeding regime alterations on microbial community dynamics, with a view to establishing some possible connections between microbial community dynamics and observed changes in biogas production and *E. coli* survival. The top 30 classes are displayed in Figure 4.5, with high levels of similarity between

the three bioreactors over the course of the trial. *Clostridiales* was the most prominent, with *Bacteroidales* next. Of the methanogens, *Methanomicrobiales* was most prominent, particularly during FR2, followed by *Methanosarcinales*. The results for Bioreactor 3 on Day 96 are not available because all four lanes failed, possibly due to library prep and/or sequencing issues.

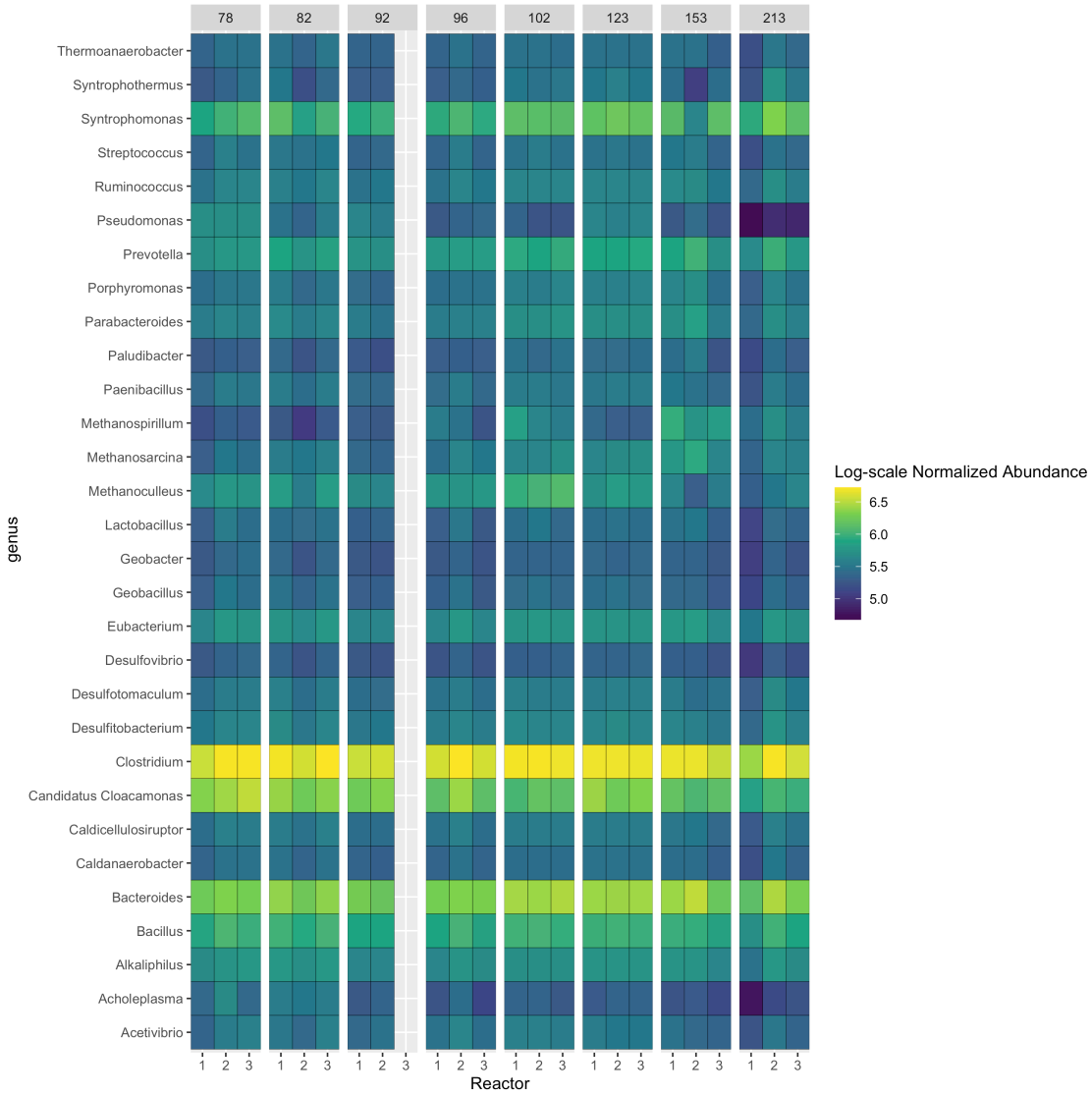


Figure 4.5: Log-scale normalised abundance for genus top 30 by Day/Reactor

In this study, FOG was the feedstock component contributing the majority of methane potential, and syntrophic β-oxidation has been identified as the principle pathway for conversion of fatty acids to methane (Sousa et al., 2009), with the efficiency of FOG degradation dependent on *Syntrophomonas* concentrations (Ziels et al., 2016). Hence, the metagenomic dataset was

examined to determine *Syntrophomonas* community development over the course of the trial. Upon examination of the present metagenomics dataset using MG-RAST a sustained increase in *Syntrophomonas* from FR1, through the transition phase into FR2 was observed (visible in Figure 4.5), corresponding with the improved methane yield recorded, as presented in Figure 4.6. A comparative examination of continuous versus 'pulse-fed' (feeding every two days) anaerobic codigestion of cattle manure with oleate similarly reported increase methane yields and identified an increase in *Syntrophomonas* as a key distinction between the metagenomic datasets of the two feeding regimes (Ziels et al., 2018, 2017). Syntrophic bacterial communities have been found to be more resilient to disturbance (Werner et al., 2011), and hence the intermittent overloading of fatty acids resulting from semi-continuous feeding (one big feed every three days at 2g VS L^{-1} d^{-1} as opposed to multiple smaller hourly or daily feeds at the same loading rate) may have resulted in biological selection of more resilient communities better suited to fatty acid degradation (Ziels et al., 2017).

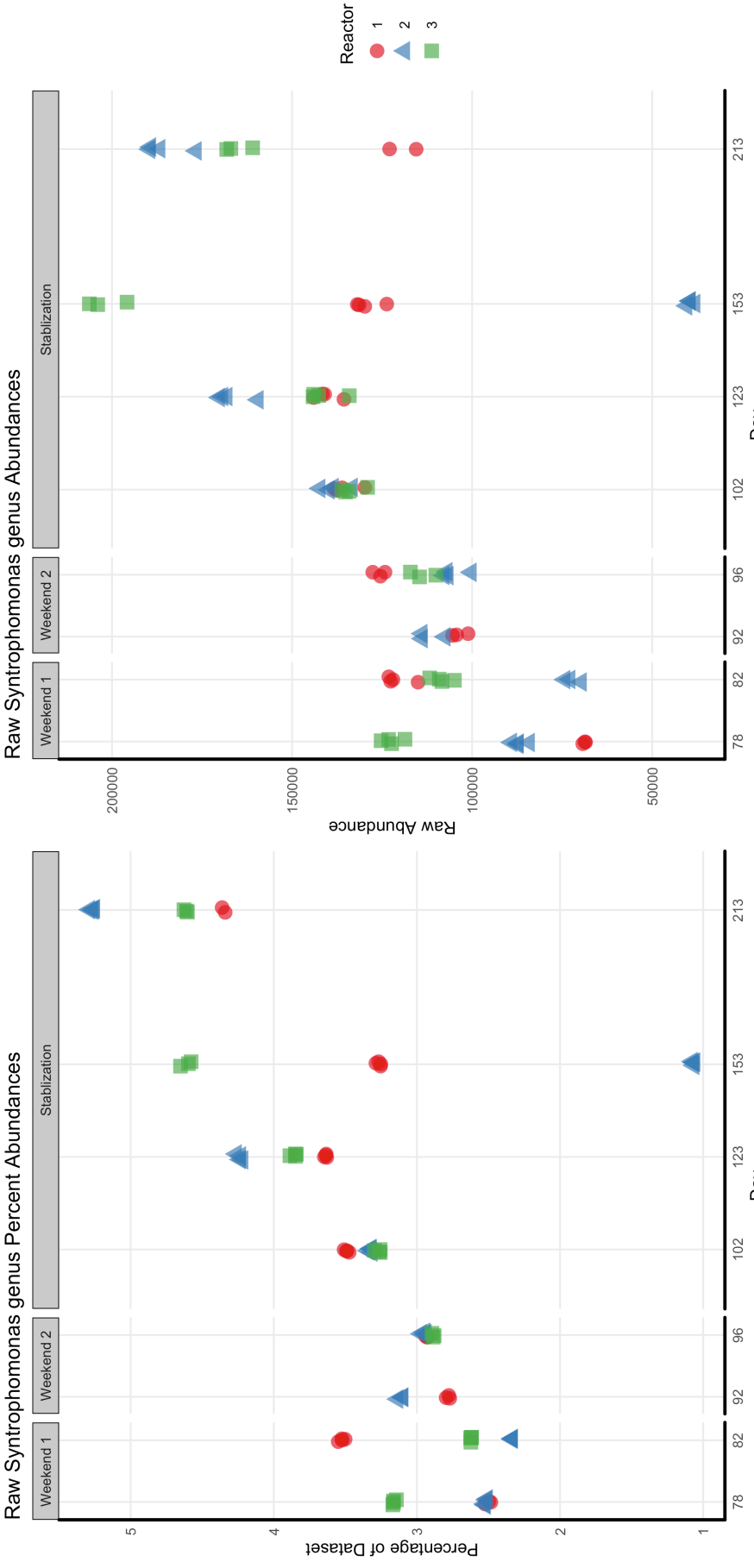


Figure 4.6: Raw Syntrophomonas abundances in samples from three continuosly stirred reactors codigesting dairy slurry with FOG during two feeding regimes.

The change in relative abundance of the top 30 genera compared with Day 78 is displayed in Figure 4.7. Here a decline in *Candidatus* Cloacimonetes is visible in correlation with the increased concentrations of *Syntrophomonas*. *Candidatus* Cloacimonetes may ferment amino acids and also oxidise propionate into H_2, CO_2 and acetate but has previously been found to be in competition with syntrophic bacteria (Braz et al., 2019).

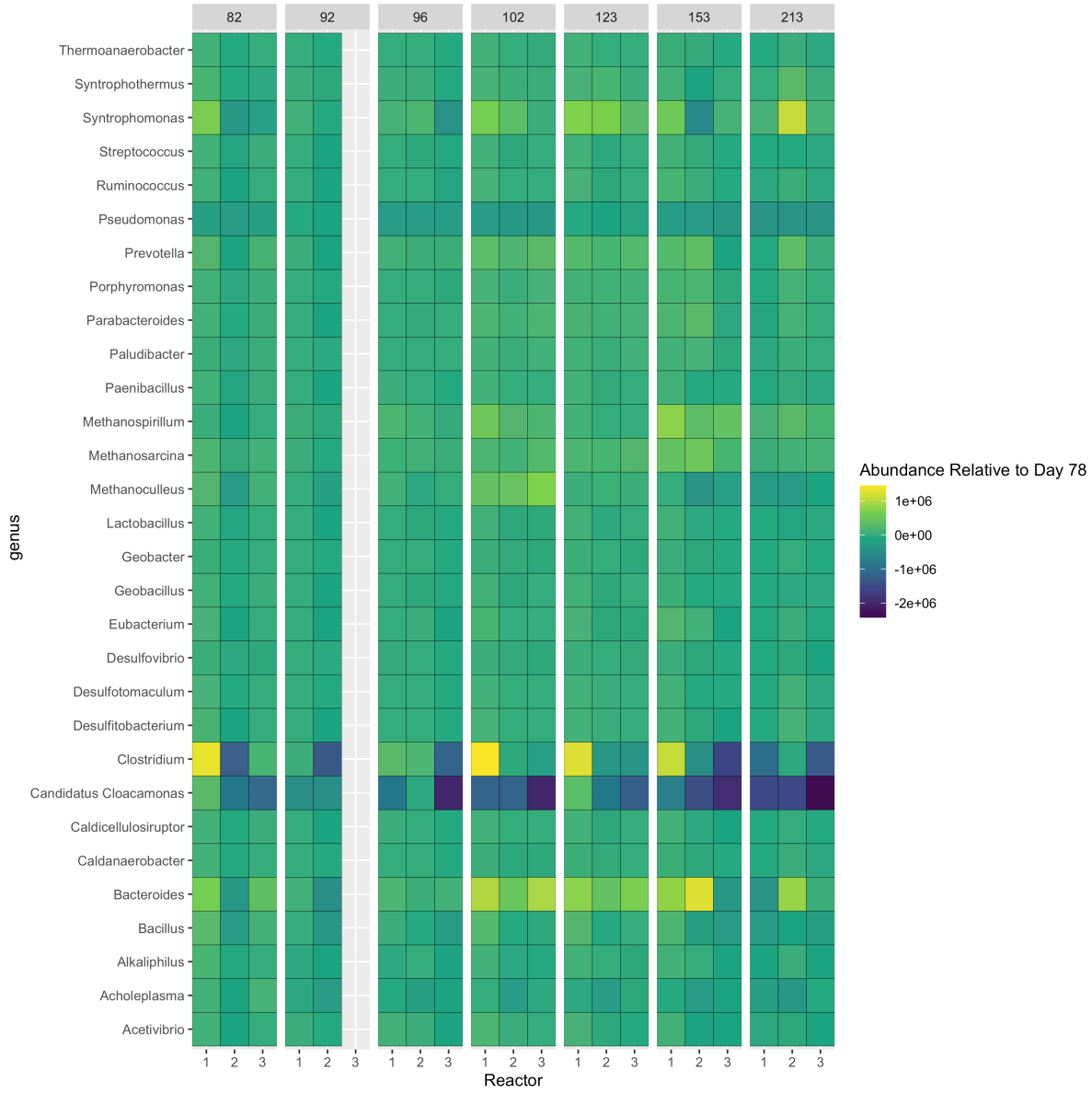


Figure 4.7: Top 30 genera relative to Day 78 showing changes over the course of the trial

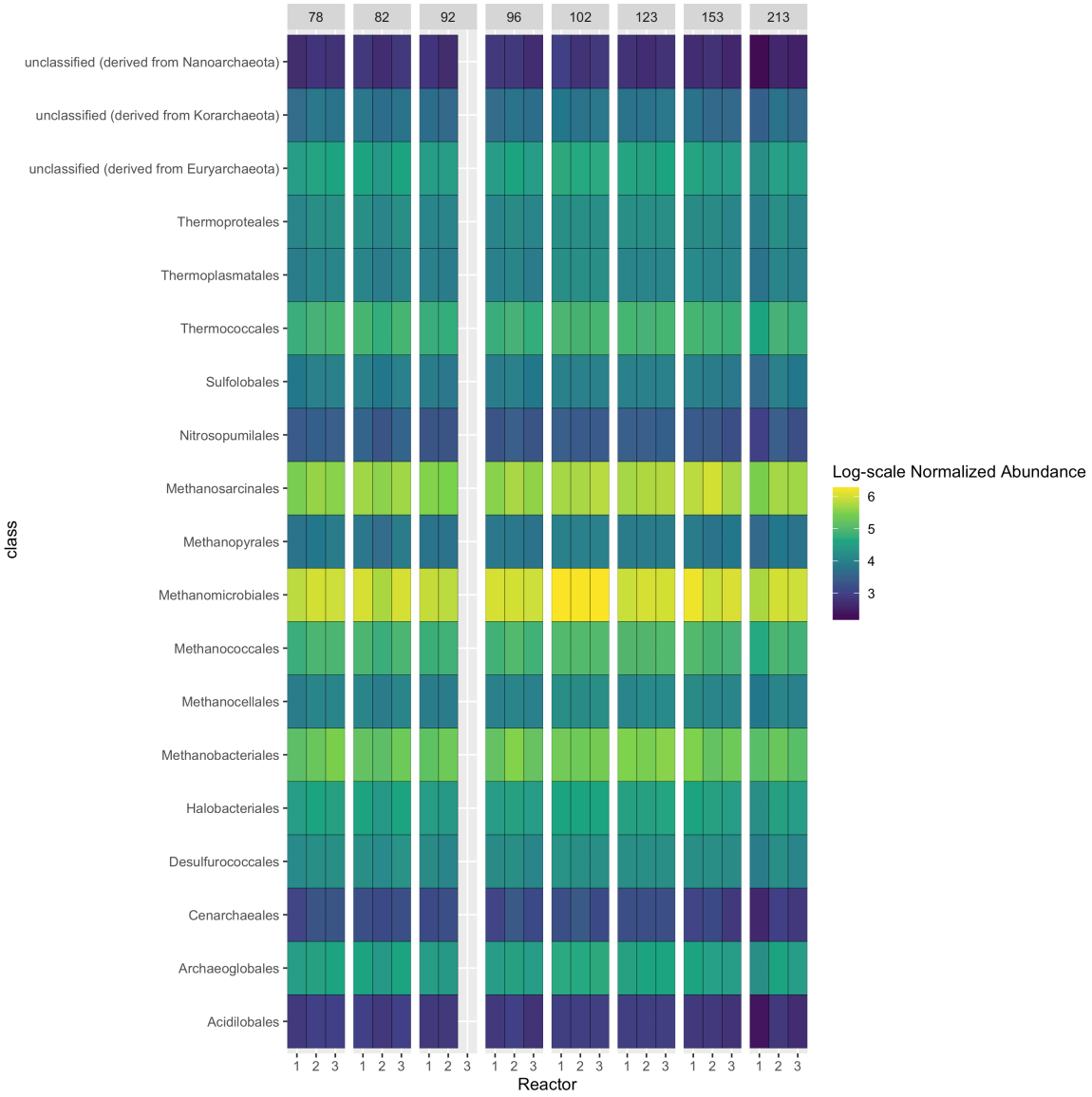


Figure 4.8: Log-scale normalised abundance for top 30 Archaea by Day/Reactor

Log-scale normalised abundances for the most prevalent archaeal classes, demonstrate a clear trend whereby *Methanomicrobiales* and *Methanosarcinales* appear to increase in relative abundance following the change to intermittent (3-day) feeding (Figure 4.8).

A closer look at the methanogenic abundance at genus level, normalised relative to total reads, indicates development of a more diverse archaeal population with higher relative abundances over time, particularly during the highest biogas production phase (Figure 4.9). The data presented in Figure 4.9 demonstrate both the increased relative abundances and the increased diversity of the

methanogenic community following transition to FR2, in line with the improved methane yield observed.

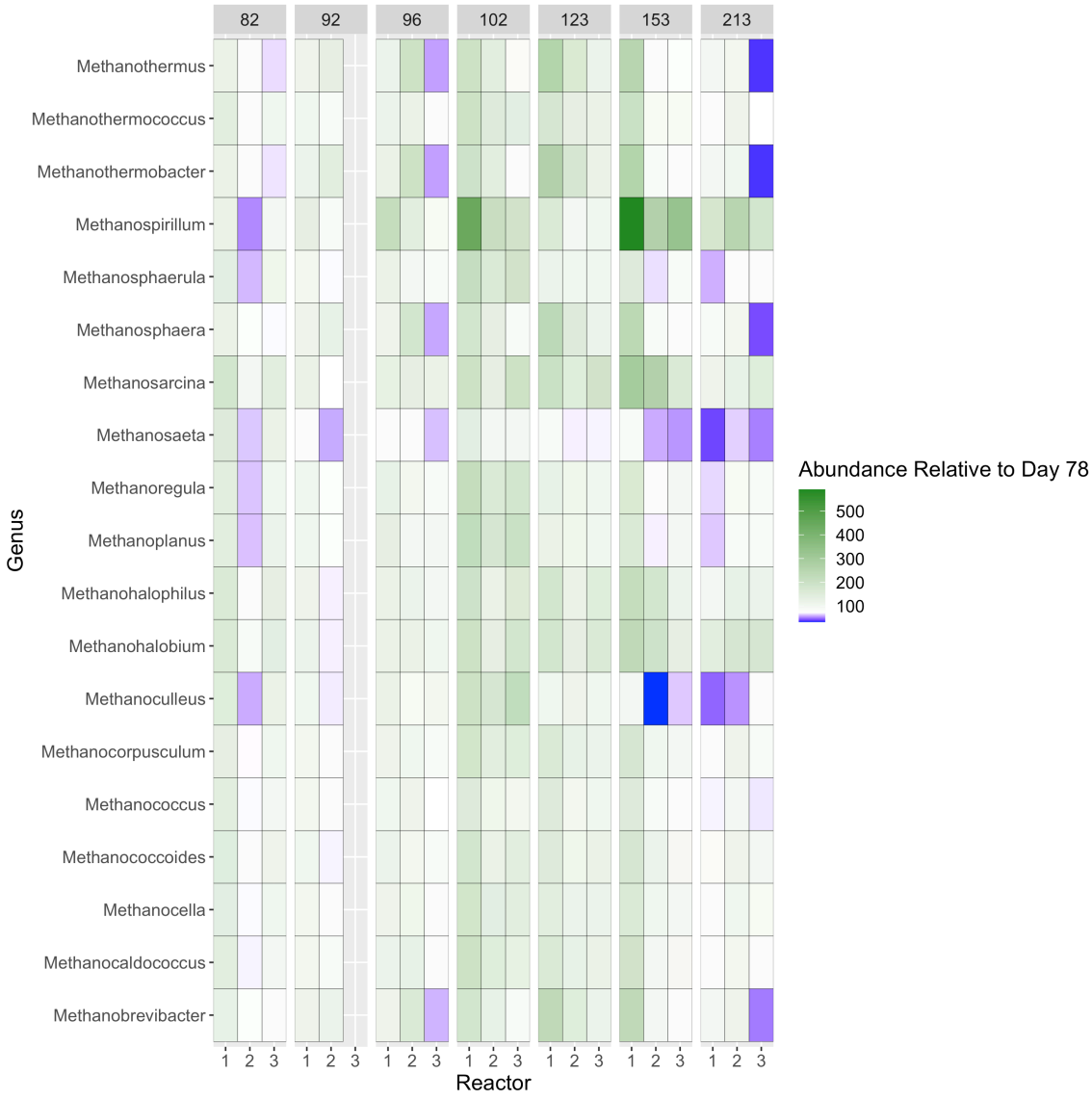


Figure 4.9: Selected Genus-level methanogenic Archaea of percent abundances relative to Day 78

There are distinct differences between the two feeding regimes in terms of methanogenic community richness, with *Methanospirilium* being 3 - 5 times more abundant, while *Methanosarcina* and *Methanohalobium* were 2 - 3 times higher by the end of FR2 compared with FR1. *Methanospirilium* is a hydrogenotrophic methanogen which also utilises CO_2, and has hence been

proposed as a potential candidate for bioaugmentation of AD systems for improved biogas quality, with a higher methane percentage (Jain et al., 2020), as well as for recovery following overloading (Tale et al., 2015). Given the correlation between the 3 - 5 fold increase in *Methanospirilium* and the improved methane yield observed in the present work, it is feasible that *Methanospirilium* played a significant role. These findings align with those of Bonk et al. (2018) and De Vrieze et al. (2013), who observed improved functional stability in AD systems when employing semi-continuous feeding with ≥ 2 day feeding intervals, compared with hourly or continuous feeding, arising from improved bacterial diversity and dynamics and resultant increased tolerance to ammonia and organic overloading (De Vrieze et al., 2013).

All methanogens examined were more abundant in the latter half of the trial except *Methanosaeta*. The increase in relative abundance of *Methanosaeta* observed after the higher volume ("weekend") feeding during FR1 was initially replicated during the perturbation period between the two feeding regimes, but was not sustained (Figure 4.9). This trend could be expected, given that pure cultures of *Methanosaeta* species have been reported to have higher fatty acid tolerance than *Methanosarcina* (Silva et al., 2016). In this case the higher FOG loading during FR1 and higher acid concentrations during the perturbation period, during which methanogenesis failed to keep pace with acidogenesis, could have led to increased *Methanosaeta* abundance, with that advantage declining as the excess concentration was converted to methane following the perturbation period. Similarly to Bonk et al. (2018), a switch from continuous to discontinuous feeding resulted in a decline in *Methanosaeta* relative abundance (Figure 4.10) and a simultaneous increase in *Methanosarcina* abundance (Figure 4.9). Bonk et al. (2018) attributed this community shift to niches created by fluctuating acetic acid concentrations.

In summary, the observed improved methane yield after shifting to intermittent feeding correlated with improved methanogenic community diversity and relative abundance, with notable increases in some key fat and hydrogen consumers, in line with recent observations in the literature.

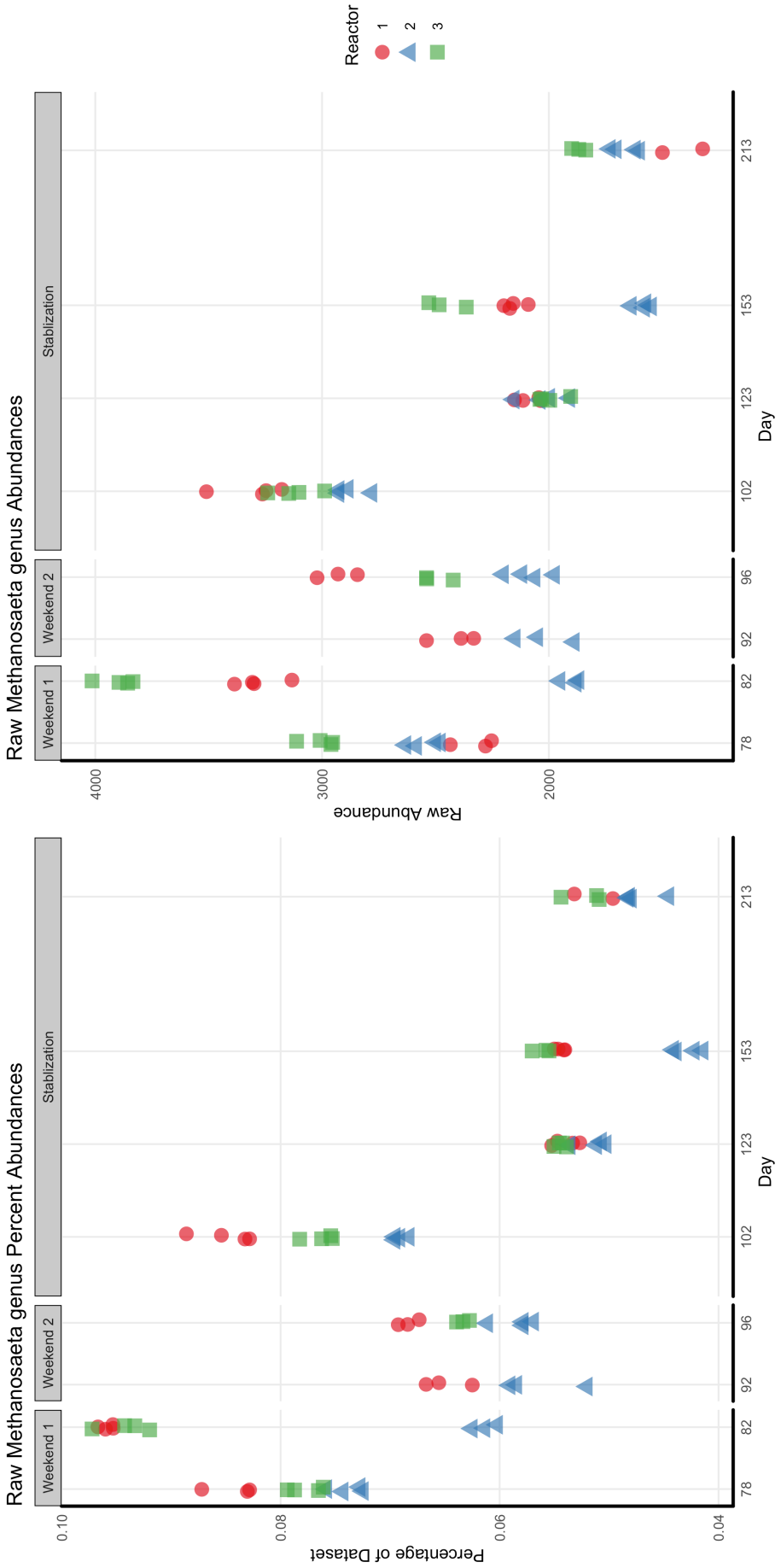


Figure 4.10: Raw *Methanosaeta* abundances in samples from three continuosly stirred reactors codigesting dairy slurry with FOG during two feeding regimes.

4.3.7 Metagenomics: *E. coli*

Although much of the experiment was structured around understanding *E. coli* counts as a function of feeding regime, there was not a clear trend in variance between feeding regimes, as the overall proportion of *E. coli* in the dataset is very low, ranging between .06 % and .13 % (Figure 4.11). The *E. coli* fragments of DNA do not appear to reflect the viability findings assessed with the IDEXX kits. This could be for a variety of reasons, including the very low abundances of *E. coli* in the reactors and the possibility of sequencing dead cells, in particular given that in the case of bacteriophage activity in particular, although the infected host is metabolically active, it is in fact genetically dead (Wang, 2006).

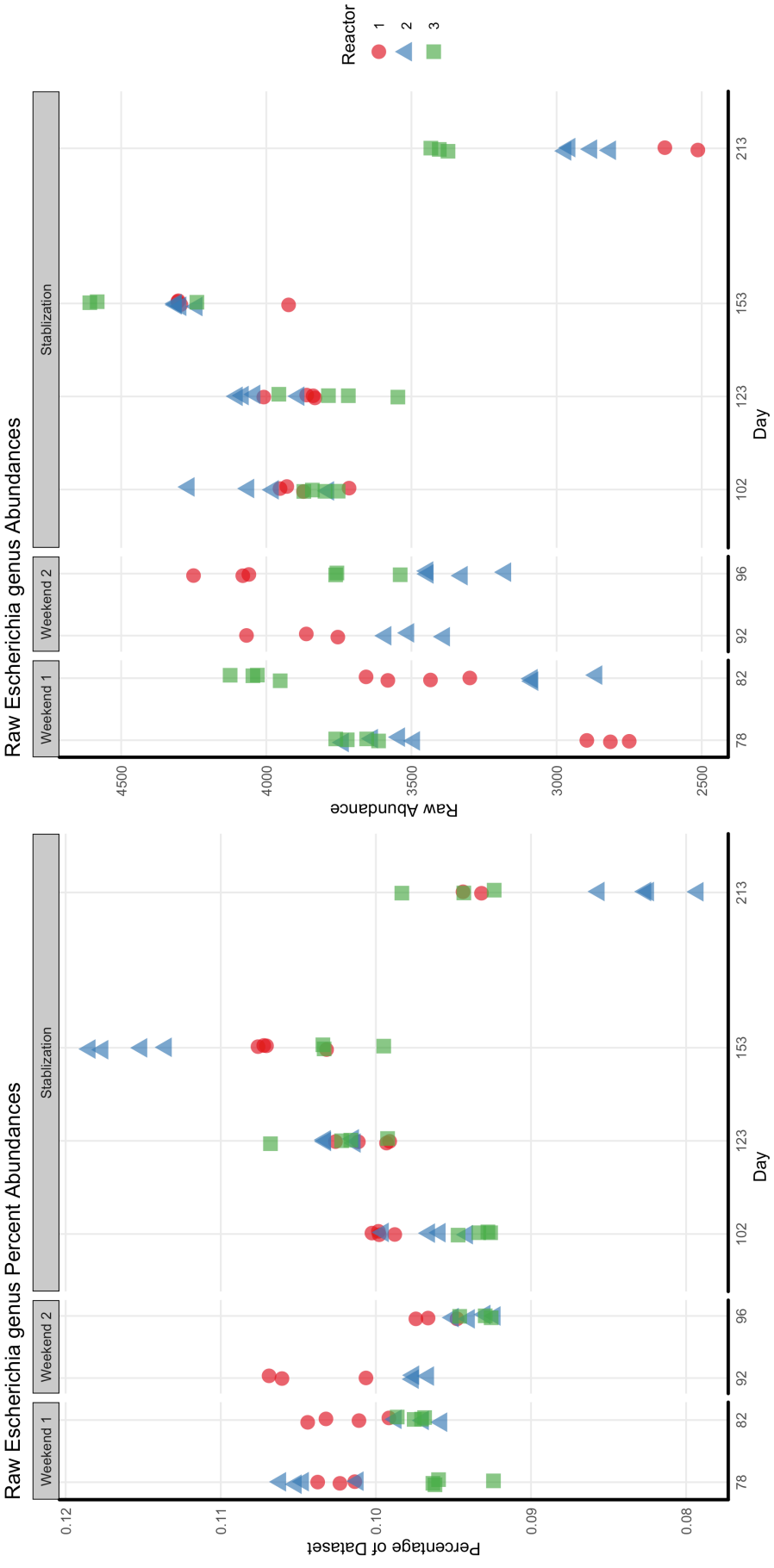


Figure 4.11: *Escherichia* genus abundance and percent abundances in samples from three CSTRs codigesting dairy slurry with FOG during two feeding regimes

4.3.7.1 Bacteriophage

Bacteriophage are ubiquitous viruses that infect bacteria and archaea and divert the host's normal synthesis of proteins or nucleic acids to instead replicate viral components which are then assembled within the host prior to lysing through the host cell wall, killing the host and infecting new hosts. The role of bacteriophage in modulation of anaerobic digestion microbial communities has received minimal attention, but is beginning to be examined (Zhang et al., 2017). A hypothesis of the present work was that alteration of the feeding regime provided conditions more suited to bacteriophage propagation, working on the theory that providing a higher host density with a longer intermittent period would allow for several cycles of infection without depletion of host (Wang, 2006), until a critical mass is accumulated. For the initial trial period where *E. coli* numbers were lower and possibly weaker due to extended cold storage, a more complete *E. coli* reduction might reasonably have been anticipated. However, if bacteriophage is indeed a primary factor, then the physiological state of the host comes into play, influencing the rate of phage progeny assembly/maturation (Wang, 2006).

The metagenomics dataset was assessed using MG-RAST, after which percent abundances and raw abundances for a number of potential bacteriophage candidates were analysed using R. Figures 4.11 - 4.15 present some of the more interesting outputs from that analysis. *Podoviridae* phages are a family of viruses containing well characterised species, some of which infect *E. coli*, such as T7-like viruses and N4-like viruses (Choi et al., 2008; Cuervo et al., 2013). From the initial analysis there appeared to be a trend of increased bacteriophage abundances following transition to FR2, particularly for Day 123 and Day 153. The lower abundances on the final time point may be a function of the efficacy of the bacteriophage activity, whereby competition for hosts may have had an effect on the population, as there are lower *Eschericia* abundances on samples from that day also (Figure 4.11).

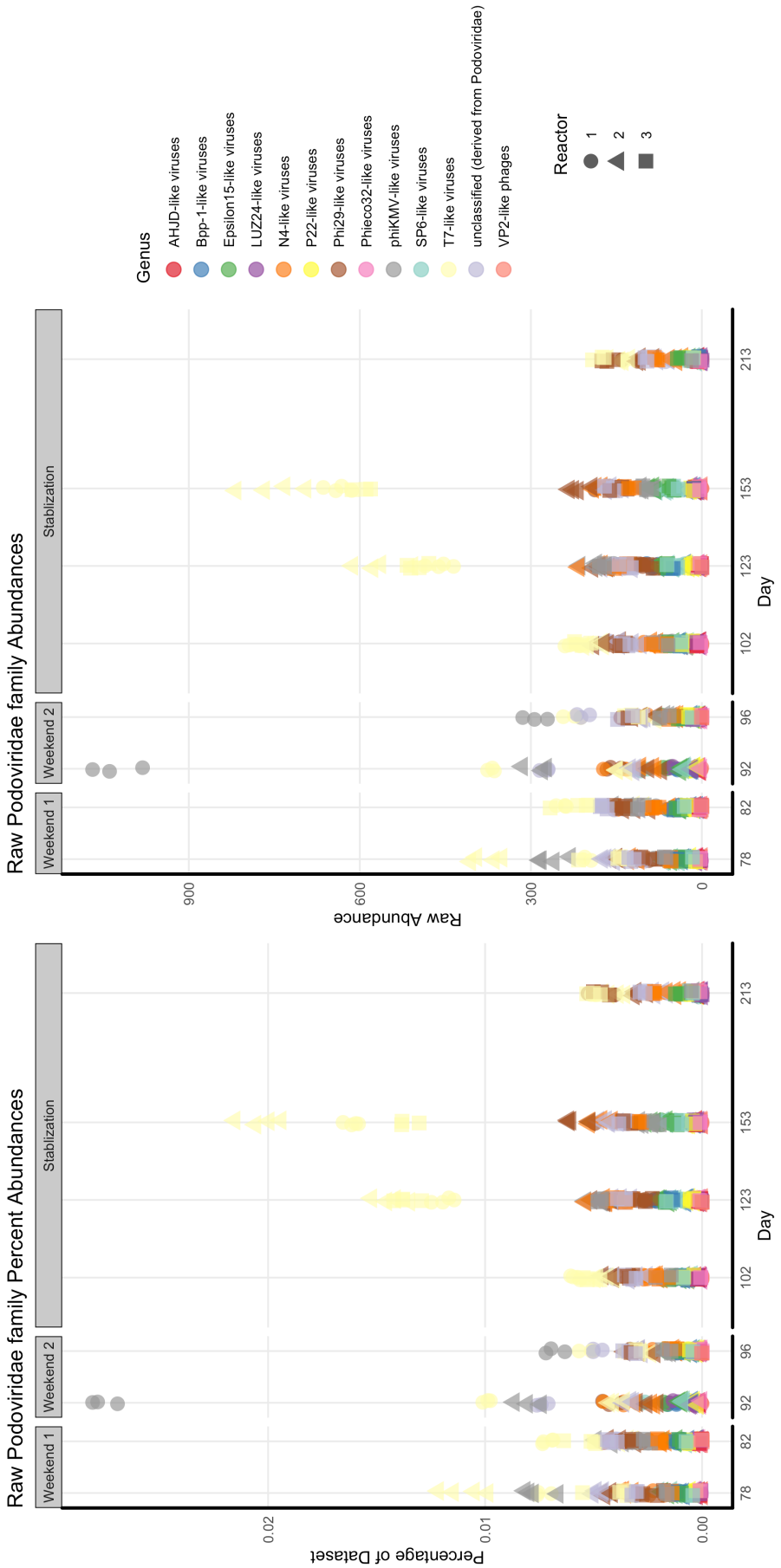


Figure 4.12: Raw *Podoviridae* family abundance and percent abundances in samples from triplicate CSTRs at eight key time points.

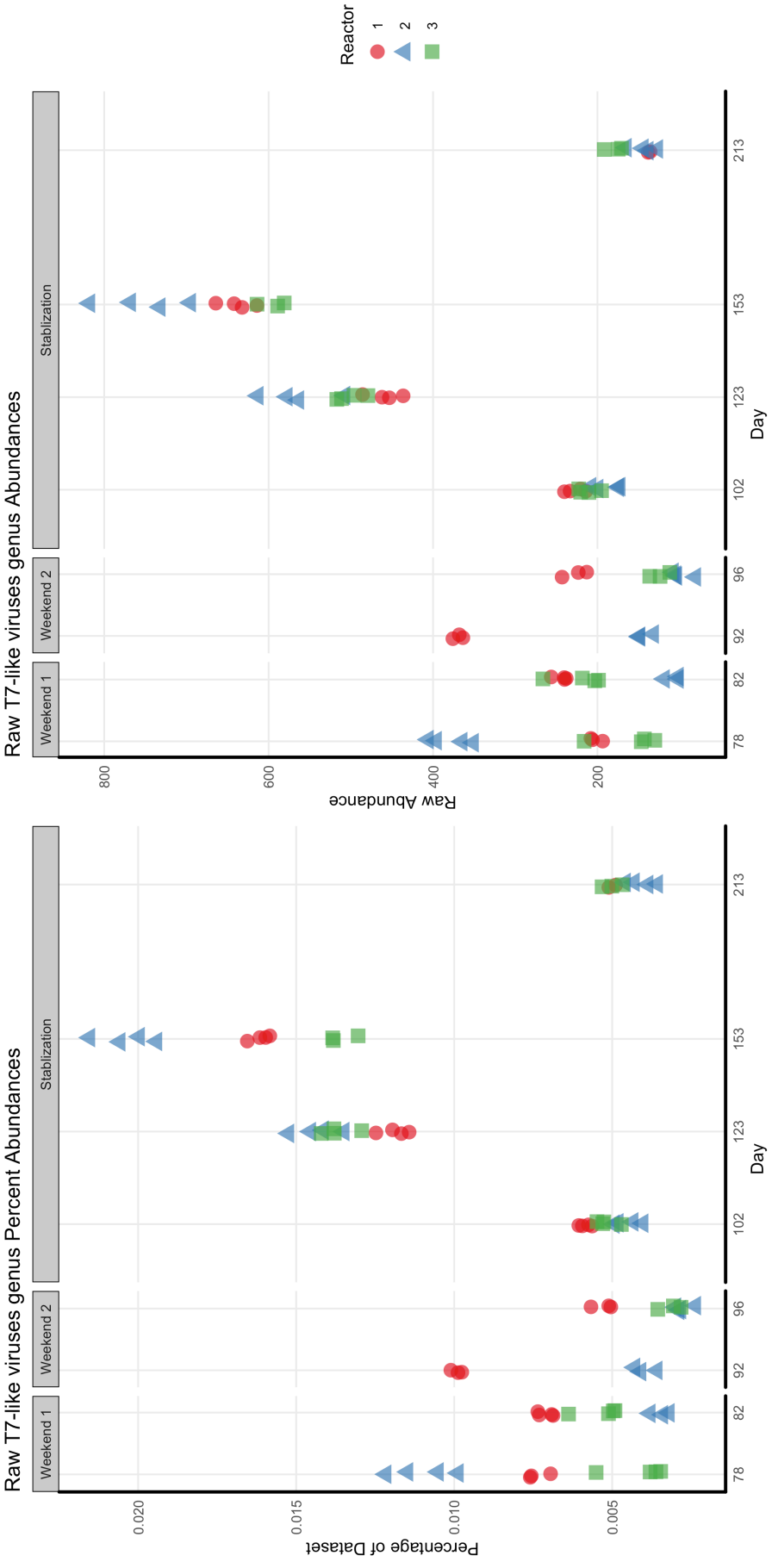


Figure 4.13: Raw T7-like virus genus abundance and percent abundances in samples from triplicate CSTRs at eight key time points.

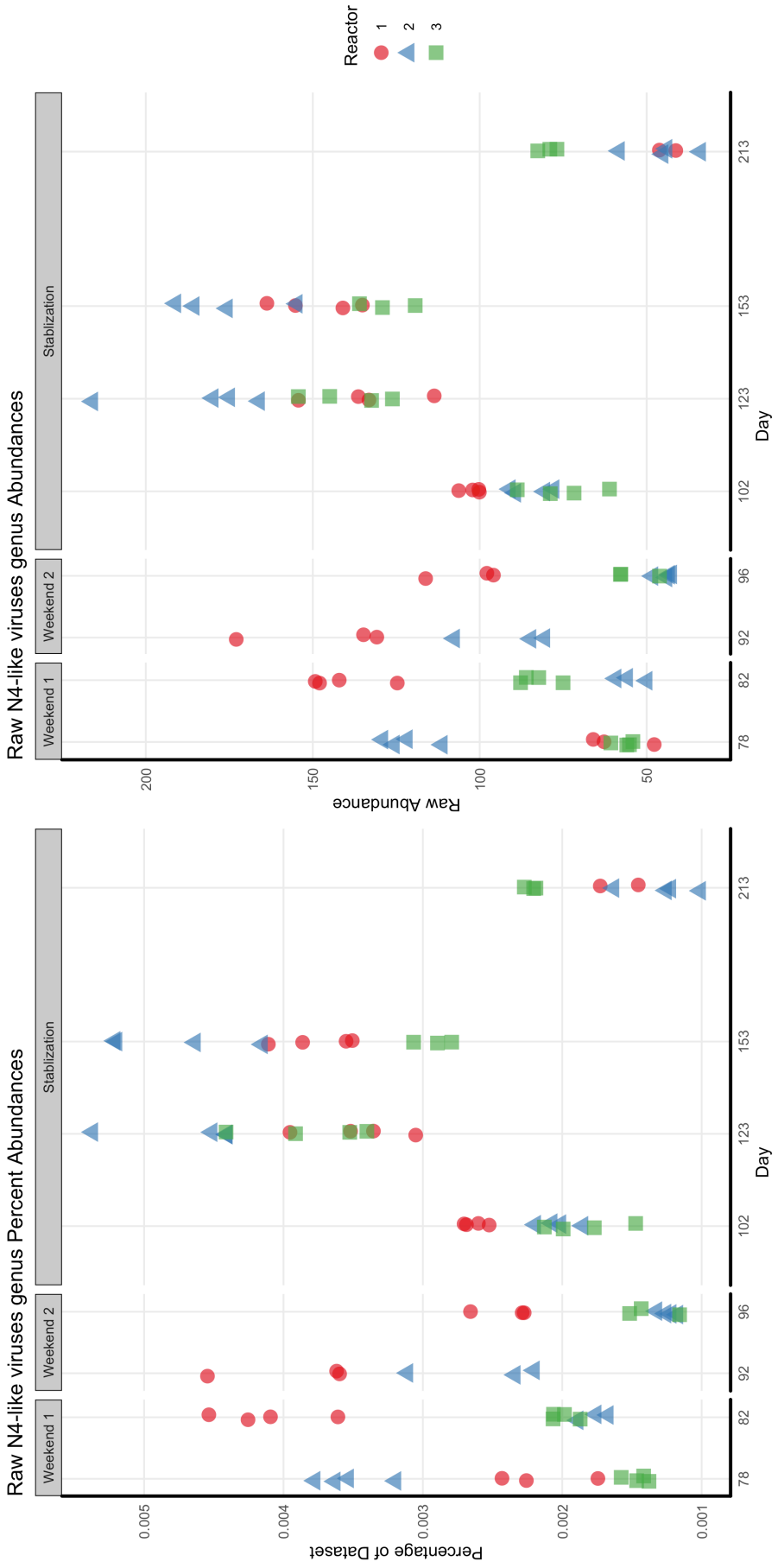


Figure 4.14: Raw N4-like virus genus abundance and percent abundances in samples from triplicate CSTRs at eight key time points.

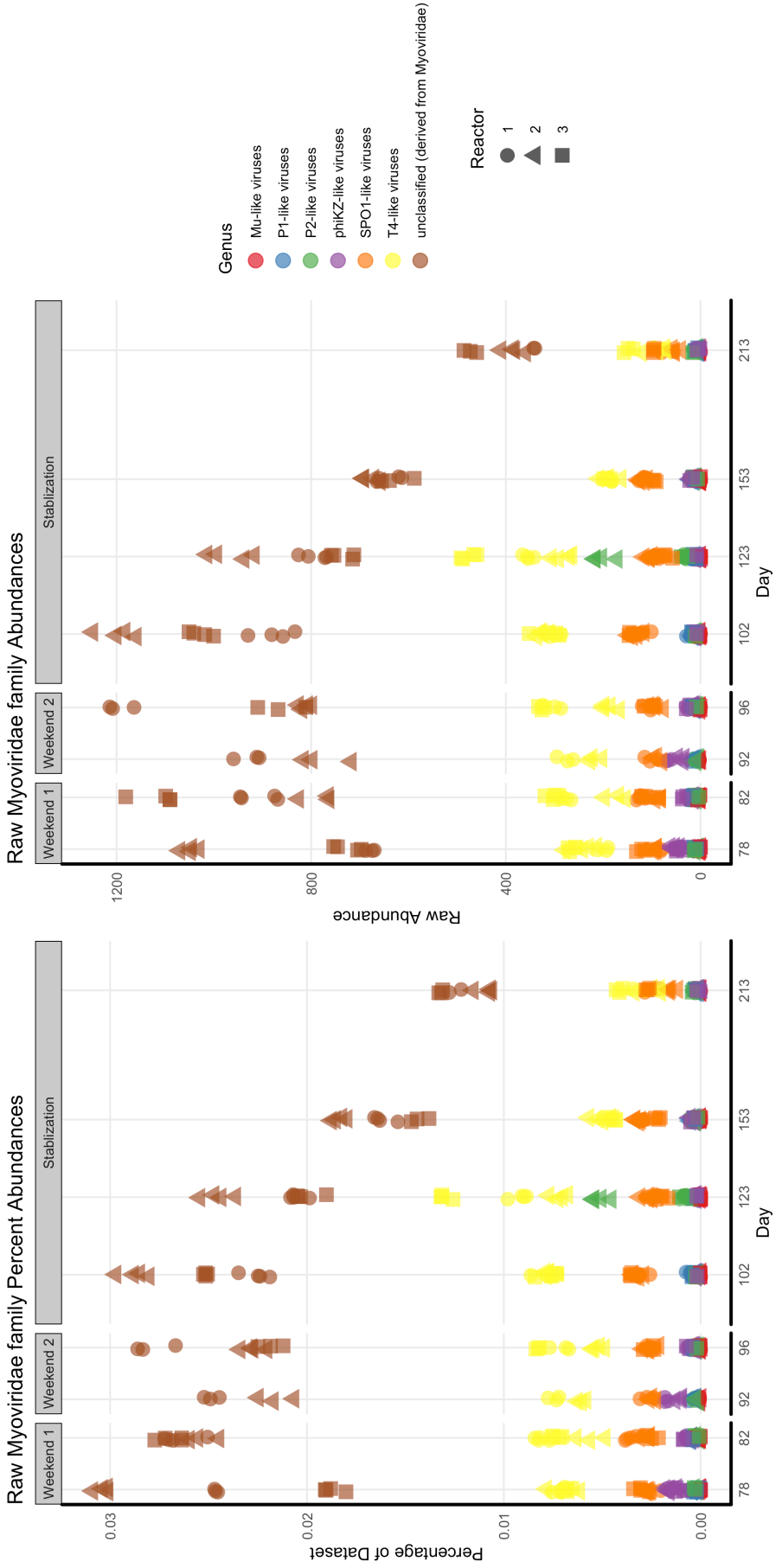


Figure 4.15: Raw *Myoviridae* family abundance and percent abundances in samples from triplicate CSTRs at eight key time points.

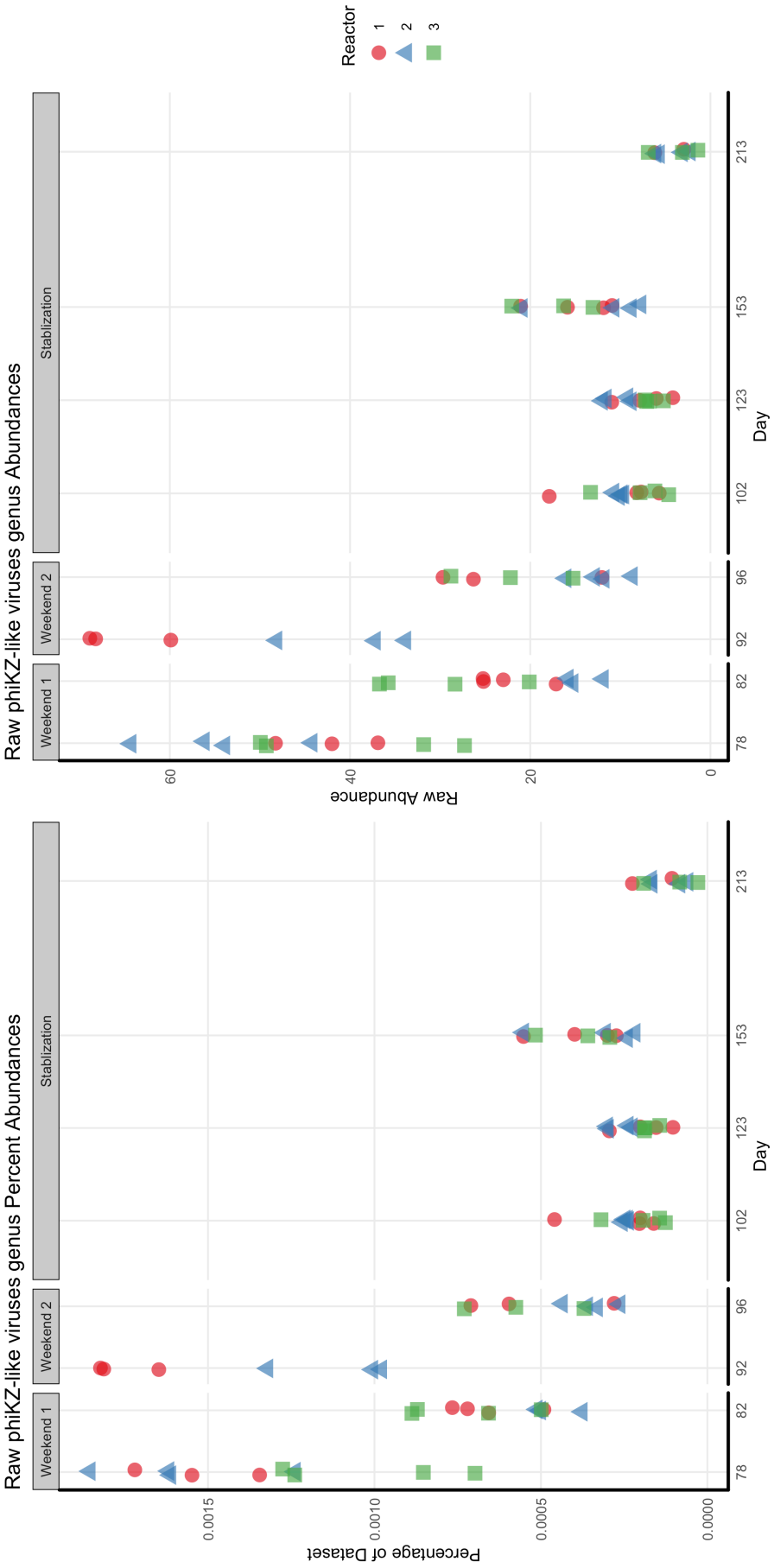


Figure 4.16: Raw phiKZ-like virus genus abundance and percent abundances in samples from triplicate CSTRs at eight key time points.

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One genus of myovirus bacteriophage associated with *Pseudamonas*, phiKZ-like virus (Krylov et al., 2007), appears to be more prevalent during FR1 in samples from daily feeding, which may indicate that manipulation of the AD system for reduction of one potential pathogen or set of pathogens could render unintended consequences for survival of other potential pathogens. This possibility requires further attention, although in this case specifically, *Pseudamonas* spp. are important to substrate degradation in agriculture-based anaerobic digestion (Buettner et al., 2019; Duran et al., 2006), and hence the reduction in phiKZ-like viruses may possibly have contributed to improved *Pseudamonas* survival and consequent improved biogas yield.

4.3.7.2 Fungi and Protozoa

Fungal toxicity has been described as a factor in microbial competition between *E. coli* and for example *Candida albicans* (Cabral et al., 2018), but an initial examination of the dataset did not present significant differences for *Candida* between reactors across time points. The possibility of a fungicidal factor in pathogen sanitisation in anaerobic digestion is worth further examination however, and has not yet received significant attention in the literature.

Protozoan grazing of *E.coli* is a significant factor in some bioremediation systems, responsible for up to 99 % of *E. coli* removal in slow sand filters (Haig et al., 2015). Furthermore, a study of dairy lagoon wastewater attributed 90 % of *E. coli* removal to predation by *Platyophyra* and *Colpoda* (Ravva et al., 2010), but initial analysis of the dataset did not identify either protozoa. Relative abundance of one protozoan genus known for bacterivory, *Tetrahymena* (Gurijala & Alexander, 1990), did increase over time by 30 - 40% in line with the change in feeding regime. The significance of this increase and the extent of its association with concomitant *E. coli* removal requires further examination.

4.4 Conclusions

FIB removal and methane production can be improved through feeding regime manipulation.

This chapter has presented results from a 216-day laboratory scale trial, which aimed to examine the potential for process optimisation, both in terms of methane yield and sanitisation in 10 L continuously stirred tank reactors processing dairy cattle slurry with fats, oils and grease. Having observed that FIB removal is not sufficient in mesophilic AD fed daily (despite reductions when compared with unprocessed slurry), and having observed improved *E. coli* removal using a longer feeding interval, feeding regime was selected as the operational parameter to be manipulated, whilst maintaining all other conditions (temperature, mixing rate, OLR, HRT, feedstock type and ratio). Following a perturbation period in the aftermath of changing to intermittent (3-day) feeding, methane production per gram VS fed increased significantly and EU standards for *E. coli* sanitisation (< 1,000 cfu g^{-1}) were consistently achieved.

Semi-continuous feeding (3-day) improves microbial community diversity and abundance compared with daily feeding. In particular, increased relative abundances of important fatty acid degrading bacteria (*Syntrophomonas*) and hydrogenotropic archaea (*Methanospirilium*) correlated closely with improved methane yield, possibly resulting from improved microbial-mediated conversion efficiencies. Future work on this dataset will examine function as well as delving deeper into taxonomy.

Bacteriophage may play an important role in reducing *E. coli* in agricultural AD systems and their prevalence and potential for manipulation requires further attention.

Having demonstrated the potential for optimisation of agriculture-based AD, both for increased sanitisation and increased renewable energy output, it is necessary to examine the pollution mitigation potential in the field following landspreading of digestate as fertiliser.

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Chapter 5

Landspreading with co-digested cattle slurry, with or without pasteurisation, as a mitigation strategy against pathogen, nutrient and metal contamination associated with untreated slurry

Having established the potential for agricultural-AD to reduce pathogen survival and transmission to the environment, it was necessary to carry out field trials examining the extent, if any, to which anaerobic digestion of slurry mitigates environmental contamination when landspread as an organic ferilitser/soil improver.

I wrote this paper with the help of my supervisors and collaborators, having carried out the field trials and data analysis. Camilla Thorn assisted with data analysis and visualization. The paper presented here is the manuscript as published in *Science of the Total Environment* using their typesetting template, in accordance with NUIG's requirements. It was published in July 2020 (Nolan et al., 2020).

5.1 Landspreading manuscript

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Landspreading with co-digested cattle slurry, with or without pasteurisation, as a mitigation strategy against pathogen, nutrient and metal contamination associated with untreated slurry

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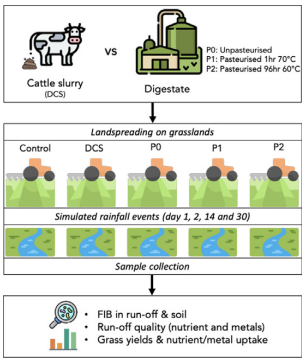
HIGHLIGHTS

- Co-digesting cattle slurry with food processing waste mitigates environmental impacts.
- Lower microbial, nutrient and metal concentrations in runoff from digestate compared with slurry.
- Reduced microbial runoff from digestate was the most prominent difference compared with slurry.
- Pasteurisation further improved the environmental benefits of amending soils with digestate.

GRAPHICAL ABSTRACT



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ABSTRACT

North Atlantic European grassland systems have a low nutrient use efficiency and high rainfall. This grassland is typically amended with unprocessed slurry, which counteracts soil organic matter depletion and provides essential plant micronutrients but can be mobilised during rainfall events thereby contributing to pathogen, nutrient and metal incidental losses. Co-digesting slurry with waste from food processing mitigates agriculture-associated environmental impacts but may alter microbial, nutrient and metal profiles and their transmission to watercourses, and/or soil persistence, grass yield and uptake. The impact of EU and alternative pasteurisation regimes on transmission potential of these various pollutants is not clearly understood, particularly in pasture-based agricultural systems. This study utilized simulated rainfall (Amsterdam drip-type) at a high intensity indicative of a worst-case scenario of ~11 mm hr^{-1} applied to plots 1, 2, 15 and 30 days after grassland application of slurry, unpasteurised digestate, pasteurised digestate (two conditions) and untreated controls. Runoff and soil samples were collected and analysed for a suite of potential pollutants including bacteria, nutrients and metals

Abbreviations: ABP, Animal by-products; AD, Anaerobic Digestion; °C, Celsius; DAFM, Department of Agriculture, Food and the Marine (Ireland); DCS, Dairy cattle slurry; EU, European Union; FIB, Faecal indicator bacteria; K, Potassium; MPN, Most probable numbers; N, Nitrogen; P, Phosphorus; TC, Total Carbon; XRF, X-ray fluorescence.

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Anaerobic digestate
Pasteurisation

following rainfall simulation. Grass samples were collected for three months following application to assess yield as well as nutrient and metal uptake. For each environmental parameter tested: microbial, nutrient and metal runoff losses; accumulation in soil and uptake in grass, digestate from anaerobic co-digestion of slurry with food processing waste resulted in lower pollution potential than traditional landspreading of slurry without treatment. Reduced microbial runoff from digestate was the most prominent advantage of digestate application. Pasteurisation of the digestate further augmented those environmental benefits, without impacting grass output. Anaerobic co-digestion of slurry is therefore a multi-beneficial circular approach to reducing impacts of livestock production on the environment.

1. Introduction

In North Atlantic Europe, grassland systems have a low nutrient use efficiency (~23% for N; Buckley et al., 2013) and high rainfall (>800 mm per annum). Historically, these grassland systems have received both inorganic and organic fertilizers. The use of cattle slurry over inorganic fertilizer has many advantages e.g. it counteracts soil organic matter depletion (Bhattacharya et al., 2016), thereby enhancing soil health (Larkin, 2015) and also provides essential plant micronutrients (Nikoli and Matsi, 2011; Slepetiene et al., 2020). However, land applied cattle slurry can become temporarily mobilised during rainfall events thereby contributing to pathogen, nutrient (nitrogen (N) and phosphorus (P)) and metal incidental losses along surface and subsurface pathways (Clagnan et al., 2019, 2018; Misselbrook et al., 1995; Peyton et al., 2016; Roberts et al., 2017).

Pre-treatment of organic fertilizers before land application attempts to mitigate such transmission of pollutants to the environment. For example, anaerobic digestion (AD) of slurry captures methane that would otherwise be emitted during storage and landspreading, thereby reducing overall greenhouse gas emissions (Amon et al., 2006). However, as slurry has a relatively low biomethane potential, it is typically necessary to co-digest with energy crops, and/or to take in the organic fraction of municipal waste, food waste or wastes arising from the processing of food (fats, oils and grease; belly grass; fish offal etc.) to ensure feasibility (Clemens et al., 2006). Utilization of externally sourced waste streams may introduce pathogens not typically found in agriculture. Digestate resulting from AD of these waste streams is landspread, potentially increasing the risk to human and animal health. Pathogenic microorganisms may survive the AD process, depending on the type of organism, the initial concentration of the organism and AD conditions, particularly temperature (Jiang et al., 2020; Sahlström, 2003; Strauch, 1991). Retention time and feedstock composition also play a role (Chen et al., 2012; Jiang et al., 2020; Nolan et al., 2018; Smith et al., 2005) along with mixing efficacy and the extent of bypass flow (Smith et al., 2005). Moreover, further pathogen die-off may occur during digestate storage before spreading (Luo et al., 2017; Paavola and Rintala, 2008).

Although digestate from AD consistently demonstrates reduced pathogen load compared with undigested slurry, the additional risk of cross-contamination between food production facilities and farms prompted European Union (EU) legislation requiring a pasteurisation step (70 °C for 1 h) in AD systems importing animal by-products (ABP) (Directive No. 142/2011). An additional allowance was made for Member States to introduce national legislation, provided that it achieved the same reductions in faecal indicator bacteria (FIB) numbers. To this end, the Department of Agriculture, Food and the Marine (DAFM) in Ireland, introduced the National Transformation parameter of pasteurisation at 60 °C for 96 h (DAFM, 2014), which was historically applied to composting. Thus, Irish AD plants handling ABP may use either the EU or national pasteurisation standard.

AD of slurry breaks down complex organic compounds, converting carbon to biogas, and mineralising N compounds to NH_4^+-N, potentially enhancing N-availability (Weiland, 2010) and soil N_{Org}-mineralisation when compared with unprocessed slurry (Möller and Müller, 2012). The impact of pasteurisation on the nutrient concentration and risk of runoff following landspreading must also be considered. While Ware and Power (2016) demonstrated increased bioavailability of soil organic matter following pasteurisation of slaughterhouse waste, the impact of digestate pasteurisation on nutrient concentration and availability has not been clearly established.

In an effort to characterise bio-based fertilizers and their impact on the environment and human health, a toolbox of techniques must be deployed during field experiments. The first of these tools are mobile, field rainfall simulators, which can be deployed to simulate heavy rainfall and attendant runoff from which to examine edge of field runoff losses of faecal indicator bacteria, nutrients and metals at several time-points (24 h, 48 h etc.) after application (Peyton et al., 2016). Such losses represent "worst case" scenarios and do not factor in attenuation further along the transfer continuum. FIB are non-pathogenic indicators of faecal contamination and as such can be more safely and easily monitored to assess risks of pathogenic infection to humans and animals in field environments (Kay et al., 2008; Oliver et al., 2009). Another tool is temporal soil and crop sampling with x-ray fluorescence (XRF) analysis to determine metal uptake in soil and plant tissues (Daly and Fenelon, 2017).

The combined positive impacts of AD (energy, carbon capture etc.) may suggest the need to mandate processing of slurry in an AD plant prior to landspreading, in line with European Union (EU) Circular Economy and European Green Deal goals of improved environmental and climate performance (COM, 2019). However, as studies to date have focused on individual environmental impacts of grassland soil amendment with unprocessed slurry compared with digestate (typically either microbial or nutrients or metals), a comprehensive approach considering multiple possible emission sources together is essential to facilitate drafting of appropriate, informed policies. Furthermore, the impact of mandatory pasteurisation to EU or Member State alternative standards on concentration of pollutants in runoff, soil persistence or grass crop is not generally understood, particularly in digestate from the same source.

Thus, the aim of this study was to undertake a comprehensive examination of FIB, nutrients and metal concentration in soil, transmission in runoff and uptake in grass after land application of a) unprocessed slurry and b) slurry co-digested with FW in an anaerobic digester, without pasteurisation and with pasteurisation at c) 70 °C for 1 h (EU Standard) and d) 60 °C for 96 h (DAFM Standard), with all of these treatments being compared with e) untreated controls. To achieve this aim, 20 micro plots were established and examined for: microbial, nutrient and metal load in runoff resulting from simulated rainfall; microbial, nutrient and metal retention in soil; nutrient and metal uptake in grass.

Hypothesis tested: microbial, nutrient and metal concentrations are lower in runoff, soil and grass following application of unpasteurised and pasteurised digestate (2 conditions) from co-digestion of slurry with FW compared with unprocessed slurry, without negatively impacting grass yield.

2. Materials and methods

2.1. Field site characterisation

The study site was a 0.6-ha mid-slope, non-grazed plot located on the beef farm at Teagasc, Johnstown Castle Environment Research

Centre, Co. Wexford, in the southeast of Ireland (latitude 52.293415, longitude −6.518497). The area has a cool, maritime climate, with an average temperature of 10.1 °C and mean annual precipitation of 879 mm. The site has been used as a grassland sward for over 25 years with organic and inorganic nutrient inputs applied as necessitated by routine soil testing. The site has undulating topography with average slopes of 6.7% along the length of the site and 3.6% across the width. The field is moderately drained with a soil texture gradient of clay loam to sand silt loam, as classified by Brennan et al. (2012). As phosphorus (P) index is used as the limiting factor in organic fertilizer amendment, to determine treatment loading rate, composite 10 cm soil cores from each section ($n = 20$) were analysed for Morgan's P (Pm) using Morgan's reagent (Morgan, 1941; Table 4).

2.2. Micro-plot installation

Micro-plots have been used in several field studies to facilitate precise rainfall simulation and collection of all runoff from each plot (Bochet et al., 2006; Brennan et al., 2012; Gillingham and Gray, 2006; Healy et al., 2017; McConnell et al., 2013; Peyton et al., 2016). Micro-plots represent edge of field runoff losses in worst case scenarios, and results from micro-plots have been validated as proxies for field-scale trials (Larsbo et al., 2008). Twenty grassland micro-plots were isolated using stainless steel frames, hammered into the soil to a depth of 50 mm (Fig. 1). Each micro-plot was 0.4 m in width and 0.9 m in length (0.36 m^2), oriented with the longer dimension in the direction of the slope. The frames isolate each plot at the back and sides, and include a runoff channel at the front with a spout for runoff to drain into sample cups (Fig. 1). Once installed, the front rim was sealed to avoid by-pass flow, and any soil disturbed during construction was washed away.

2.3. Soil characterisation

Composite soil core (10 cm) samples were taken from the four corners outside each plot prior to treatment (t_0), and within each plot post-treatment (Day 15 and Day 30), prior to the rainfall simulation on those days. Representative subsamples for each plot were used for soil physico-chemical characterisation including dry matter (105 °C for 24 h) and soil pH, which was determined using a 2:1 ratio of deionised water to soil (Peyton et al., 2016). Samples were ground to 2 mm before being analysed for total P (TP) using the microwave-assisted acid digestion method (US EPA, 1996). Total nitrogen (TN) and total carbon (TC) were determined using the high-temperature combustion method by a LECO TruSpec CN analyser (Table 4). Soil concentrations of Al, Fe, Ca and trace metals (cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb), zinc (Z)) were determined using an Agilent 5100 synchronous vertical dual-view inductively coupled plasma optical emission spectrometer (Agilent 5100 ICP-OES) following the microwave-assisted acid digestion method (US EPA, 1996; Supplementary Table 1).

Additionally, composite samples were tested for the presence and enumeration of FIB, namely total coliforms, *E. coli* and enterococci. Samples were suspended in sterile deionised water (1:9 w/vol), vortexed briefly and shaken in an end-over-end shaker for 30 min. Following serial dilution, most probable numbers (MPN) of total coliforms and *Escherichia coli* were quantified using IDEXX Colisure with Quanti-Tray/2000 incubated at 35 °C for 24 h. MPN of enterococci were determined using IDEXX Enterolert kit with Quanti-Tray/2000 incubated at 41 °C for 24 h (Table 4).

2.4. Treatment characterisation

Five treatments were examined in this study: untreated controls; dairy cattle slurry (DCS); and three types of AD digestate, namely unpasteurised (P0); pasteurised for 1 h at 70 °C (P1) and; pasteurised, 96 h at 60 °C (P2). All digestates were sourced from the same semi-continuously fed, mesophilic, continuously stirred tank bioreactors, which were co-digesting DCS with waste from a food processing facility (FW). DCS was collected from a dairy farm in Co. Galway, Ireland, following mechanical agitation of the underground slurry tank. Fresh DCS and digestates were collected in sealed, 10 L-capacity plastic storage containers and transported to the field site location where they were briefly stored at 4 °C prior to application.

Dry matter was determined by drying fresh samples in an oven at 105 °C for 24 h, after which samples were placed at 550 °C for 2 h in a furnace to determine organic matter (loss on ignition). Treatment pH was determined with a pH meter (Mettler-Toledo Inlab Routine). Following freeze drying and microwave-assisted acid digestion (US EPA, 1996), samples ($n = 3$) from the four treatments were analysed for concentrations of nutrients (phosphorus (P), potassium (K), magnesium (Mg), sulphur (S), sodium (Na), and calcium (Ca)), and metals (arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb), zinc (Zn), aluminium (Al), iron (Fe), cobalt (Co), molybdenum (Mo) and manganese (Mn)) using an Agilent 5100 synchronous vertical dual view inductively coupled plasma optical emission spectrometer (Agilent 5100 ICP-OES). Freeze dried samples were also analysed for TC and N using a LECO TruSpec CN analyser. The FIB numbers in the

Fig. 1. Depiction of experimental set-up, using micro-plots (40 × 90 cm) to which different treatments (slurry or digestate) were applied before plots were subjected to a number of rainfall simulation events, where specialized frames allowed collection of runoff water.

four treatments were determined as outlined for soil (Table 1). Prior to application, all treatments were thoroughly mixed to re-suspend solids.

2.5. Treatment application and replication in micro-plots

The five treatments (untreated control, DCS and three digestates; P0, P1 and P2), used in this study were each replicated four times (5×4) through assignment to 20 micro-plots. These were divided into four 'replicate blocks' each containing five micro-plots to which one of the five treatments was randomly assigned. To aid logistics of rainfall simulation, sample collection and processing, replication was performed over time, with one week between the application of treatments to each of the four treatment blocks.

Application of DCS and digestate to the micro-plots was governed by the P content of the treatments and the P index of the soil. For comparable results, all micro-plots were classified into Index 2 P soil, which meant that all treatments were applied to all plots at a rate of 40 kg P ha^{-1} (Wall and Plunkett, 2016). As a result of the P content (highest in P2 treatment) and the DM of each individual digestate, application rates per individual plot were 1644 g of P0, 1547 g of P1 and 1440 g of P2. The DCS was spread at 3830 g per individual plot.

DCS and digestate were surface applied in rows to each micro-plot using a watering can to replicate normal trailing shoe application (Fig. 1). To ensure even distribution, each micro-plot was divided into four quadrants (each 0.09 m^2 in area) and a proportionate amount of treatment was applied in each quadrant.

2.6. Rainfall event simulation and application

As replication was performed over time (one week between each), 'rainout' shelters and a rainfall simulator were used to ensure each replicate run received the same rainfall (Fig. 1). This also allowed regulated runoff from the plots for comparative assessment of nutrient, metal and FIB load in runoff. In order to simulate rainfall events with controlled intensity and duration, an Amsterdam drip-type rainfall simulator, similar to that described by Bowyer-Bower and Burt (1989) was used, with the addition of wheels for easier movement. It was designed to form droplets with a median diameter of 2.3 mm, spaced 30 mm apart in a 1000 mm × 500 mm × 8 mm Perspex plate over a 0.5 m^2 simulator area. The rainfall simulator was calibrated to deliver a rainfall intensity of 11 mm hr^{-1}, as was the case in other studies such as Peyton et al. (2016).

For better control of rainfall simulations and to prevent runoff losses caused by natural rainfall events, individual micro-plots were covered from the time of treatment application to the end of the third rainfall event by 'rainout' shelters (large plastic shelters on steel frames that prevent direct rainfall onto soil, while allowing air circulation). The first rainfall simulation event (RS1) occurred 24 h after treatment application, so as to demonstrate losses representative of a worst-case breach of regulations which stipulate that spreading of organic manure should not be carried out within 48 h of forecast heavy rain. The second rainfall event (RS2) was performed 48 h after initial application, which was representative of current legislation, the third (RS3) after 15 days and the fourth (RS4) 30 days after initial application, representing normal animal exclusion time from treated fields.

Volumetric moisture content (MC) of the soil in each plot ($n = 3$) was measured immediately prior to and after each rainfall event using a time domain reflectometry device (Delta-T Devices Ltd., Cambridge, UK), which was calibrated to measure resistivity in the upper 50 mm of the soil in each plot.

2.7. Runoff sample collection and analysis

Surface runoff was deemed to occur once 50 mL of water was collected in the sample collection cup. The collection of the first 50 mL ($t = 0$) was used to indicate time to runoff (TR), and was used for part of the microbial analysis. Samples for nutrient and metal analysis were collected every 10 min ($t = 10$, t = 20, $t = 30$) from TR to allow for the flow weighted mean concentration (FWMC) to be calculated (Brennan et al., 2012; Peyton et al., 2016). Following this, another 50 mL of surface runoff water was collected so that it could be combined with the first 50 mL of runoff to create a 100 mL composite sample for microbial analysis. The rainfall simulator was then switched off and a final sample was collected until no runoff occurred, to determine the final runoff ratio. Immediately after collection, all samples were stored in cool boxes with ice until they were returned to the laboratory for analysis.

The 100 mL composite samples designated for FIB analysis were serially diluted with sterile water and analysed using kits as described for soil (Section 2.3). An aliquot of each runoff water sample was filtered through 0.45 μm filter paper and a sub-sample was analysed calorimetrically for P, nitrite (NO_2^-N), dissolved organic N (DON) and ammonium (NH_4^+–N) using a nutrient analyser (Aquachem Labmedics Analytics, Thermo Clinical Labsystems, Finland). Unfiltered runoff water samples were analysed for total P with an acid persulphate digestion as well as P, total C, total N and total organic carbon (TOC) using the Aquachem Analyser. Metal and nutrient analysis (Al, Ca, Cd, Cl, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb and Zn) was carried out on the filtered samples using inductively coupled plasma optical emission spectroscopy (ICP-OES). All samples were tested in accordance with the Standard Methods (APHA, 2005).

2.8. Grass sample collection and analysis

Prior to treatment application, the grass on all micro-plots was cut to 50 mm. Nitrile gloves were worn during sample collection, and were changed between plots to avoid cross-contamination. Thereafter, grass was collected on days 14, 30, 57, 85 and 112 to determine yield as well as metals and nutrient uptake. Collected grass was weighed, then dried in an oven at 60 °C for 48 h to determine solids content. Dried samples were ground and analysed using energy-dispersive X-ray fluorescence (EDXRF) spectroscopy as described by Daly and Fenelon (2017), using a Rigaku NEX CG EDXRF spectrometer equipped with a nine-place sample changer with spin function using slow and steady spinning mode. Grass samples were analysed for % N, P, K, S, Ca, Na, Mg, as well as mg/kg of Al, As, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, and Zn using EDXRF as described by (Daly and Fenelon, 2017, 2018). As higher levels of Mo were detected as function of some treatments, the ratio of Cu:Mo was calculated in order to determine if these levels were toxic to animals.

2.9. Data analysis

The data was a blocked one-way classification (5 treatments) with repeated measures over the course of experimental time (corresponding to rainfall simulation events for runoff and soil data) (Peyton et al., 2016). Variables measured for each sample type collected, and those grouped together for statistical analyses and graphical representation are detailed in Supplementary Table 2. Data were tested for normality (Shapiro Wilk test), and some variables were identified as non-normally distributed. Therefore, for each dataset a Friedman test (repeated measures for non-parametric data) was performed, to determine if differences were seen as a function of treatment. If differences were seen, then for each variable, at each sample day, statistical differences in means as a function of treatment were tested using the non-parametric Kruskal-Wallis test. Where statistical differences were seen ($p < 0.01$), a post-hoc test was performed using Fisher's Least Significant Difference (LSD). Where multiple variables were presented on the same figure, p-values were corrected for multiple comparisons using the false discovery rate (FDR) approach. Statistical tests were performed as implemented in the agricolae package (de Mendiburu, 2020), while data was plotted using ggplot2 (Wickham, 2016) in R (R Core Team, 2019). Points on all plots represent the mean of each variable

Table 1
FIB (log_{10} cfu/g), organic matter, N, P, K and S analysis of four treatments, with standard error (n = 4). For each variable, shared letters denote no difference while different letters denote statistically significant differences ($p < 0.05$) as a function of treatment.

	Coliforms	*E. coli*	Enterococci	OM	N	P	K	S
	log_{10} cfu/g	log_{10} cfu/g	log_{10} cfu/g	%	%	%	%	%
Slurry	6.7 ± 0.57^{a}	6.6 ± 0.61^{a}	6.0 ± 0.11^{a}	8.61 ± 0.3^{a}	2.53 ± 0.05^{a}	0.4 ± 0.01^{a}	6.12 ± 0.05^{a}	0.47 ± 0.01^{a}
Digestate P0	$3{,}2 \pm 0.76^{b}$	2.8 ± 1.73^{b}	3.3 ± 0.13^{b}	6.41 ± 0.04^{b}	2.62 ± 0.1^{a}	1.34 ± 0.16^{b}	3.39 ± 0.5^{b}	0.60 ± 0.02^{b}
Digestate P1	0.5 ± 0.50^{c}	0.0 ± 0.00^{c}	2.8 ± 0.59^{c}	6.35 ± 0.08^{b}	2.48 ± 0.02^{a}	1.53 ± 0.01^{c}	2.87 ± 0.02^{b}	0.63 ± 0.01^{c}
Digestate P2	0.0 ± 0.00^{c}	0.0 ± 0.00^{c}	1.7 ± 1.18^{d}	6.26 ± 0.07^{c}	2.35 ± 0.08^{a}	1.47 ± 0.01^{b}	2.75 ± 0.01^{c}	0.62 ± 0.01^{b}

over the 4 repeated rainfall simulations performed and error bars show standard error of the mean ($n = 4$). Letters within the points illustrate statistical differences as a function of treatments at a given time point, where shared letters denote no difference ($p > 0.05$), and unshared letters denote a statistical difference within a group ($p < 0.05$).

3. Results and discussion

3.1. Microbial load of the four organic amendments tested

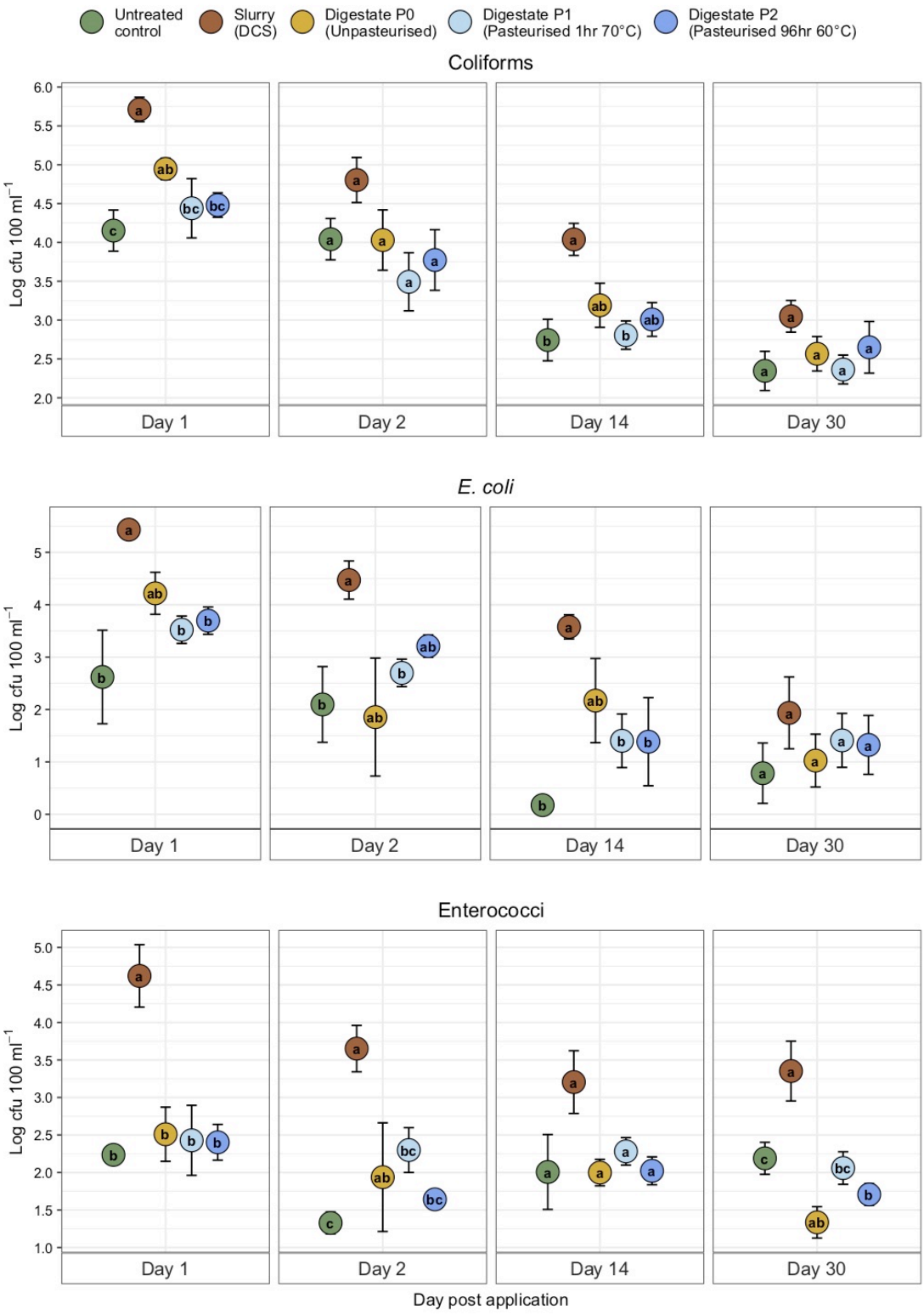
The unprocessed DCS contained significantly higher numbers of all three FIB tested prior to application (Table 1). Processing in AD without pasteurisation resulted in >4 log_{10} reduction of coliforms and *E. coli*, as well as a 2 log_{10} reduction in enterococci numbers. The results above are in line with previously reported sanitization effects of AD on slurry (Nag et al., 2019; Sahlström, 2003). This reduction in FIB partially results from a dilution effect, whereby slurry with high FIB is mixed with FW with low FIB, and then fed into an anaerobic digester with low background FIB numbers, but this does not account for the total FIB decrease (Nolan et al., 2018). Furthermore, studies of pathogen survival in mesophilic anaerobic digestion, have consistently identified a significant sanitization effect beyond that of dilution, attributed to free volatile fatty acid and free ammonia concentration and competition for resources (Jiang et al., 2020; Nolan et al., 2018; Sahlström, 2003; Smith et al., 2005; Zhao and Liu, 2019). Protozoan grazing and fungal antimicrobial activity are also factors that have not received much attention (Avery et al., 2014), while the major role played by bacteriophage in other bacterial spheres makes it a good candidate for further research with a view to manipulation for improving sanitization effect. However, the extent of pathogen inactivation is affected by a variety of physical factors as outlined previously, and hence a precautionary pasteurisation step has been deemed necessary. Pasteurisation at both the EU and Irish standards (P1 and P2 respectively) resulted in a further reduction of coliforms and *E. coli* below the limit of detection (100 cfu g^{-1}) with no significant difference between pasteurisation conditions. Enterococci were reduced by both pasteurisation conditions below the 1000 cfu g^{-1} required for landspreading, but proved to be more resilient to pasteurisation than *E. coli*, and hence a more conservative indicator bacteria where pathogen persistence is a concern, as previously highlighted in the literature (Nolan et al., 2018; Sahlström, 2003).

3.2. Microbial load in runoff and persistence in soil

At every time point post-application (24 h and 2, 15 and 30 days), all FIB tested were highest from the plots treated with DCS (Fig. 2). Coliform and *E. coli* numbers were significantly higher in runoff from slurry treated plots (6.43 ± log_{10} per 100 mL) than plots treated with pasteurised digestate (both conditions) and untreated controls after 24 h. Coliform numbers in runoff from rainfall simulation 24 h post-treatment displayed a lower trend for unpasteurised digestate (P0) than slurry-amended plots. High mobilization of FIB during the first rainfall event may be attributed to solubilization of unattached cells in the organic amendments (Chadwick et al., 2008), and likely would have been lower had FIB been exposed to sunlight for more than one day (Oladeinde et al., 2014). By the second rainfall simulation (2 days post-application), coliform and *E. coli* numbers in all digestate treatments aligned with the untreated controls, while *E. coli* numbers from DCS-treated plots were significantly higher. These results agree with those observed by (Peyton et al., 2016), who reported more resilient coliform survival in DCS than in biosolids, resulting in higher numbers in runoff from rainfall simulations 15 days post application. Although *E. coli* numbers in runoff declined steadily over time, by Day 30 runoff from slurry-treated plots still contained higher *E. coli* numbers than those recorded from untreated controls after the first rainfall simulation (465 versus 188 cfu per 100 mL). Background coliform and *E. coli* in the untreated control plots decreased over time, indicating that heavy rainfall events could wash out residual low-level indicator organisms.

The difference between treatments was most stark for enterococci (Fig. 2, panel 3), which were significantly higher (2–3 log_{10} cfu 100 mL^{-1}) in runoff from slurry-treated plots than all other treatments, which would be expected given the >2 log_{10} higher starting numbers in slurry (Table 1). Amendment with digestate (pasteurised or unpasteurised) did not increase enterococci levels in runoff above those recorded for the untreated controls. The high (>5 log_{10} cfu 100 mL^{-1}) enterococci numbers observed in runoff from Day 1 rainfall simulation highlights the importance of adherence to regulations around proximity of landspreading with forecast heavy rainfall to prevent incidental losses (Chadwick et al., 2008). Furthermore, the elevated enterococci numbers in Day 30 runoff from slurry-treated plots indicate detachment of residual FIB (Tyrrel and Quinton, 2003), and the prolonged risk requiring remedial action to reduce agricultural impact on the environment through contamination of watercourses resulting from landspreading of raw slurry. Processing slurry in AD would significantly reduce the risk of microbial pollution of watercourses, while pasteurisation may further reduce that risk for bacteria susceptible to heat treatment.

Soil samples were taken prior to application of the treatments, as well as 14 days and 30 days after application. Background FIB numbers in the soil prior to application were higher in some plots, affecting the significance of the results. This was possibly due to historical slurry spreading, as *E. coli* have been found to persist in soil for more than nine years following a single manure application (Brennan et al., 2010). Despite this variability, soil from slurry-amended plots had higher *E. coli* numbers than the digestate-amended plots (pasteurised and unpasteurised: Supplementary Fig. 2 panel B). After 14 days the number of coliforms in the soil was 2 log_{10} cfu g^{-1} higher in slurry-treated soil compared with untreated controls, and 1 log_{10} higher than soil amended with digestate (Supplementary Fig. 2 panel A). There was no significant difference in coliform or *E. coli* levels in soil treated with unpasteurised or pasteurised digestate after 14 days. By Day 30, plots treated with unpasteurised and pasteurised digestate had similar faecal coliform numbers to untreated plots, while slurry-treated plots had at least 1 log_{10} cfu g^{-1} higher survival (Table 3), However, modeling of *E. coli* survival after landspreading of DCS indicates that an additional 10 days (40 day exclusion) would be sufficient to reduce risk to grazing animals (Ashekuzzaman et al., 2018). The results support the findings of Goberna et al. (2011), who similarly observed lower *E. coli* numbers in


Untreated control
Slurry (DCS)
Digestate P0 (Unpasteurised)
Digestate P1 (Pasteurised 1hr 70°C)
Digestate P2 (Pasteurised 96hr 60°C)
Coliforms
E. coli
Enterococci
Log cfu 100 ml⁻¹
Day 1
Day 2
Day 14
Day 30
Day post application

soil treated with digestate than manure 30 days after application, which they attributed to the activity of the indigenous soil microbial community. The results may however also indicate that coliforms and *E. coli* did not infiltrate the soil and were predominantly washed off in runoff during the rainfall simulations. As the main purpose of this study was the runoff pathway, the slope was required. If flat land and well-drained soil were used for the trial the DCS in particular would likely have remained on the surface (due to high solids). In that case as surface application was used, exposure to ultraviolet radiation would likely have reduced FIB numbers in the treatments (Oladeinde et al., 2014), with similar studies finding a decline to background numbers within 17 days (Hodgson et al., 2016).

Enterococci in the soil were higher for slurry and unpasteurised digestate-treated soils on Day 14 than on Day 0, and lower for both pasteurised digestate treatments (Supplementary Fig. 2 panel C). By Day 30 however, enterococci numbers in soil treated with unpasteurised digestate were similar to those for pasteurised digestate and untreated controls. Slurry application resulted in a steady increase in soil-borne enterococci over time, so that by Day 30 enterococci numbers remained higher than all other treatments (Table 3). Desiccation and ultraviolet radiation are the primary factors in microbial survival following landspreading (Lu et al., 2012) and as each plot was subjected to similarly intensive rainfall, the likely differentiating factor contributing to the observed higher survival in DCS is that higher fibrous solids shielded bacteria from UV radiation (Table 1 and Fig. 1),

3.3. Fate of metals and nutrients – soil and runoff concentrations

As hypothesised, nutrient and metal concentrations were found to be lower in runoff and soil following application of unpasteurised and pasteurised digestate (2 conditions) from co-digestion of slurry with FW compared with unprocessed slurry. Application of DCS or digestate (all pasteurisation conditions) did not result in samples exceeding metal limit values in soil tested 14 and 30 days post-application (EU Directive 86/278/EEC), with no substantial differences in soil concentrations observed in that timeframe (Supplementary Table 1). These results are in line with a recent examination of metal accumulation in grassland soils amended with wastewater treatment sludge (Ashekuzzaman et al., 2019). Some metals are required in the AD process (Fermoso et al., 2015: Ca, Co, Cu, Fe, Mo), and the reduced levels of some of those (Ca, Cu, Fe, Mo) found in the digestate used in this trial compared with DCS indicate a reduced risk of accumulation of those elements in the environment following landspreading of digestate compared with slurry. A recent study of cattle slurry and digestate from four full scale Irish AD plants recorded results of elemental analysis over two years and similarly found reduced Cu levels in the AD plant co-digesting slurry with food waste, but no significant difference in Ca or Fe between digestate from that AD plant and slurry (Coelho et al., 2020). Similarly to Coelho et al. (2020) however, some elements (Al, Cr, Ni, Pb, S, Zn) had higher levels in the digestate used in this study compared with DCS, and although there was no significant soil accumulation compared with controls in this trial, elevated Cd, Cr, Ni and Pb soil results from other studies indicate that it may be necessary to incorporate metals into the analysis suite for regular (5 year) soil tests (Dragicevic et al., 2018; Tang et al., 2020). This may be particularly necessary for digestate from plants co-digesting municipal wastewater treatment sludge with food waste, given the comparatively high results for Al, Ca, Cr, Cu, Fe, Pb and Zn reported by Coelho et al. (2020).

Iron (Fe; 200 μg L^{-1}) and manganese (Mn; 50 μg L^{-1}) in runoff samples from DCS, P0 and P2 exceeded EU drinking water limits (Council Directive 98/83/EC; Statutory Instrument, 2014) during the first rainfall simulation. However, landspreading within 24 h of forecast heavy rain constitutes a breach of best practice, and hence an uncommon, worst-case scenario. Fe and Mn limits were again exceeded only in runoff from DCS during RS2 (48 h post-application), while metals in runoff from all other treatments satisfied maximum acceptable concentrations (MACs) or environmental quality standards (EQS) established by regulations such as the Drinking Water Directive and Surface Waters Regulations (EC, 1998; EPA, 2001; EU, 2013; S.I. No. 272/2009;Table 5). Although Peyton et al. (2016) observed no breach of metal limits in runoff from DCS-amended plots, they did not report Fe or Mn results.

Slurry application resulted in significantly elevated Mn in runoff for 15 days post-treatment. Mn in runoff from digestate-amended plots (all pasteurisation conditions) was higher than untreated controls at RS1 (1 day post application), but had returned to the same level as untreated controls 2 days post application (RS2). Digestate application resulted in lower Mn runoff than slurry (69% lower for RS1 and 91% lower for RS2), and on day 14 (RS3) Mn concentrations in runoff from slurry treated plots were still 4 times higher than those from digestate treated and untreated control plots (4.7 ± 2 vs 1.1 ± 0.5 μg L^{-1}). Despite breaching EU limits during RS1 and RS2 (DCS), Mn concentrations from all treatments did not come close to WHO health-based values (400 μg L^{-1}) at any point throughout the trial (WHO, 2006). Although a 2018 survey identified 73 of 104 countries with tighter Mn standards than the WHO, this significant discrepancy may be cause for re-evaluating WHO standards, particularly in agricultural regions where landspreading of unprocessed slurry is practiced, given the breaches observed in the present work (WHO, 2018).

Application of all organic amendments resulted in higher Zn in runoff during RS1 (1 day post application) compared with the untreated control (Fig. 3). The Zn runoff from the slurry-treated plots remained elevated during RS2 (2 days post application) compared with all digestate treatments (21 ± 9 vs $5–8 \pm 0.86$ μg L^{-1}). By Day 30 (RS4), Zn runoff from all treatments had reached background levels seen in the untreated control plots (Fig. 3).

Concentrations of Mg and Ca in runoff were higher from slurry than digestate or untreated controls during RS1, but there was no significant difference thereafter (Fig. 3). Na runoff was higher for all organic amendments during RS1, but returned to the same level as the untreated controls for digestate (P1) by RS2, while slurry-associated Na remained higher in RS2 and RS3. By Day 30 Na concentration in runoff was still significantly higher for slurry, although maximum acceptable concentrations (MAC) were not exceeded from any treatment throughout the trial. Although higher Al was detected in runoff from unpasteurised digestate plots than for slurry and untreated control plots, the results were skewed significantly by high readings in one of the four treatment blocks, as evidenced by the large error bars. This may be associated with the ~15% higher than average Al concentration in soil from that plot prior to treatment application (15.2 ± 0.29 vs 17.7 g/ kg). By 14 days post application (RS3), only runoff from slurry-amended plots contained significantly elevated Al concentrations, and by Day 30 all treatments had fallen to the same level seen in runoff from untreated control levels (Fig. 3.). No organic fertilizer used in this trial resulted in Al runoff concentrations exceeding typical limits (WHO, 2018; 200 μg L^{-1}).

For the elements detected in higher concentrations in digestates used for plot amendments, namely Al (3×), Cr (>2×), Ni (1.5×) and Zn (1.5×), digestate application resulted in levels of these elements similar to that seen in runoff from DCS at RS1 (1 day after application; Table 2). However, while they remained relatively high in DCS-derived runoff, their levels fell rapidly in runoff from digestate (P0, P1 and P2) treated plots (Fig. 3). Peyton et al. (2016) also reported higher runoff of Cr from DCS than from digestate, similarly peaking in runoff during RS1, although their reported peak doubled that of the present work (3.89 vs 1.6 μg L^{-1}). DCS application resulted in significantly

Fig. 2. Faecal indicator bacteria (log_{10} cfu mL^{-1}) in runoff from five treatments from rainfall simulation 1, 2, 14 and 30 days after application. Error bars indicate standard error of the mean ($n = 4$). Numbers of Coliforms, *E. coli* and Enterococci detected in runoff (log10 cfu/mL^{-1}). Statistically significant differences ($p < 0.05$) are represented below each panel, where coloured dots correspond to the treatment against which differences in FIB were significant.

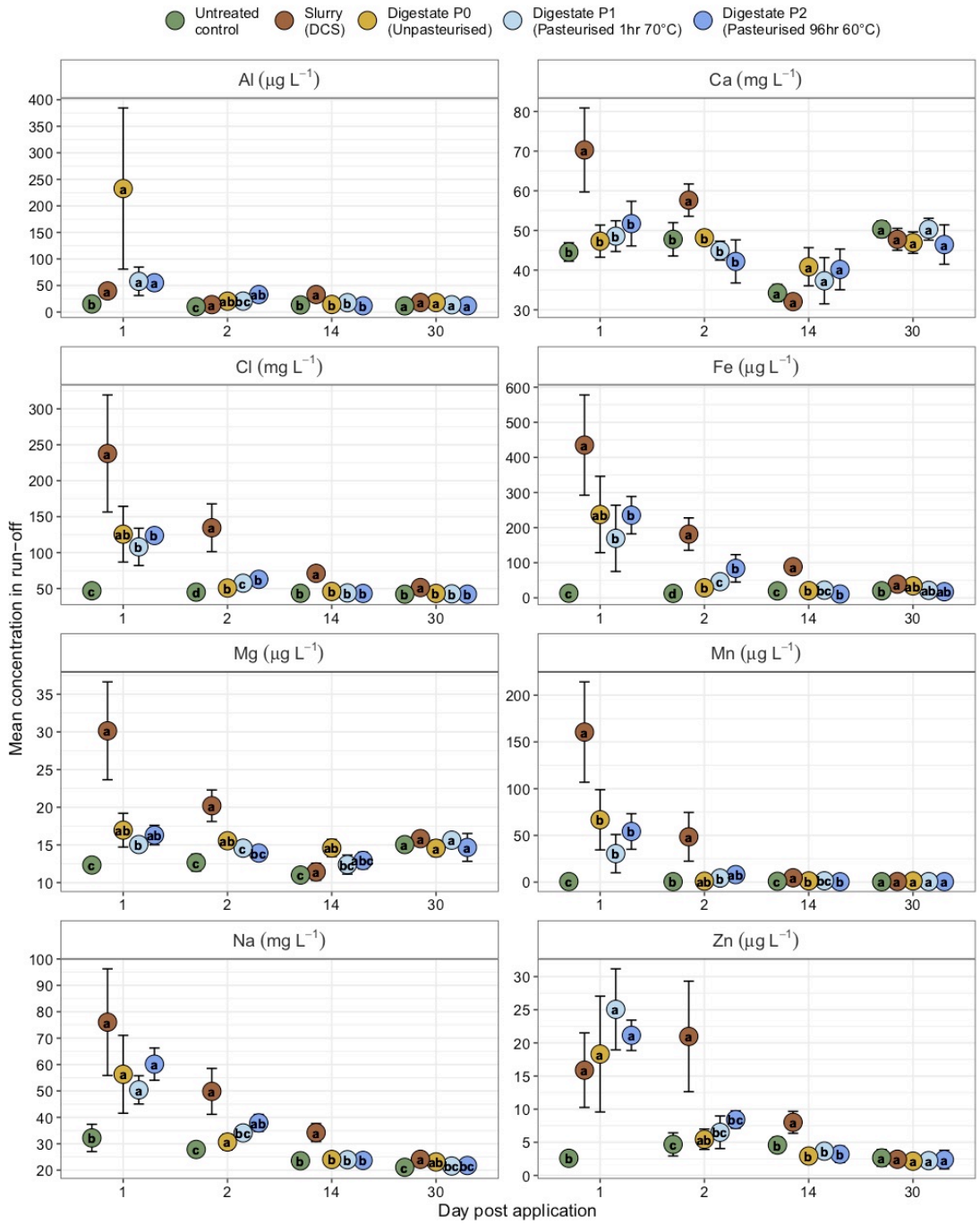


Fig. 3. Al, Fe, Mg, Mn, Zn (µg L^{-1}) and Ca, Cl, Na (mg/L) following five treatments (T1 = untreated control (no organic amendment); T2 = DCS; T3 = unpasteurised digestate; T4 = pasteurised digestate (70 °C); T5 = pasteurised digestate (60 °C)) from rainfall simulation 1, 2, 14 and 30 days after application. Error bars indicate standard error of the mean (n = 4).

higher and sustained runoff of Na, Mn, Mg, Cl, Fe, Ca, than application of digestate with or without pasteurisation. By Day 14 concentrations of metals and nutrients in runoff from all treatments began to equilibrate (with DCS still highest), and by Day 30 there was no significant difference between treatments with the exception of higher Fe from DCS. Dry matter content has been identified as the main determining factor affecting infiltration of slurry into soil (Misselbrook et al., 2006). The reduction of dry matter by anaerobic digestion (Table 1) logically

Table 2
Nutrient and metal analysis of four treatments, with standard error (n = 4). For each element, shared letters denote no difference while different letters denote statistically significant differences ($p < 0.05$) as a function of treatment.

	Al	Ca	Cd	Co	Cr	Cu	Fe	Mg	Mn	Mo	Ni	Pb	Zn
	g/kg	%	mg/kg	mg/kg	mg/kg	mg/kg	g/kg	%	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Slurry	4.1 ± 0.1^{a}	4.6 ± 0.07^{a}	0.37 ± 0.02^{a}	1.39 ± 0.06^{a}	9.8 ± 0.90^{a}	106 ± 1.3^{a}	3.74 ± 0.3^{a}	0.69 ± 0.01^{a}	249 ± 2.6^{a}	9.49 ± 0.15^{a}	9.66 ± 1.0^{a}	3.01 ± 0.05^{a}	106 ± 1.3^{a}
Digestate P0	11.6 ± 1.3^{b}	4.0 ± 0.06^{b}	0.47 ± 0.04^{a}	1.92 ± 0.12^{a}	23.2 ± 2.60^{b}	70.7 ± 5.8^{b}	3.08 ± 0.1^{a}	0.74 ± 0.01^{b}	245 ± 0.48^{a}	7.77 ± 0.28^{b}	15.1 ± 1.2^{b}	4.12 ± 0.09^{b}	143 ± 6.2^{bc}
Digestate P1	12.9 ± 0.1^{b}	4.0 ± 0.02^{b}	0.51 ± 0.02^{a}	2.03 ± 0.04^{a}	26.5 ± 0.17^{c}	65.0 ± 0.5^{b}	3.17 ± 0.01^{a}	0.75 ± 0.01^{b}	246 ± 0.78^{b}	7.48 ± 0.02^{b}	16.9 ± 0.16^{c}	4.11 ± 0.15^{a}	150 ± 0.67^{c}
Digestate P2	12.7 ± 0.1^{ab}	3.8 ± 0.03^{c}	0.49 ± 0.01^{a}	1.98 ± 0.02^{a}	24.6 ± 0.02^{b}	62.6 ± 0.2^{c}	3.04 ± 0.01^{a}	0.71 ± 0.01^{a}	235 ± 0.66^{ab}	7.18 ± 0.05^{c}	15.6 ± 0.19^{b}	3.86 ± 0.09^{ab}	145 ± 1.1^{b}

Table 3
FIB averages ($\log_{10}$ cfu g^{-1}) in soil pre-application of organic fertilizers, 14 days post-application and 30 days post-application (n = 4). For each FIB, on the 3 different sample days, shared letters denote no difference while different letters denote statistically significant differences ($p < 0.05$) as a function of treatment.

	Coliforms			*E. coli*			Enterococci		
Day	0	14	30	0	14	30	0	14	30
Untreated Ctrl	2.7 ± 1.06^{a}	1.6 ± 0.22^{a}	1.9 ± 0.52^{a}	1.5 ± 0.91^{a}	0.7 ± 0.24^{a}	0.3 ± 0.25^{a}	3.2 ± 0.20^{a}	3.5 ± 0.21^{a}	3.1 ± 0.22^{a}
Slurry (DCS)	3.8 ± 0.50^{a}	2.7 ± 0.28^{a}	3.1 ± 0.42^{a}	2.2 ± 0.80^{a}	2.4 ± 0.17^{ab}	2.2 ± 0.22^{ab}	3.1 ± 0.24^{a}	3.4 ± 0.07^{a}	3.5 ± 0.14^{a}
Digestate (P0)	4.1 ± 0.28^{a}	1.8 ± 0.47^{a}	1.7 ± 0.62^{a}	2.5 ± 0.93^{a}	1.5 ± 0.36^{ab}	1.1 ± 0.59^{ab}	3.5 ± 0.20^{a}	3.6 ± 0.28^{a}	3.1 ± 0.17^{a}
Digestate (P1)	3.5 ± 0.61^{a}	1.7 ± 0.55^{a}	2.0 ± 0.61^{a}	2.8 ± 0.60^{a}	0.8 ± 0.55^{b}	0.3 ± 0.25^{b}	3.6 ± 0.22^{a}	3.3 ± 0.09^{a}	3.1 ± 0.04^{a}
Digestate (P2)	3.7 ± 0.49^{a}	1.7 ± 0.30^{a}	2.1 ± 0.56^{a}	1.7 ± 0.55^{a}	1.5 ± 0.21^{b}	0.9 ± 0.33^{b}	3.5 ± 0.18^{a}	3.2 ± 0.20^{a}	3.0 ± 0.17^{a}

facilitates improved infiltration, reducing risk of surface runoff. Although this increased infiltration did not result in statistically significant differences in soil metal concentrations in this study, repeated application may result in accumulation and necessitates regular soil testing, as previously discussed (Nkoa, 2014).

DCS contained 17.5, 18.8 and 15.3% more Fe than unpasteurised, P1 and P2 digestates, respectively (Table 2), yet runoff from DCS-amended plots had twice as much Fe than digestate-amended plots at RS1, 3.4 times more at RS2 and 5 times more at RS3 (88.63 ± 20.4 vs 17.7 ± 3.3 μg L^{-1}) (Fig. 3). By Day 15 there was no difference between digestate (all pasteurisation conditions) and untreated controls. By Day 30 there were no significant differences between organic amendments and untreated controls (Fig. 3). A study examining alum amendment of poultry litter for reduction of incidental P losses observed an attendant significant reduction of Fe in runoff (Moore et al., 1998). The elevated levels of Al in digestate may be in part responsible for the significantly lower Fe in runoff observed from digestate in these trials compared with DCS.

Table 4
C, N, P, K and pH data of soil prior to (Day 0) and following application of DCS, unpasteurised digestate (P0) and pasteurised digestate (two conditions), ± standard error of the mean (n = 4). No statistically significant differences were seen as a function of treatment for any variable on any testing day.

Day	Tmt	C %	N %	P mg/kg	K g/kg	pH
0	Ctrl	3.47 ± 0.15	0.330 ± 0.02	639 ± 22.6	2.44 ± 0.49	5.55 ± 0.02
	DCS	3.68 ± 0.08	0.366 ± 0.01	570 ± 58.0	2.21 ± 0.31	5.51 ± 0.02
	P0	3.52 ± 0.20	0.317 ± 0.02	603 ± 63.3	2.47 ± 0.40	5.54 ± 0.07
	P1	3.64 ± 0.22	0.349 ± 0.02	575 ± 41.3	2.22 ± 0.27	5.49 ± 0.05
	P2	3.68 ± 0.05	0.340 ± 0.00	567 ± 27.8	2.60 ± 0.30	5.54 ± 0.05
14	Ctrl	3.63 ± 0.11	0.348 ± 0.01	634 ± 34.1	2.50 ± 0.26	5.70 ± 0.06
	DCS	3.56 ± 0.18	0.339 ± 0.02	605 ± 45.5	2.76 ± 0.30	5.74 ± 0.06
	P0	3.76 ± 0.24	0.349 ± 0.03	603 ± 49.4	2.55 ± 0.28	5.77 ± 0.08
	P1	3.68 ± 0.14	0.350 ± 0.02	616 ± 37.5	2.51 ± 0.50	5.71 ± 0.08
	P2	3.63 ± 0.16	0.338 ± 0.02	620 ± 34.5	2.80 ± 0.30	5.73 ± 0.10
30	Ctrl	3.69 ± 0.22	0.347 ± 0.02	601 ± 37.8	2.21 ± 0.21	5.75 ± 0.05
	DCS	3.72 ± 0.10	0.346 ± 0.02	582 ± 49.4	2.47 ± 0.42	5.84 ± 0.06
	P0	3.78 ± 0.34	0.344 ± 0.03	598 ± 70.0	2.36 ± 0.28	5.86 ± 0.03
	P1	3.56 ± 0.15	0.337 ± 0.02	601 ± 51.0	2.51 ± 0.27	5.83 ± 0.06
	P2	3.47 ± 0.20	0.311 ± 0.01	560 ± 22.7	2.40 ± 0.42	5.88 ± 0.11

Elemental analysis of runoff from the five treatments showed no significant differences between any treatments for Cd, Cr or Pb in the runoff and these data are therefore not shown. The effect of mandatory digestate pasteurisation on nutrient and metal runoff potential has not been considered in the literature, and the present work found no significant impact on nutrient and metal runoff.

Incidental P losses account for between 50 and 90% of all P losses from soil to water (Withers et al., 2003) and occur when slurry is spread in close proximity to a heavy rainfall event, allowing insufficient time for slurry to infiltrate soil (Brennan et al., 2011). Digestate (all pasteurisation conditions) resulted in lower incidental P losses than slurry in runoff from all rainfall simulations (Fig. 4), despite having significantly higher P in the starting substrate (3× higher; Table 2). TP in runoff from digestate for RS1 was on average 25% lower than slurry, 28% lower for RS2 and 47% lower for RS3. Two factors may account for the lower incidental P losses from digestate; firstly, the faster assimilation of P into the soil, due to the lower solids and viscosity, is visible in the soil test results from Day 14 (Table 4), where soils treated with digestate have 32% higher P, compared with 25% for slurry and − 11% for untreated controls. A second factor may be the higher levels of Al in digestate (Table 2). Al (in alum form) has been used as a slurry amendment to form stable Al-P precipitates (Brennan et al., 2011), reducing P solubility and by extension, incidental P losses, particularly in soils with high background P (Kalbasi and Karthikeyan, 2004). Additional amendment of digestate with alum may further reduce incidental P losses. As with Peyton et al. (2016), the concentration of nutrients in runoff decreased across successive rainfall events (Fig. 4).

3.4. Grass yield and elemental accumulation following application of slurry compared with unpasteurised and pasteurised digestate

Grass yield was significantly higher for all treated plots than the untreated controls (Fig. 5), with the treatment effect still evident 85 days post-treatment application. By Day 112, grass yields across treatments were no longer significantly different to untreated controls, although grass growth generally had by then declined. Yields at Day 14 post-application suggest that all three digestates supported a more rapid growth response than DCS, with average yield of 814 kg DM ha^{-1} from digestates comparing favourably with 662 and 480 kg DM ha^{-1}

Table 5
Summary of FIB, metal and nutrient concentrations in water, soil and grass following five treatments (T1 = untreated control (no organic amendment); T2 = slurry; T3 = unpasteurised digestate; T4 = pasteurised digestate (70°C); T5 = pasteurised digestate (60°C)).

		Untreated Controls				Slurry DCS				Digestate P0				Digestate P1				Digestate P2			
Rainfall Sim		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
FIB	Coliforms	✗	✗	✓	✓	✗	✗	✗	✗	✗	✗	✓	✓	✗	✗	✓	✓	✗	✗	✓	✓
	E. coli	✓	✓	✓	✓	✗	✗	✗	✓	✗	✓	✓	✓	✗	✗	✓	✓	✗	✓	✓	✓
	Enterococci	✓	✓	✓	✓	✗	✗	✗	✗	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Nutrients	N[a]	✓	✗	✓	✗	✓	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✗	✓	✗	✓	✗
	$N\text{-}NO_2$[d]	✓	✓	✓	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	$N\text{-}NH_4$[d]	✓	✓	✓	✓	✗	✗	✗	✓	✗	✓	✓	✓	✗	✗	✓	✓	✗	✗	✓	✓
	TP	✓	✓	✓	✓	✗	✗	✓	✓	✗	✓	✓	✓	✗	✗	✓	✓	✗	✓	✓	✓
	TOC[b]	✓	✓	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Elements/Metals	Al[d]	✓	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Cd[c]	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Cl[d]	✓	✓	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Cr[d]	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Cu[d]	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Fe[d]	✓	✓	✓	✓	✗	✗	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓	✓
	Mn[d]	✓	✓	✓	✓	✗	✗	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓	✓
	Na[d]	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Ni[c (i)]	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Pb[c]	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Zn	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

		Untreated Controls			Slurry DCS			Digestate P0			Digestate P1			Digestate P2		
Soil	Day	0	14	30	0	14	30	0	14	30	0	14	30	0	14	30
	Cd	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Cu	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Ni	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Pb	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Zn	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

		Untreated Controls						Slurry DCS						Digestate P0						Digestate P1						Digestate P2					
Grass	Day	0	14	30	57	85	112	0	14	30	57	85	112	0	14	30	57	85	112	0	14	30	57	85	112	0	14	30	57	85	112
	Cu	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Ni	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Pb	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Zn	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

[a]N: 1 mg L^{-1} is limit for A1, 2 for A2 and 3 for A3 (European Communities Environmental Objectives (Surface Waters) Regulations, 2009 (S.I. No. 272 of 2009)), threshold set at >3 mg L^{-1} for this table as worst case scenario.
[b]"No abnormal change" – (European Drinking Water Directive 98/83/EC).
[c]Cd, Ni, Pb: Max admissible concentration (MAC) Environmental Quality Standard (EQS) as per Directive 2013/39/EU.
[(i)]Exceeds annual average (AA) on RS1 for all organic amendments but not MAC.
[d]European Drinking Water Directive 98/83/EC.
[e]EU Surface Water Regulations (1989) as outlined in EPA (2001).

for DCS and untreated plots respectively. The impact of the rapid and highly available N in digestate aligns with the findings of Alburquerque et al. (2012a) examining the fertilizer potential of digestate for horticulture, while also flagging the need to ensure that this highly available N is not lost to the environment. In a study comparing digestates with mineral fertilizer for growing tomatoes, Barzee et al. (2019) also demonstrated that digestate is as good as or better than mineral N fertilizer for horticulture crops, while Walsh et al. (2012) and Coelho et al. (2019) observed a similar finding for grasslands. Coelho et al. (2019) did however note that digestate from an AD plant processing sewage sludge did not perform as well in terms of grass growth as digestate from co-digestion of animal slurry with FW. Similarly, after examining 12 agriculture based digestates, Alburquerque et al. (2012b) concluded that agriculture-based AD digestate has good fertilizer potential, which they attributed to the high NH_4-N content. The concern raised in that study about the potential need for post-treatment of the digestate prior to application has been addressed in the present work, with results indicating that post-AD pasteurisation does not negatively affect the fertilizer potential of the digestate. In fact, the opposite may be hinted at by the slightly higher grass yield from the digestate pasteurised to EU Standard (70 °C for 1 h) compared with unpasteurised, particularly evident on Day 30 (1147 vs 968 kg DM ha^{-1}). It may be the case that this short heat treatment alters the digestate nutrient bioavailability (Ware and Power, 2016), improving fertilizer potential, although as none of the nutrient or metal parameters examined are obviously different across treatments, this theory would require further examination. The results of the present research indicate that as hypothesised, digestate from co-digestion of slurry with FW, whether pasteurised or not, is at least as good as untreated slurry as an organic fertilizer for growing grass (Figs. 5 and 6).

Alburquerque et al. (2012b) raised a further concern about the higher Zn content typical of agriculture-based AD, citing disease-prevention additives in animal diets as the source. Although the levels of Zn in the digestates used in this trial were within acceptable limits (WRAP PAS110), digestate from AD plants processing pig slurry may have higher concentrations, given the more widespread use of Zn in

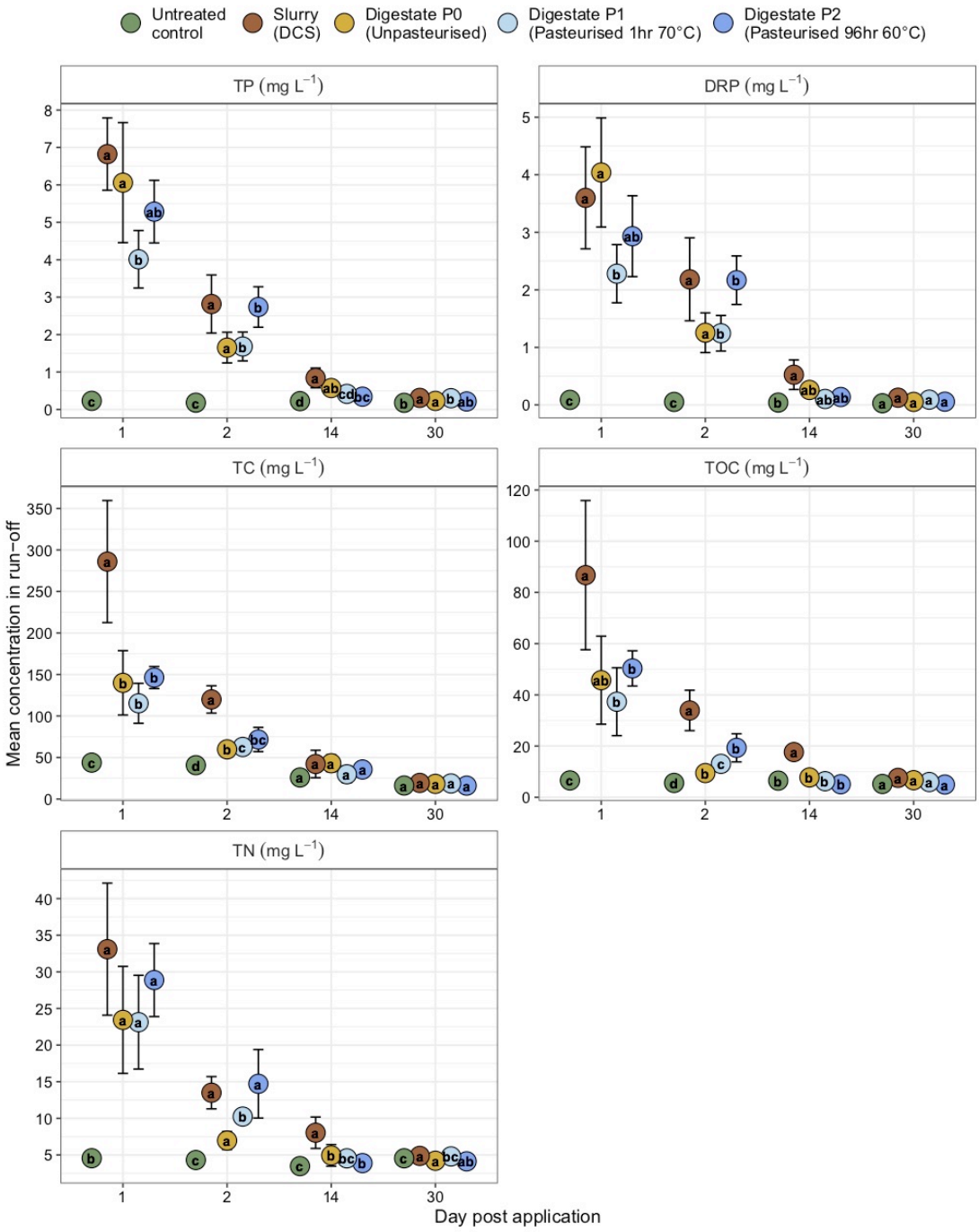


Fig. 4. Nutrients (TP, DRP, TC, TOC and TN) in unfiltered runoff (mg/L) following five treatments (T1 = untreated control (no organic amendment); T2 = slurry; T3 = unpasteurised digestate; T4 = pasteurised digestate (70 °C); T5 = pasteurised digestate (60 °C)) from rainfall simulation 1, 2, 14 and 30 days after application. Error bars indicate standard error of the mean (n = 4).

that industry. There was no significant difference between slurry and digestate treated plots in terms of Zn uptake in grass, nor were any treatments significantly different from untreated plots.

Mo concentration in grass from the DCS and P0 treatments was higher than pasteurised digestate (P1 and P2) and the untreated control after 14 days (Fig. 7). While Mo concentration in grass from all three

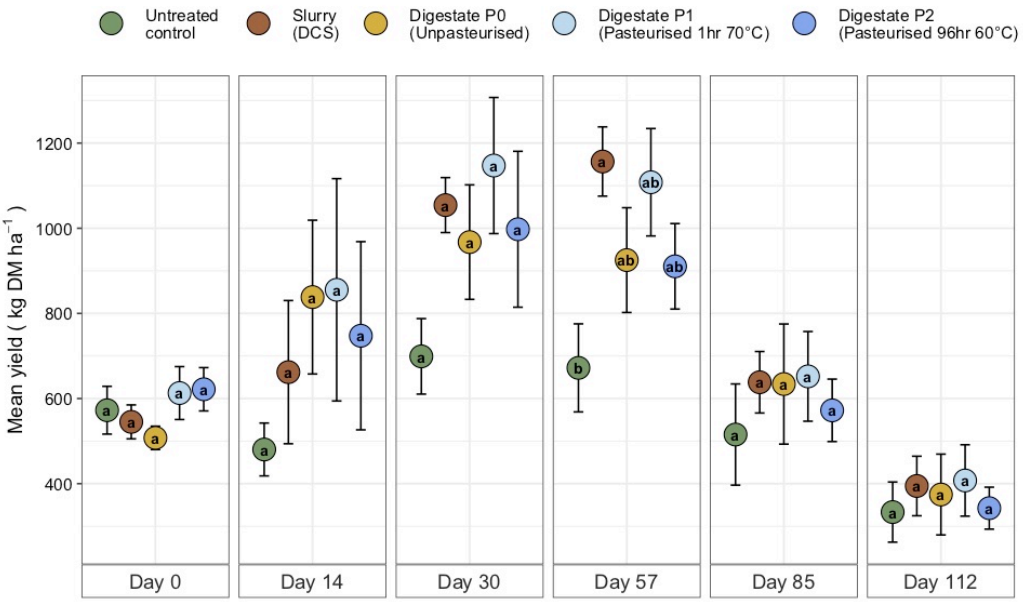


Fig. 5. Grass yield following application of DCS, unpasteurised digestate and pasteurised digestate (two conditions) and an untreated control. Error bars indicate standard error of the mean (n = 4).

digestates declined over time, grass from DCS plots saw a steady Mo increase in concentration for two months after treatment, stabilizing at 2.2–2.3 mg/ kg throughout the remainder of the trial, a level 3–4 times higher than the untreated plots. Molybdenum is an essential element for nitrate reductase (Kaiser et al., 2005), but can be problematic at concentrations above 2 mg/ kg (Brogan et al., 1973). At these concentrations, Mo reduces Cu availability in the rumen by forming Mo-S compounds called thiomolybdates which pass through the rumen wall

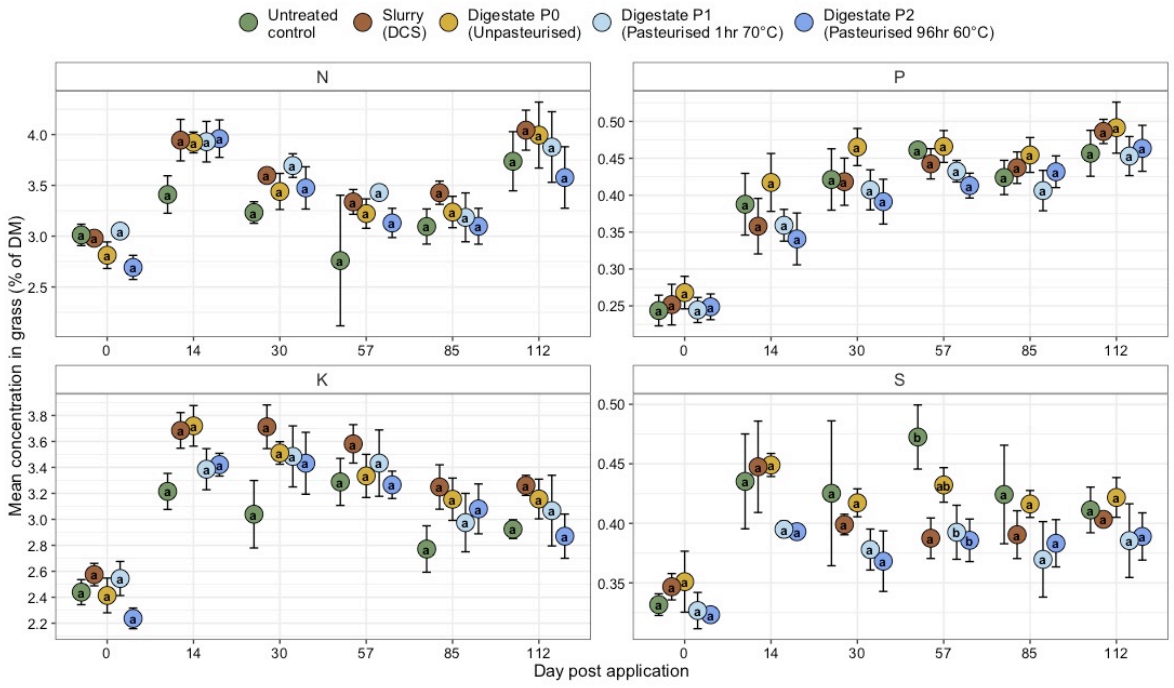


Fig. 6. N, P, K and S content in grass harvested before (Day 0) and following application of DCS, unpasteurised digestate and pasteurised digestate (two conditions), and an untreated control plot. Error bars indicate standard error of the mean (n = 4).

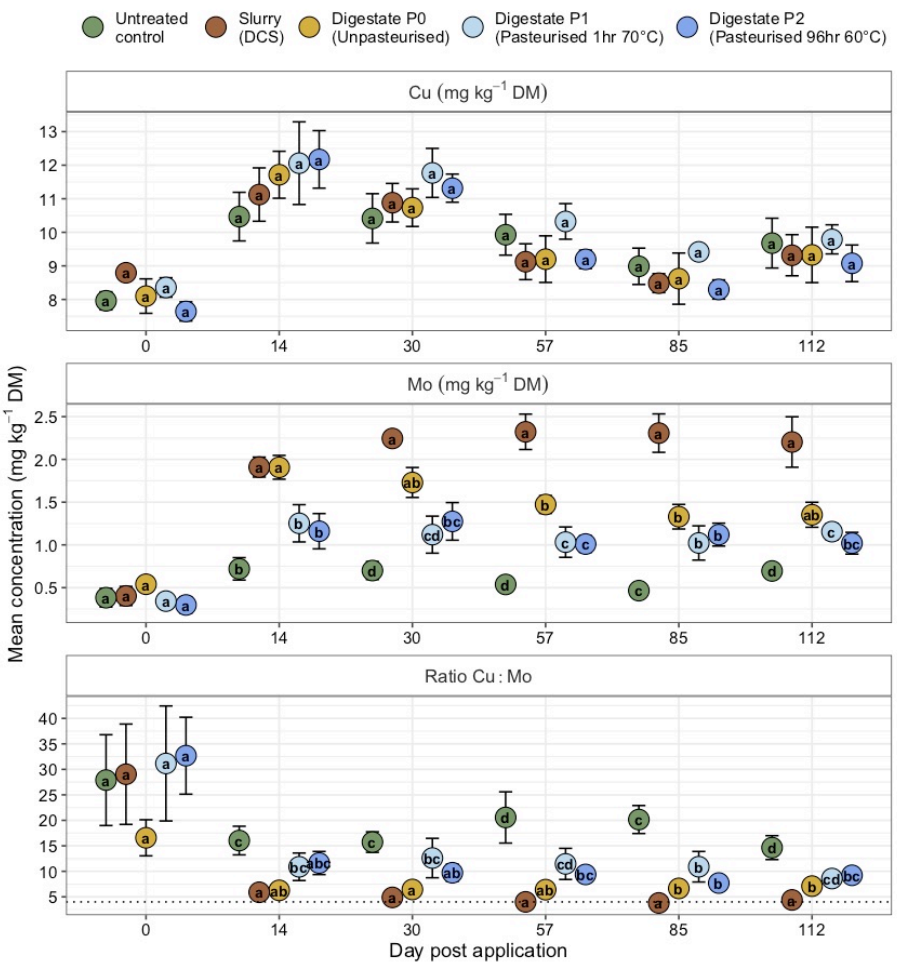


Fig. 7. Cu and Mo content and Cu:Mo ratio in grass harvested before (Day 0) and following application of DCS, unpasteurised digestate and pasteurised digestate (two conditions), and an untreated control plot. Error bars indicate standard error of the mean (n = 4).

and readily bind to Cu, causing toxicity with clinical signs similar to Cu deficiency (Gould and Kendall, 2011). This negative affect is compounded if the ratio of 4:1 Cu:Mo, considered safe, is breached (Pitt, 1976), with acute Mo toxicosis possible from a ratio below 2:1 (Miltimore and Mason, 1971). Although none of the samples tested dropped below the critical 2:1 ratio, forage collected from the DCS plots breached the 'safe' 4:1 ratio on Day 57 and Day 85 (3.9 and 3.7 respectively; Fig. 7), likely owing to the higher initial Mo concentration in DCS compared with all three digestates tested (Table 2).

The lower initial Mo concentrations in digestate compared with DCS may be explained in part by experiments reporting benefits of Mo supplementation for biogas production (59% increase), indicating that Mo in slurry is utilized by anaerobic microbial consortia (Cai et al., 2018). It may also indicate an additional benefit of processing slurry in AD, given the reduced risk of Mo-induced hypocupraemia.

3.5. Summary table of treatments

For ease of comparison results are tabulated with a red cross indicating a breach of regulatory or recommended limits and a green tick indicating satisfactory levels (Table 5). The most significant difference was visible between treatments for runoff of FIB, particularly enterococci, which remained high from slurry-treated plots beyond 30 days. Pasteurisation did not significantly alter runoff of FIB, accumulation or uptake of metals and nutrients, but one pasteurisation condition (P2: 60 °C for 96 h) did result in higher P and N runoff on Day 2, similar to that recorded from slurry. Clearly anaerobic co-digestion of slurry with FW is an approach that facilitates shifting the focus "from compliance to performance", improving water quality and significantly reducing use of chemical fertilizers in line with European Green Deal goals (COM, 2019). However, to prevent the risk of 'pollution swapping' a holistic assessment that includes gaseous emissions and fertilizer replacement value is necessary. Finally, the data from these trials should be incorporated into models that assess benefits and risks to human health from improved water and air quality in order to inform synergistic manure and waste management policies,

4. Conclusions

This study compared the traditional practice of landspreading untreated slurry with landspreading of unpasteurised and pasteurised digestate from agriculture-based AD. Our results indicate that for each

environmental parameter tested: microbial, nutrient and metal runoff losses; accumulation in soil and uptake in grass, digestate from anaerobic co-digestion of slurry with FW resulted in reduced potential for pollutant transmission to watercourses, soil and grass than traditional landspreading of slurry without treatment. Reduced microbial runoff from digestate was the most prominent advantage of digestate application. Pasteurisation of the digestate further augmented those environmental benefits, without impacting grass output. Anaerobic co-digestion of slurry is therefore a multi-beneficial circular approach to reducing impacts of livestock production on the environment.

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CRediT authorship contribution statement

S. Nolan: Investigation, Data curation, Writing - original draft. **C. Thorn:** Validation, Formal analysis, Data curation, Visualization. **S.M. Ashekuzzaman:** Methodology, Resources. **I. Kavanagh:** Investigation. **R. Nag:** Conceptualization, Investigation. **D. Bolton:** Project administration, Funding acquisition. **E. Cummins:** Writing - review & editing. **V. O'Flaherty:** Funding acquisition, Writing - review & editing. **F. Abram:** Writing - review & editing. **K. Richards:** Supervision, Funding acquisition. **O. Fenton:** Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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5.2 Future work

Management of slurry is a significant factor in greenhouse gas and ammonia emissions from agricultural production. Hence, having examined faecal indicator bacteria, nutrient and metal runoff and persistence in soil from plots amended with unprocessed slurry compared with unpasteurised and pasteurised digestate, it is necessary to also examine gaseous emissions in order to establish a more holistic perspective.

The food processing waste used in agriculture-based AD may contain plastic packaging that currently requires physical removal and separation prior to processing in the AD system. Whether depackaging is carried out manually or using machine (paddle) depackers, some plastic packaging may get through into the AD system. The mandatory 12 mm screen required as part of the ABP transformation regulations also serves to remove large plastic particles from the digestate prior to dispatch as an organic fertiliser/soil improver. Yet plastic packaging may be partially degraded to micro- or nano-plastics, which could conceivably pass through the system and be landspread. The fate of these plastics following landspreading, particularly following heavy rainfall, is not clearly understood and requires attention in future work.

Development of AD as a biorefinery, capable of converting food waste to high value intermediates for use in pharmaceuticals or bioplastic production, is gathering pace. As bioplastics are increasingly utilised for food packaging, they too will inevitably enter the AD system. The fate or extent of degradation of these bioplastics in AD, and their subsequent persistence in the environment is not yet established. The true extent of the sustainability of such systems must be determined with field trials examining fate and persistence in agricultural food production systems and the surrounding environment.

Chapter 6

Fate of carbon and nitrogen following landspreading of anaerobically co-digested slurry to grassland

Having carried out the field trials, I wrote this chapter with the help of my supervisors and collaborators, particularly Prof Owen Fenton. The ammonia and greenhouse gas data were analysed with Ian Kavanagh and data visualisation was carried out with Camilla Thorn. A version of this chapter is being prepared for submission to *Science of the Total Environment.*

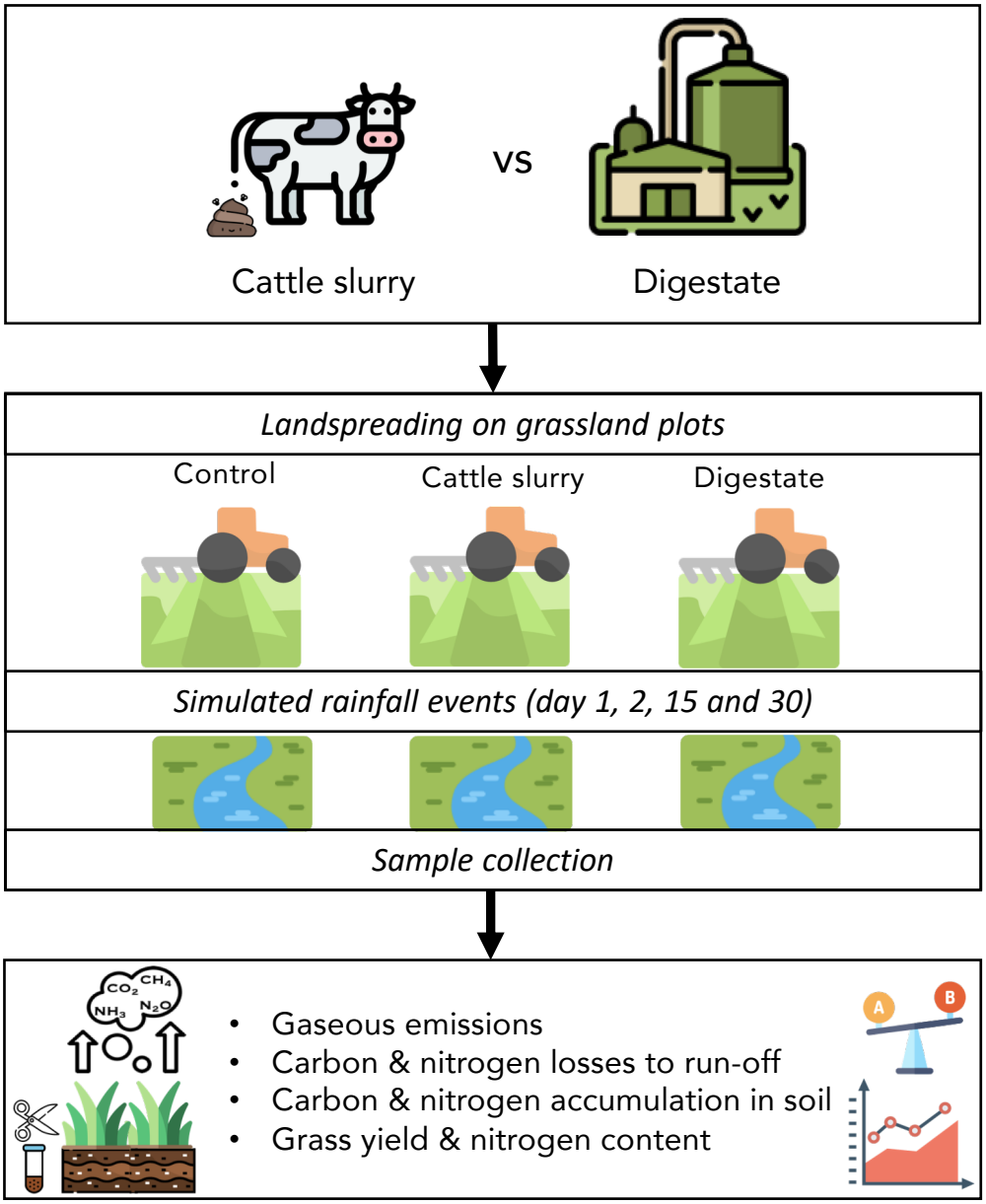
Abstract

Agriculture is responsible for up to 98 % of ammonia and 10 % of greenhouse gas (GHG) emissions within the European Union (EU), 16.8 % of which comes from manure management. Anaerobic digestion (AD) of slurry is seen as a mitigating measure for GHG emissions from agriculture, converting carbon (C) to usable energy, leaving a nutrient-rich digestate by-product which is landspread as an organic fertiliser. However, the relatively low methane (CH_4) potential of slurry necessitates co-digestion with various organic waste streams, such as food processing waste, This alters the nutrient profile of the slurry and may have knock on implications for emissions of C and nitrogen (N) directly after land application or over time. Such emissions may also be influenced by rainfall events, particularly heavy rainfall soon after landspreading. Herein, replicated grassland plots were set up to assess gaseous emissions after application of AD digestate from co-digestion of dairy cattle slurry (DCS) with food processing waste compared with unprocessed DCS and untreated controls over time (117 days) which included an examination of emissions after four simulated heavy ($\sim$11 mm hr^{-1}) rainfall events, 1, 2, 15 and 30 days post-application. Ammonia (NH_3) emissions were monitored using a dynamic chamber system coupled to a photoacoustic field gas-monitor. The GHG emissions were measured using square (40 x 40 cm) static chambers. Gas samples were analysed for CH_4, carbon dioxide (CO_2) and N_2O using gas chromatography. Daily fluxes were calculated from the increase in headspace concentration over four sampling times (0, 10, 20 and 30 min after enclosure). Runoff from the treated plots, as well as soil and grass were also analysed to further inform the fate of C and N following land application.

A 72 % reduction in CH_4 emissions was observed from plots treated with digestate compared with slurry, more than 90 % of which occurred on the day of spreading. Given the predominantly aerobic field environment, methane emissions following land application primarily result from release of trapped bubbles in the substrate, which may be exacerbated by rainfall droplet surface disturbance of the organic matrix. N_2O emissions were two times higher from slurry than digestate, but peaked later in slurry-treated plots. There was no significant difference between digestate-treated and untreated control plots in terms of total N_2O emissions. One

replicate of the untreated controls had significantly higher N_2O emissions, which was attributed to fungal activity visible as mushroom growth in that plot. CO_2 was measured for 117 days, but no significant difference was found between treatments, although the slurry treatment was slightly lower than untreated controls and digestate treated plots, perhaps as an artifact of the denser slurry "blocking" respiration. The higher NH_3 emissions from digestate immediately post-landspreading are in part attributable to higher N availability in digestate, and NH_3 losses/emissions from slurry during storage. Rainfall simulation increased CH_4 and N_2O emissions from slurry and digestate but suppressed NH_3 emissions for both. CO_2 emissions were higher post-RS for slurry, but lower for digestate, likely due to improved infiltration characteristics. Grass yield was higher from digestate than slurry treatment. Anaerobic co-digestion of slurry with food processing waste is a viable means of reducing agricultural GHG emissions whilst maintaining fertiliser potential, thereby contributing significantly to development of a circular economy.

Graphical Abstract



Keywords: Landspreading; Greenhouse gas mitigation; Ammonia; Slurry; Anaerobic Digestate

6.1 Introduction

The European Green Deal aims to achieve carbon neutrality by 2050, through reduction of greenhouse gas (GHG) emissions, while also improving air and water quality and maintaining a sustainable level of agricultural production (COM, 2019). Air quality receives significant attention, with ammonia a major contributing factor to poor air quality because of its contribution to PM2.5 (Behera and Sharma, 2010).

Agriculture is responsible for 98 % of ammonia and 10 % of GHG emissions within the EU. For agriculture-dependant countries such as Ireland, that contribution rises to 34 % (EPA, 2019), 16.8 % of which comes from manure, predominantly stored in wet 'slurry' form (Pérez Domínguez et al., 2016). This unprocessed slurry is typically landspread as an organic fertiliser, resulting in further fugitive gaseous carbon (C) and nitrogen (N) losses (Chantigny et al., 2009, 2001; Misselbrook et al., 2005), in the form of methane (CH_4), carbon dioxide (CO_2), nitrous oxide (N_2O) and ammonia (NH_3). Measures are therefore required which address the simultaneous EU ambitions of maintaining sustainable agricultural production levels while reducing associated emissions.

An EU-funded Joint Research Council (2016) report identifies anaerobic digestion (AD) as the most effective means of economically reducing slurry GHG emissions, with potential reduction of between 9.1 to 12.5 million tonnes of CO_2 equivalents (Pérez Domínguez et al., 2016). The EU's revised Renewable Energy Directive 2018/2001/EU (EU RED II, 2018) entered into force in late 2018, and formally recognised the potential for AD as a GHG mitigation strategy, particularly where animal manure is used as a substrate. Annex VI of the Directive lists rules for calculating the GHG impact of biomass fuels, allowing for a GHG emissions saving value of up to 202 % for biomethane used in transport and 246 % for electricity generated from biogas when mono-digesting wet manure, with a declining sliding scale tracking reduced percentages of slurry in co-digestion AD. Much of this emissions savings potential (44.16 kg CO_2 eq/ton) is attributed to reduced methane (CH_4) emissions (JRC, 2017).

Methane has a 100-year greenhouse warming potential 23 - 28 times that of CO_2, and manure management accounts for approximately 24.4 % of agricultural methane emissions in the EU (Pérez Domínguez et al., 2016). AD of slurry captures methane that would otherwise be emitted during storage and landspreading, thereby

reducing overall GHG emissions (Amon et al., 2006), but as slurry has a relatively low biomethane potential, it is typically necessary to co-digest with energy crops or food production waste to ensure viability (Clemens et al., 2006). Yet, codigestion of food production wastes such as fats, oils and grease (FOG) may result in partially degraded solids in the digestate and hence an increased residual biomethane potential above that of unprocessed slurry (Alburquerque et al., 2012a). Furthermore, the impact of AD of slurry on NH_3 emissions from subsequent landspreading has not been definitively determined, with some studies finding no significant difference (Amon et al., 2006; Chantigny et al., 2004), while others have found an increase (Ni et al., 2012) or a decrease (Rubæk et al., 1996), depending on weather conditions and application method. Hence, examination of a range of co-digestion feedstocks to establish a broad understanding of GHG and NH_3 mitigation potential is required.

Anaerobic digestion of slurry breaks down complex organic compounds, converting C to biogas, and mineralising N compounds to NH_4^+-N, potentially enhancing N-availability (Weiland, 2010) and soil N_{0rg}-mineralisation when compared with unprocessed slurry (Möller and Müller, 2012). When compared with mineral fertiliser, land application of digestate is beneficial in closing nutrient cycles and improving soil fertility (Slepetiene et al., 2020), but may increase the risk of C and N loss to the environment in cases of improper application (Alburquerque et al., 2012b; Nkoa, 2014). Strict rules are in force around timing of land application in close proximity to forecast rain to minimise risk of runoff, but occasional heavy rainfall events may occur. Although the potential for runoff of C and N resulting from heavy rainfall soon after grassland application of organic amendments has received some attention, the effect of rainfall on C and N gaseous emissions is not clearly understood.

Finally, the RED II AD emission savings value is predicated on a "soil carbon accumulation" (e_{sca}) bonus of 45 g CO_2 eq/MJ manure used in AD, which equates to 54 kg CO_2 eq/ton fresh matter, explicitly for the directly avoided GHG emissions through improved manure management (JRC, 2017). Improved manure management through AD processing is anticipated to lead to soil C accumulation, but requires "solid and verifiable evidence...that it can reasonably be expected to have increased" (COM, 2010), where "measurements of soil carbon can constitute such evidence" (COM, 2010), and should be included in any study where organic fertilisers are

landspread. This is still a knowledge gap with soil C accumulation following grassland spreading of digestate from AD of co-digested slurry with FOG, particularly where that grass is being regularly harvested.

Thus, the overall aim of this study was to compare emissions of CH_4, CO_2, N_2O and NH_3 across three treatments (DCS, digestate and untreated control) from application to 117 days after application. At days 1, 2, 15 and 30 the effect of rainfall simulations on gaseous emissions was explored. Runoff and soil samples were examined to further establish the fate of C and N, while grass yield and N uptake were also examined. For this experiment nine microplots were set up and randomly assigned to treatments.

Hypotheses tested

1. Methane emissions are higher from DCS than from digestate-treated plots.
2. Ammonia emissions are higher from digestate compared with DCS-treated plots.
3. During rainfall events ammonia emissions from DCS and digestate-treated plots are suppressed.
4. Digestate application leads to soil carbon accumulation.

6.2 Materials and Methods

6.2.1 Field site characterisation

The study site was a 0.6-ha mid-slope non-grazed plot located on the beef farm at Teagasc, Johnstown Castle Environment Research Centre, Co. Wexford, in the southeast of Ireland (latitude 52.293415, longitude −6.518497). The area has a cool maritime climate, with an average temperature of 10.1°C and mean annual precipitation of 1,002 mm. The site has been used as a grassland sward for over 25 years with organic and inorganic nutrient inputs applied as necessitated by routine soil testing. The site has undulating topography with average slopes of 6.7 % along the length of the site and 3.6 % across the width. The field is moderately drained with a soil texture gradient of clay loam to sand silt loam, as classified by Brennan et al. (2012). Soil nutrient status in the upper, middle and lower sections was characterised by taking composite soil samples from each section (n = 20). The soil nutrient status at these locations (Morgan's P (P_m), K, and magnesium (Mg)) was

determined using Morgan's extractant (Morgan, 1941). Soil pH was determined using a pH probe (Mettler-Toledo Inlab Routine) and a 2:1 ratio of deionised water to soil as previously described in Peyton et al. (2016).

6.2.2 Treatment characterisation

Three treatments were examined in this study: dairy cattle slurry (DCS), unpasteurised AD digestate and untreated control. Digestate was sourced from bioreactors which co-digest DCS with food processing waste, namely fats, oils and grease at a 2:1 ratio. DCS was collected from a dairy farm in Co. Galway, Ireland following mechanical agitation of the underground slurry tank. Fresh DCS and digestate was collected in sealed, 10 L-capacity plastic storage containers and transported to the field site location where they were briefly (< 48 h) stored at 4°C prior to application. Fresh samples of both organic fertilisers were analysed for pH using a pH meter, and then dried to determine total solids (OM) in an oven at 105°C for 24 hours, and then volatile solids were determined using a furnace at 550°C for 2 hours (Table 6.1). Freeze dried samples (n = 3) were also analysed for nitrogen (N), using a LECO TruSpec CN analyser (Table 6.1), and following microwave-assisted acid digestion concentrations of phosphorus (P) was determined to dictate application rate using an Agilent 5100 synchronous vertical dual view inductively coupled plasma optical emission spectrometer (Agilent 5100 ICP-OES). Prior to application, all treatments were thoroughly mixed to re-suspend solids. Potassium (K), sulphur (S) and pH were also analysed using standard methods (Table 6.1).

Table 6.1 Organic fertiliser/soil improver analysis

	OM %	N %	P %	K %	S %	pH
Slurry	8.61±0.3	2.53±0.05	0.4±0.01	6.12±0.05	0.47±0.01	7.3±0.2
Digestate	6.41±0.04	2.62±0.1	1.34±0.16	3.39±0.5	0.60±0.02	7.93±0.02

6.2.3 Experimental micro-plot setup

Nine micro-plots, each 0.4 m wide and 0.9 m long were set up similarly to those described in Nolan et al. (2020) using stainless steel frames, hammered into the soil to a depth of 50 mm, oriented with the longer dimension in the direction of the slope. The frames isolate each plot at the back and sides, and include a runoff channel at the

front with a spout for runoff collection into sample cups during rainfall simulation (Nolan et al., 2020), with modifications allowing for gaseous emission collection.

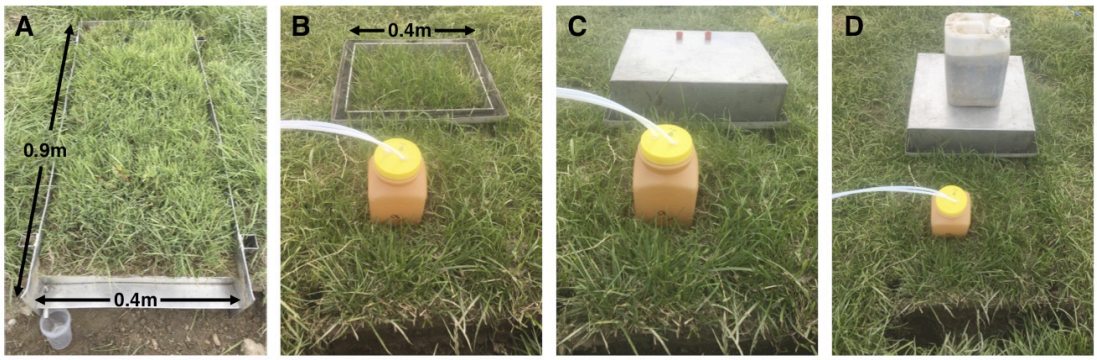


Figure 6.1 Field trial gas analysis experimental set up, from left to right: Frames for runoff collection during rainfall simulation; Static chamber open, closed and weighed down to ensure seal, with ammonia sample collection container in the foreground, showing tubing to photoacoustic analyser

The three treatments (digestate, DCS and soil-only control) used in this study were randomly assigned to the nine micro-plots to compare GHG and ammonia emissions. Application of DCS and digestate to the micro-plots was carried out using a watering can spout to simulate trailing shoe application and was governed by the P content of the treatments and the plant available P of the soil as measured by Morgan's P. For comparable results, all micro-plots were classified into Index 1 P soil (Morgan's P of 5 mg L^{-1}), which meant that all treatments were applied to all plots at a rate of 40 kg P ha^{-1} (Wall and Plunkett, 2016). As a result of the P content and the DM of each individual digestate, application rates per individual plot were 1,644 g of digestate and 3,830 g of DCS per individual plot. As N concentration in both treatments was similar, this resulted in higher N application to the slurry plots than digestate plots.

6.2.4 Gaseous emissions

GHG emissions were measured using square (40 cm x 40 cm) static chambers, sealed with a rubber septum with weighted drums ensuring a complete seal (Figure 6.1). Stainless steel non-insulated, non-vented covers (10 cm high) were used to form a headspace chamber for measurement of GHGs, with headspace volume of approximately 16 L as per Krol et al. (2015). Gas samples were collected for analysis prior to treatment application, immediately after application, and thereafter on days

1, 2, 3, 4, 5, 7, 14, 16, 21, 30, 75 and 117, with the increasing gap between days resulting from a significant decline in gaseous emission activity.

Gas samples (10 mL) were extracted at 0, 10, 20 and 30 min after the container was closed using a polypropylene syringe (BDPlastipak, Oxford, U.K.) fitted with a hypodermic needle (BD Microlance 3; Becton Dickinson). The samples were then injected into pre-evacuated (1,000 mbar) 7 mL screw cap septum glass vials (Labco, High Wycombe, U.K.). The syringe was flushed once with ambient air before collecting sample from the chamber. The resulting gas samples were analysed for CH_4, CO_2 and N_2O concentrations using gas chromatography (GC) (Varian CP3800 GC; Varian, Walnut Creek, CA USA) and Bruker SCION 456 GC, with high-purity helium used as a carrier gas. Eight samples of ambient air were collected on each gas-sampling event and were used as time zero (t0) for GHG concentration for flux calculations from the chambers. Linearity of headspace GHG concentrations was checked at each sampling occasion by collecting five headspace samples per chamber from each treatment throughout the 60 min closed period (Chadwick et al., 2014). The increase in CH_4, CO_2 and N_2O concentrations in the containers over time was used to determine the gas flux. Daily fluxes were calculated for each gas and each treatment from the increase in headspace concentration over four sampling times (0, 10, 20 and 30 min after enclosure) following Eq. (6.1), adapted from Kavanagh et al. (2019):

Eq. (6.1)

$$F(daily) = \left(\frac{\partial C}{\partial T}\right) \times \frac{M \times P}{R \times T} \times \left(\frac{V}{A}\right)$$

where ∂C is the change in gas concentration in the chamber headspace during the enclosure period in parts per billion by volume (ppbv) or $\mu l l^{-1}$; ∂T is the enclosure period expressed in days, M the molar mass of the gas element; P is the atmospheric pressure in Pa; and T is the temperature in Kelvin. Carbon dioxide (CO_2) equivalents for all gases measured were determined after Birch (2014) to enable comparison of results.

Mean N_2O emission factors (EFs) were calculated by subtracting cumulative control emissions from cumulative emissions from individual organic fertiliser amended plots, according to the IPCC equation (IPCC, 2006) presented in Eq. (6.2):

Eq. (6.2)

$$EF = \left(\frac{(Cumulative\ N_2O\ flux\ (kg\ N_2O{-}N) - cumulative\ N_2O\ flux\ from\ control\ (kg\ N_2O{-}N)}{N\ applied\ (kg\ N)}\right)$$

Indirect N_2O emissions resulting from downstream deposition of NO_3^- or NH_4^+ were estimated according to the IPCC (2006) assumed figure of 1 % of volatilised NH_3. Indirect N_2O emissions from leached N were calculated according to IPCC (2006) assumptions that 30 % of applied N is lost via leaching and that 0.75 % of leached N is re-emitted as N_2O.

Ammonia emissions were monitored using a dynamic chamber system as per Kavanagh et al. (2019), prior to treatment application, immediately after application, and on days 1, 2, 3, 4 and 5, with a return to background levels signalling the end of sampling. Concentrations of NH_3 in the air entering and leaving each container, over a period of 16 min per sampling, were monitored using a photoacoustic INNOVA 1412 field gas-monitor (LumaSense Technologies, Denmark) coupled to a Gasmux multiplexer GM3000 (IMT Vohenstrauss, Germany). Glass wool soaked with oxalic acid (0.05 M) was used to strip moisture from the background air entering the photoacoustic monitor. Gas fluxes (Fj in mg m^{-2} h^{-1}) for NH_3 were calculated according to (Dinuccio et al., 2008):

Eqn. (6.3)

$$Fj = Q\frac{(Cex.j - Cin.j)}{A}$$

where Q is the airflow rate through the chamber (m^3 h^{-1}), Cex.j is the NH_3, concentration of air outlet from the chamber (mg m^{-3}), Cin.j is the NH_3 concentration of air into the chamber (mg m^{-3}), and A is the area of emitting surface covered by the chamber (m^2). Mean NH_3 emission factors (EF) for each treatment were calculated according to the IPCC (2006) method, whereby cumulative control plot emissions are subtracted from cumulative emissions from individual organic fertiliser amended plots as displayed in Eq. (6.4):

Eq. (6.4)

$$EF = \left(\frac{Cumulative\ NH_3\ flux\ (kg\ NH_3{-}N) - Control\ plot\ cumulative\ NH_3\ flux\ (kg\ NH_3{-}N)}{N\ applied\ (kg\ N)}\right) \times 100$$

$$EF = \left(\frac{Cumulative\ NH_3\ flux\ (kg\ NH_3{-}N)}{N\ applied\ (kg\ N)}\right) \times 100$$

6.2.5 Rainfall simulation

Rainfall simulations were carried out using a modified Amsterdam-drip type rainfall simulator, 24 h, 48 h, 15 d and 30 d post-application at a rate of ~ 11mm hr^{-1} to replicate worst-case scenario conditions. Rainout shelters were used between rainfall simulations to avoid any natural rainfall falling on the plots. Volumetric moisture content (MC) of the soil in each plot (n = 3) was measured immediately prior to and after each rainfall event using a time domain reflectometry device (Delta-T Devices Ltd., Cambridge, UK), which was calibrated to measure resistivity in the upper 50 mm of the soil in each plot.

Gaseous emissions were measured immediately prior to and within 10 minutes post-rainfall simulation in the same manner as outlined above. Runoff from the microplots was collected as described in Nolan et al. (2020). An aliquot of each runoff water sample was filtered through 0.45 µm filter paper and a sub-sample was analysed calorimetrically for nitrite (NO_2^-N), total organic N (TON) and ammonium (NH_4^+–N) using a nutrient analyser (Aquachem Labmedics Analytics, Thermo Clinical Labsystems, Finland). Unfiltered runoff water samples were analysed for TC, TN and total organic carbon (TOC) using the Aquachem Analyser. All samples were tested in accordance with the Standard Methods (APHA, 2005).

6.2.6. Soil collection and analysis

Soil samples were collected one day before organic fertiliser application (D0) and analysed for nutrient status, pH and dry matter. Composited soil samples were ground to 2 mm before being analysed for total Phosphorous (TP), total Nitrogen (TN), total Carbon (TC) and pH (Table 6.2). Analysis was carried out at Teagasc Johnstown Castle Environment Research Centre using standard methods. Soil C and

N analysis was also repeated on samples collected on Day 15 and Day 30 of the experiment to determine the extent of soil C and N accumulation over time (Table 6.2).

Table 6.2 Soil sample analysis on Day 0, 15 and 30, with standard error (n=3)

		TC %	TN %	P mg/kg	K mg/kg	pH
D0	Ctrl	3.2±0.28	0.250±0.001	481±12	2194±228	5.3±0.10
	Slurry	2.9±0.09	0.168±0.085	500±14	2338±98	5.4±0.15
	Digestate	2.9±0.21	0.247±0.017	505±24	2206±155	5.5±0.05
D15	Ctrl	2.8±0.20	0.243±0.018	461±29	1328±177	5.6±0.13
	Slurry	2.7±0.09	0.235±0.012	511±16	1515±92	5.8±0.12
	Digestate	2.8±0.19	0.269±0.007	558±32	1711±90	5.8±0.06
D30	Control	2.8±0.09	0.253±0.0023	458±29	1176±146	5.8±0.07
	Slurry	3.0±0.35	0.262±0.028	526±23	1616±120	6.1±0.06
	Digestate	3.3±0.12	0.286±0.008	563±39	1338±197	6.0±0.06

6.2.7. Grass yield and N uptake

Prior to treatment application, the grass on all micro-plots was cut to 50 mm. Thereafter, grass was collected on days 14, 30, 57, 85 and 117 by cutting to 50 mm, to determine yield as well as N uptake. Collected grass was weighed, then dried in an oven at 60°C for 48 hours to determine solids content. Grass samples were then ground and analysed for N content using a Rigaku NEX CG EDXRF spectrometer equipped with a nine-place sample changer with spin function using slow and steady spinning mode. as described by Daly and Fenelon (2017) and reported in Nolan et al. (2020). Grass N content was assumed to be representative of N uptake as per Bell et al. (2016).

6.2.8 Statistical analysis

Statistical differences as a function of treatment were tested for using either ANOVA or the non-parametric Kruskal-Wallis test, as appropriate for the dataset. Where $p < 0.01$, post-hoc tests were performed and if multiple tests were performed on the same data, p-values were corrected for multiple comparisons using false discovery rate

(FDR) approach. All the above were performed as implemented in the agricolae package in R (de Mendiburu, 2020).

6.3 Results and Discussion

6.3.1 Methane and CO_2 emissions from slurry compared with digestate and untreated controls

6.3.1.1 CH_4

Methane emissions were an average of 72 % lower from plots treated with digestate compared with slurry (Figure 6.2). Similar to Clemens et al. (2006), >9 0 % of methane emissions occurred immediately after spreading for both treatments, with only DCS still producing significantly higher methane by Day 2. The rainfall simulation on Day 2 resulted in a spike in methane emissions from both slurry (1.5x or 0.177 g/m^2 pre-RS vs 0.263 g/m^2 post-RS) and digestate (9x or 0.0016 vs 0.14 g/m^2 post-RS), although the cumulative emissions were not significantly affected by rainfall. This is in line with Sänger et al. (2010) who found temporal but not cumulative increases in gaseous emissions resulting from three different simulated rainfall patterns each averaging 3 mm d^{-1}, resulting in > 80 % water filled pore space (WFPS). Given the predominantly aerobic field environment, methane emissions following land application primarily result from release of trapped bubbles in the substrate, which may be exacerbated by rainfall droplet surface disturbance of the organic matrix. Although the results of the present work demonstrate the efficacy of AD as a means of reducing methane emissions from slurry, digestate-treated plots still produced more methane in total than those receiving no organic amendment (1.9 ± 0.2 g/m^2 versus 0.2 ± 0.03 g/m^2 respectively). This indicates that a final treatment step may be necessary if complete elimination of methane emissions from organic fertiliser application is to be achieved.

6.3.1.2 CO_2

CO_2 fluxes were measured for 117 days, with the highest treatment-induced flux occuring in the hours after landspreading. CO_2 fluxes from digestate-amended plots were higher than those from DCS in the first five days post-treatment, but following a high flux on Day 5, declined to background levels by Day 7. Increased CO_2 fluxes following application of organic amendments have been attributed to accumulation

of carbonate in anaerobic storage conditions (Chantigny et al., 2001), which is rapidly released upon application of the more alkaline treatment (DCS: pH 7.81 ± 0.05; digestate: pH 7.99 ± 0.01) to the acidic soil (pH 5.53 ± 0.01). Cumulative CO_2 emissions from slurry amended plots displayed a slightly lower trend than untreated controls and digestate treated plots, a disparity primarily resulting from one DCS replicate being significantly lower than the others, while one digestate replicate was significantly higher (Figure 6.2). A second factor may be the denser slurry "blocking" the escape of gases produced during respiration, through formation of a crust on the grass/soil surface, however, it would be anticipated that this effect would be reduced over time as slurry is incorporated into soil. However over the course of the 117-day trial no significant differences in cumulative CO_2 were found as a function of treatment. These results contradict those of Köster et al. (2015) who observed CO_2 emissions six times higher in cattle slurry amended soil than untreated controls. In that case however, there was no plant cover in the laboratory controlled trials, and sieving may have interfered with the microbial community, as the bulk of CO_2 emissions are related to soil microbial activity and root respiration (Kuzyakov and Larionova, 2006).

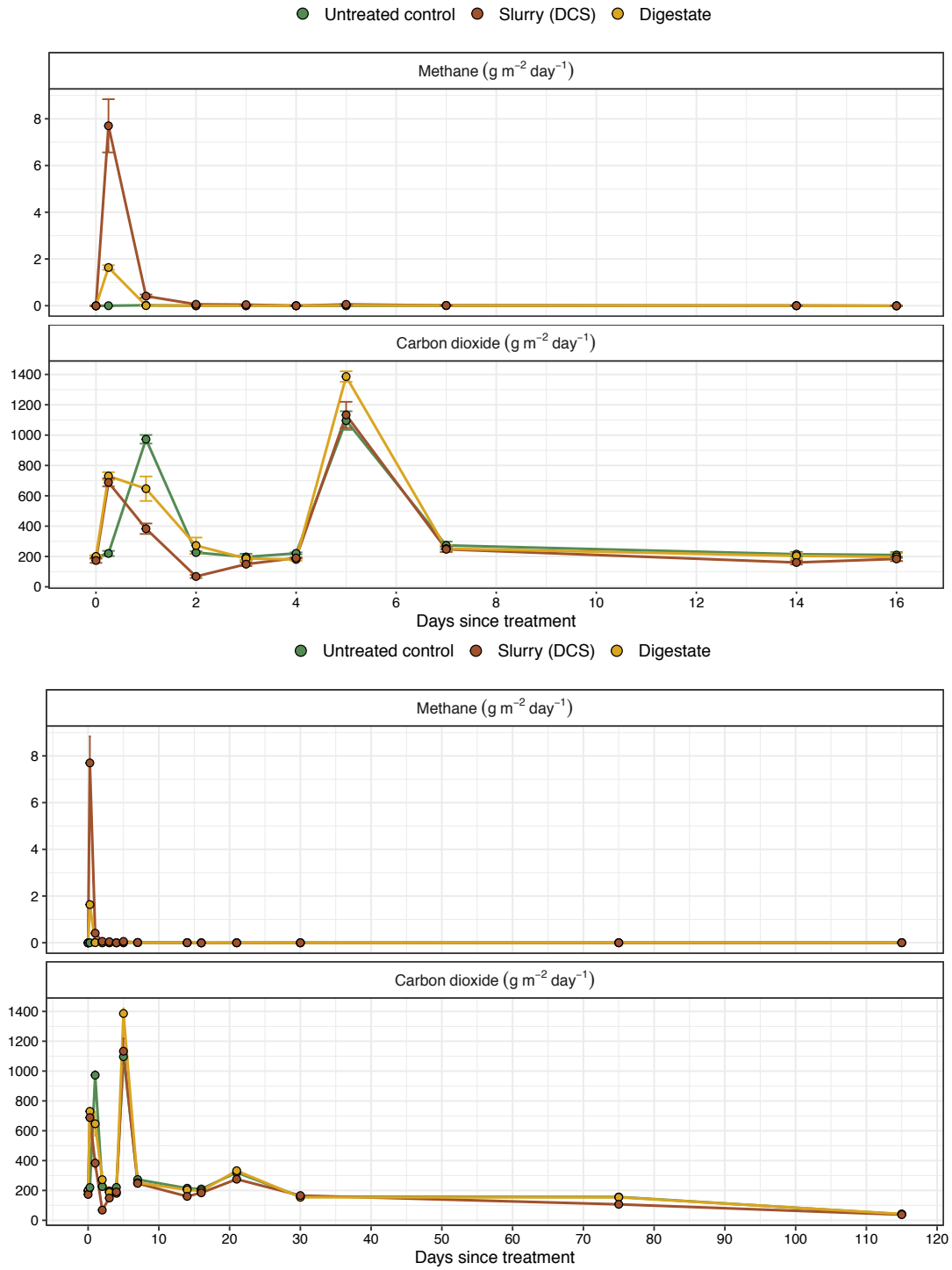


Figure 6.2A&B: Carbon gas fluxes from three treatments (n = 3), for 16 days post-treatment (A) and shown over the course of 117 days post-treatment (B)

Biotic CO_2 emissions arising from microbial activity associated with manure management are not generally quantified given the assumption that C in manure has previously been captured by dietary crops (Aguirre-Villegas and Larson, 2017). Furthermore, lifecycle inventory analysis of anaerobic digestion, particularly co-

digestion with byproducts, has been demonstrated to result in negative CO_2 emissions (Poeschl et al., 2012).

6.3.1.3 Effect of rainfall simulation on C gas emissions

Although rainfall simulation events had minimal effects on the total gaseous emissions over the course of the 117-day trial, significant short-term effects were recorded for both CH_4 and CO_2 emissions from all treatments (Figure 6.3).

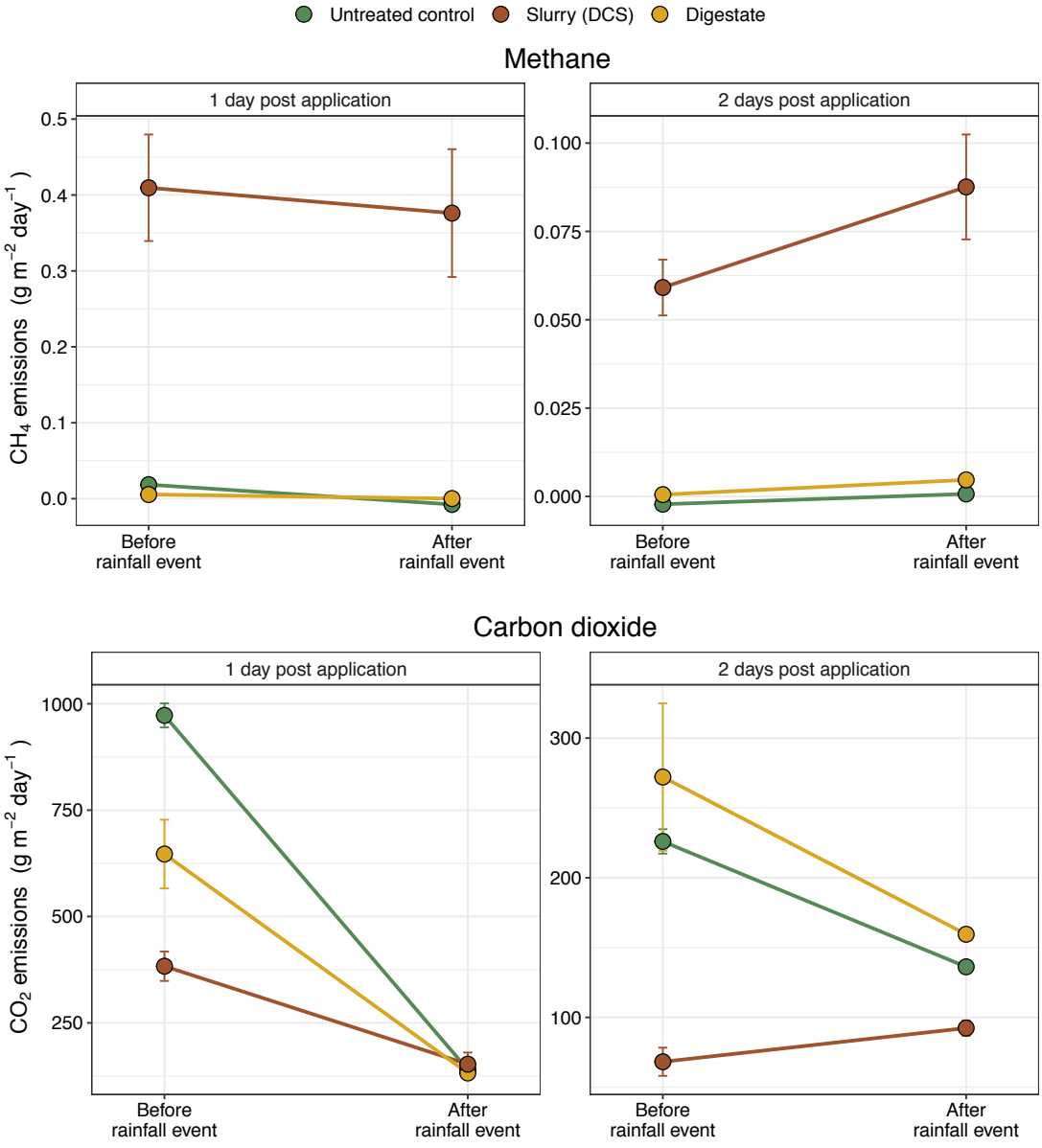


Figure 6.3 Effect of rainfall simulation on methane and carbon dioxide emissions from three treatments (n = 3), 1 and 2 days post-application

Rainfall simulation had negligible influence on CH_4 emissions from untreated controls on either Day 1 or 2. RS-24hr (24 hours) reduced methane emissions from digestate-amended plots almost completely (99 % reduction, Figure 6.3), while

causing an 8 % decrease in methane emissions from DCS. RS-48hr did however have a significant impact on methane emissions from both organic amendments, resulting in a 1.5x increase in DCS emissions and a 9x increase in methane emissions from digestate. The digestate emissions started from a notably lower baseline however, and remained less than half those from slurry even after the RS-induced increase (Figure 6.3: 0.0047 vs 0.09 g/m^2).

RS-24hr reduced CO_2 emissions from untreated controls, DCS and digestate by 86 %, 60 % and 80 % respectively, compared with emissions immediately preceding the rainfall event. This adds weight to the theory expressed previously that the majority of CO_2 emissions recorded are generally from soil microbial activity, which was suppressed by the heavy rainfall event (11 mm hr^{-1}). Rapid loss of carbonate in the initial hours immediately following amendment application may have been a factor in the decline of CO_2 emissions being measured 24 hr post-application. However but as readings were taken immediately before and after RS, the reduction in CO_2 emissions following rainfall is likely as a result of increased WFPS, which affects microbial activity through reduction of oxygen diffusion (Davidson et al., 2000; Sänger et al., 2010; Wood et al., 2013).

The suppression effect of RS-24hr was still visible in results recorded prior to RS-48hr where CO_2 emissions had not yet returned to the levels recorded prior to RS-24hr. RS-48hr further reduced CO_2 emissions from digestate (34 % reduction) and untreated controls (12 %), but resulted in a slight increase from DCS-amended plots. Hence, RS-48hr increased C-based greenhouse gas emissions from slurry, possibly as a result of release of stored gas resulting from breaking down the organic matrix in the higher-solid treatment.

6.3.1.4 Soil carbon accumulation and C runoff

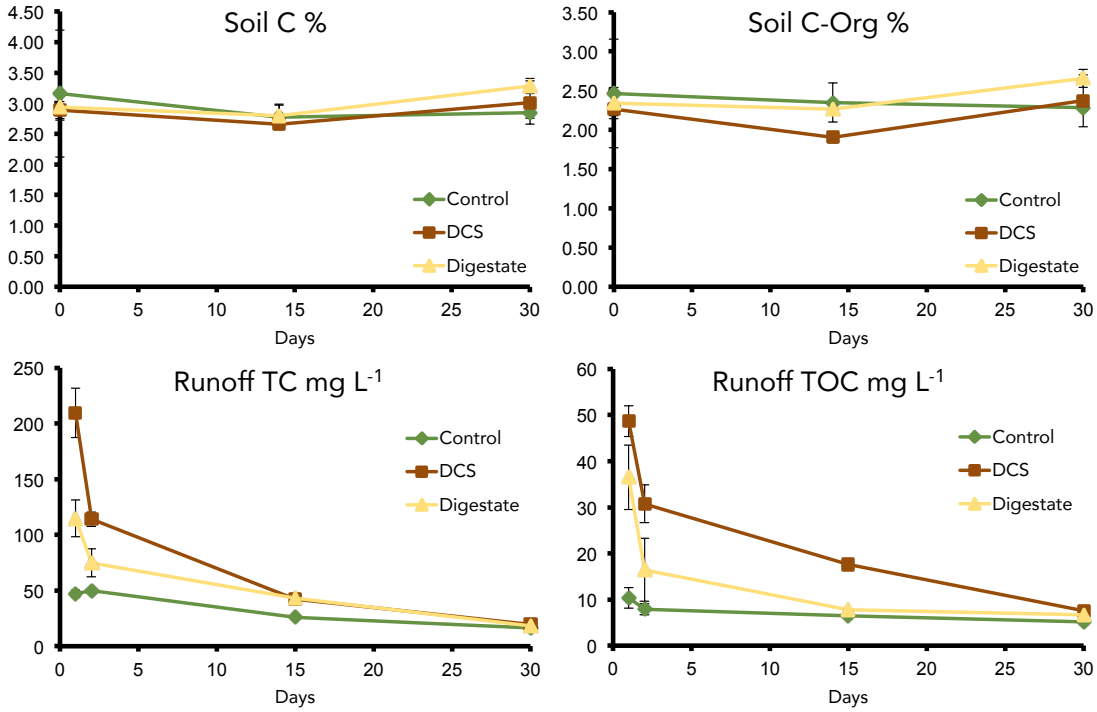


Figure 6.4 Total and organic carbon in soil from three treatments (n=3) on Day 0, 15 and 30, and in runoff from those plots on Day 1, 2, 15 and 30

Soil samples were analysed for total and organic C on Day 0 (prior to treatment application), Day 15 and Day 30. Soil C content was similar across all plots for Day 0, and decreased slightly over time in untreated control plots. Soil C initially declined in DCS-treated plots, but by Day 30 had increased by 5 % relative to Day 0. Total and organic-C content in soil from digestate-amended plots was similar on Day 0 and 15, but by Day 30 had increased by 15 %, indicating improved infiltration of digestate-derived C into soil compared with C from DCS treatments (Figure 6.4), and reflective of the lower C losses in runoff from digestate-amended plots.

Runoff from rainfall simulations on Days 1, 2, 15 and 30 was collected and analysed for total carbon (TC) and total organic carbon (TOC: mg L^{-1}). DCS amended plots resulted in 180 % more TC in runoff from the first rainfall simulation than digestate-amended plots, and 33 % higher TOC. The same trend existed following the rainfall simulation two days post-treatment, where DCS application resulted in runoff with TC and TOC concentrations 52 % and 87 % higher than that detected in digestate-derived runoff (TOC: 30.7 ± 4.1 vs 16.5 ± 6.8 mg L^{-1}). By Day 15, TC in runoff from both digestate and DCS treated plots had returned to untreated

control levels, however TOC remained elevated in runoff from both treatments, but was more than 2 times higher in DCS than digestate (Figure 6.4: 17.7 ± 1.16 vs 7.8 ± 0.04 mg L^{-1}).

6.3.2 N_2O emissions from slurry compared with digestate and untreated controls

N_2O fluxes from digestate-treated plots peaked two days post-treatment (c. 161 g N_2O-N/ha/day) and five days post-application for DCS (c. 187 g N_2O-N/ha/day, Figure 6.5). Within 13 days, digestate-derived N_2O emissions had returned to background levels (c. 7 g N_2O-N/ha/day, Figure 6.5). Although N_2O fluxes from DCS-amended plots remained significantly higher than digestate-treated and untreated control plots (> 3.5x) on day 16, by day 21 there was no significant difference between treatments (Figure 6.5). Similarly, Nicholson et al., (2017) reported that most N_2O emissions from digestate and slurry applied in different seasons and soils occurred within the first few weeks after application, with approximately 75 % of direct emissions occurring within 4-6 weeks after application. However in another study comparing N_2O emissions from food waste AD with cattle slurry, Köster et al. (2015) found that onset of N_2O emissions took up to 15 days, possibly an artifact of the interference with soil structure and moisture in a controlled laboratory setting (sieved to 4 mm and soil moisture adjusted to approximately 90 % WFPS).

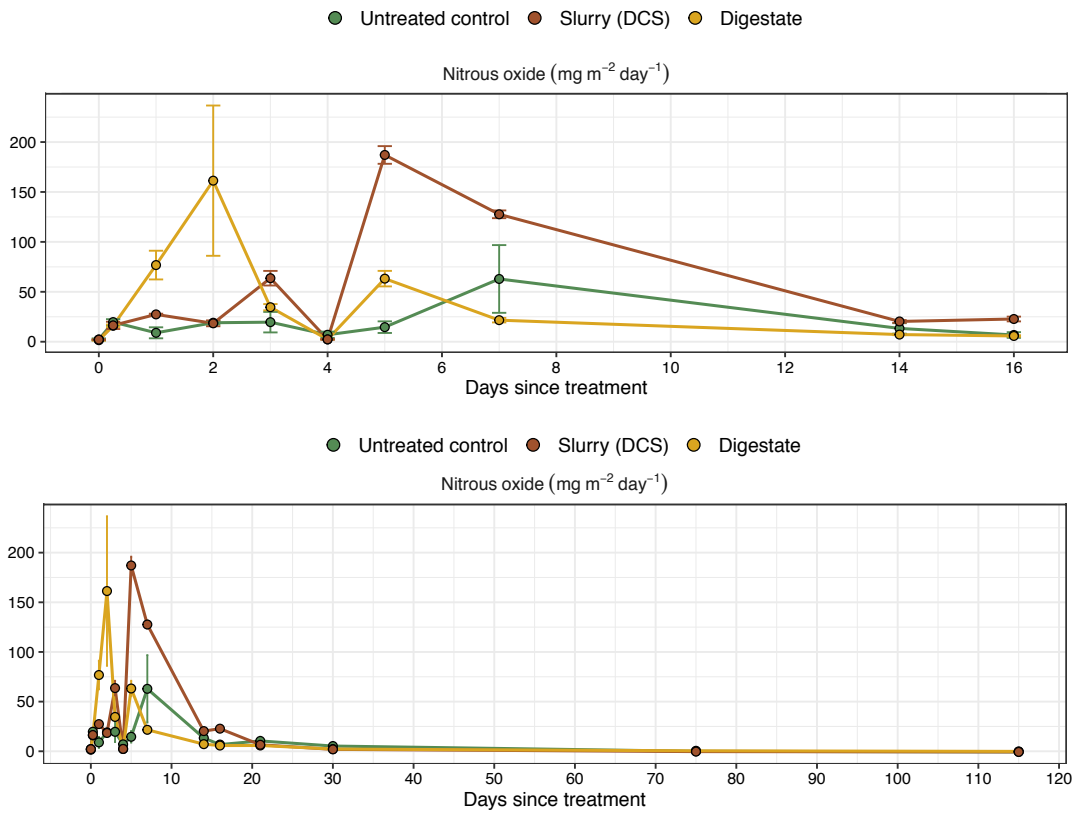


Figure 6.5A&B N_2O fluxes from three treatments (n=3), for 16 days post-treatment (A) and shown over course of 117 days post-treatment (B)

Net cumulative N_2O emissions were two times higher for DCS than digestate (1,242 vs 668 mg/m^2 respectively), similar to the three times higher observation reported by Köster et al. (2015). However, as with the work carried out by Köster et al. (2015), significantly more N was applied in the DCS treatment than digestate, in this case as application was a function of P-loading rate. Hence, when compared as a percentage of applied N both organic treatments were similar, at 0.39 % and 0.52 % of total N applied for DCS and digestate respectively. The emission factors (EF) for both DCS and digestate are lower than the IPCC Tier 1 default value of 1 % of total N applied. The digestate EF is however skewed by a single outlier flux on Day 3 for one replicate (422 g N_2O-N/ha/day), without which the EF for digestate (0.45 ± 8%) would be almost identical to that reported by Nicholson et al., (2017; 0.45 ± 15%), which were obtained for food-based digestate in the UK, a similar climate to Ireland. In a two-year examination of nine grassland sites, Soussana et al. (2007) reported average N_2O fluxes of 13 g CO_2-C equiv. m^{-2} yr^{-1}. The data from the present work (co-digested DCS and FOG) could be combined with data from studies of other potential co-digestion feedstocks, such as maize silage or food waste (Bell et al.,

2016; Nicholson et al., 2017) to build a Tier 2 inventory of N_2O EFs resulting from field-application of various digestates, in accordance with IPCC guidelines (IPCC, 2006).

6.3.2.1 N_2O and fungus

One of the untreated control replicates had higher N_2O emissions than any other treatment, 7x the average from the other control plots (1,689 vs 244 ± 42 mg/m^2), and 3x higher than the average N_2O emissions from digestate-treated plots (541 ± 96 mg/m^2). On closer examination, this plot was found to have significant fungal growth which was not evident at the start of the trial, with several mushrooms sprouting over the course of the trial. Mushroom growth regularly occurs in grassland in Ireland, and the use of replicated trials provides for more usable data in biological trials. The contribution of fungal activity to N_2O emissions in grassland has been demonstrated (Crenshaw et al., 2008; Laughlin et al., 2009; Laughlin and Stevens, 2002), attributable primarily to a lack of N_2O reductase, which normally regulates microbial conversion of N_2O to N_2 (Shoun et al., 1992). Interestingly, the grass DM yield was significantly higher in the untreated control plot with fungal growth, particularly between Day 30 and 60 when yield was 1.3x higher than slurry and digestate-treated plots, 2.5x more than the other untreated controls.

The grass from these plots was analysed for nutrient and metal concentrations, with the only significant difference between plots being a sustained higher concentration of manganese in the fungi-dominated control plot (data not shown). Thompson et al., (2005) observed Mn oxidation by soil inhabiting fungi, and Mn is essential for photosynthesis, ATP synthesis and biosynthesis of chlorophyll (Millaleo et al., 2010). The incidentally observed correlation between fungal growth, improved grass yield, higher Mn concentrations and increased N_2O emissions requires further examination, as application of different organic fertilisers may alter fungal/bacterial biomass ratios and by extension, nutrient uptake efficiency (de Vries et al., 2006). An interesting result arising from the rainfall simulations was the 20x spike in N_2O observed in the fungus-affected control plot following RS1, indicating the importance of moisture to N_2O-producing fungi.

6.3.2.2 Effect of rainfall on N_2O emissions

The first rainfall simulation (24 hours post-application) had a significant impact on N_2O emissions from both DCS and digestate, with reductions of 67 % and 78 % respectively, but had no notable effect on control plots (Figure 6.6). RS-48hr reduced N_2O emissions by 23 % from control plots, but increased N_2O by 27 % in slurry-amended plots (Figure 6.6). Similarly, Clemens and Huschka, (2001) observed increased N_2O emissions as WFPS increased from 35 to 75% in soils amended with slurry. The effect of rainfall was most pronounced in digestate-amended plots (79 % reduction), but these results were skewed by a large spike (pre-RS) in one replicate (422 mg/m^2), which was suppressed to 46.23 mg/m^2 post-RS (Figure 6.6).

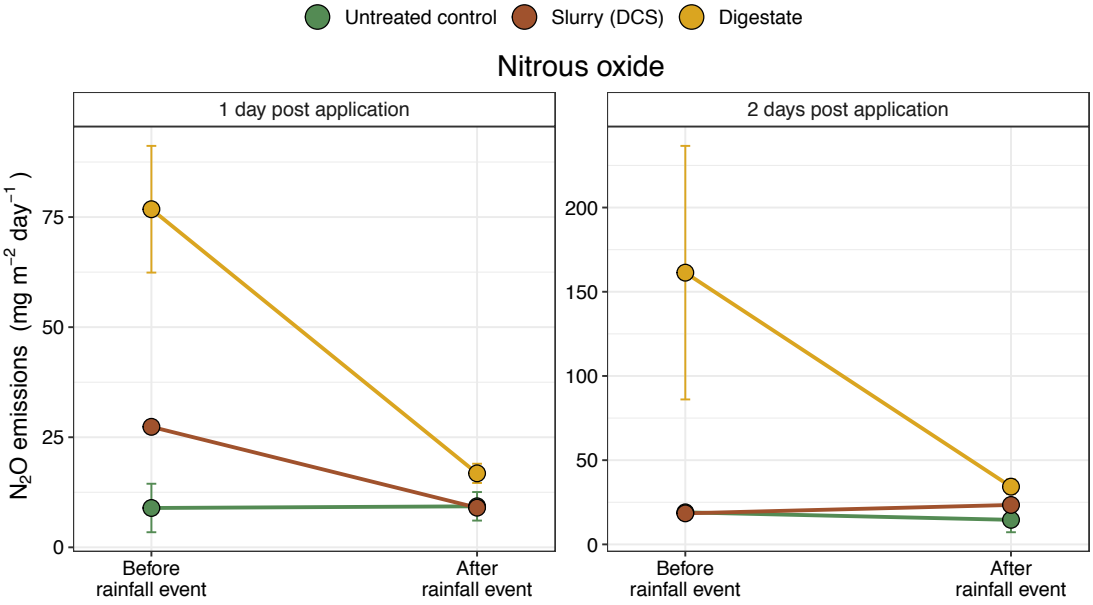


Figure 6.6: Effect of rainfall simulation on gaseous N emissions from three treatments (n=3), 1 and 2 days post-application.

As soil moisture is an important controlling factor of N_2O emissions (Baral et al., 2017; Chantigny et al., 2001; Ruser et al., 2006), soil moisture content was recorded for all plots throughout, but was not found to be a determining factor in total gaseous emissions between treatments, as all plots received the same amount of moisture in this controlled trial and rainout shelters were used between rainfall events.. Despite the immediate effect of rainfall simulation on DCS-associated N_2O emissions, similarly to Sänger et al. (2010) cumulative emissions over the course of the trial were not significantly affected (1242 ± 59 mg^{-2} N_2O m^{-2} (pre-RS) vs 1228 ± 51 mg^{-2} N_2O m^{-2} (post-RS) in DCS-amended plots). Unlike Sänger et al. (2010) however, cumulative N_2O emissions from digestate were significantly reduced (28 %) by

rainfall simulation. This N_2O reduction may have been a result of improved infiltration into the soil.

6.3.3 Ammonia emissions from slurry compared with digestate

Ammonia fluxes from both organic amendments were highest one hour immediately after application (3.17 ± 0.23 and 3.37 ± 0.26 kg/ha/day for DCS and digestate, respectively) and declined steadily until returning to background levels three days after treatment application (Figure 6.7). While analysing NH_3 emissions from two different cattle slurry application methods in spring and autumn applications, Bell et al. (2016) similarly observed peak NH_3 emissions in the first hour post-application, while Nicholson et al. (2017) report peak NH_3 emissions from food-based digestate in the first six hours following landspreading. Processing of DCS in AD resulted in higher pH as per previous studies (Table 6.2; Sommer et al., 1997), and higher pH is typically associated with increased ammonia volatilisation, particularly over pH 8 (Nicholson et al., 2018), although pH in the digestate in this study was <8 (Table 6.2).

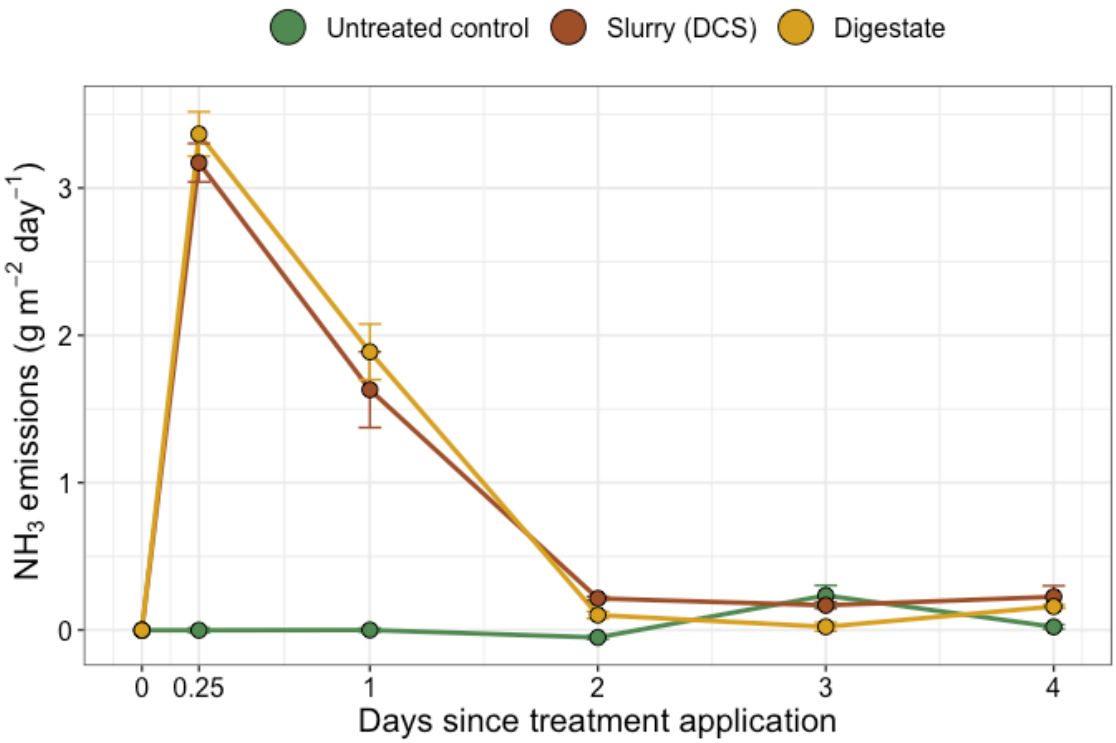


Figure 6.7: NH_3 emissions following landspreading of digestate and slurry, showing standard error (n=3)

Cumulative ammonia emissions were similar for both slurry and digestate at 5.28 ± 0.87 and 5.30 ± 0.51 kg ha^{-1}, respectively. Although there was no significant difference in cumulative NH_3 emissions between DCS and digestate as applied at the recommended rate of 40 kg P ha^{-1} (Wall and Plunkett, 2016), because P was significantly higher in digestate, less N was applied. Despite this, grass growth was similar between organic treatments, averaging 1.9 ± 0.19, 3.2 ± 0.18 and 3.2 ± 0.12 kg DM per plot for untreated controls, DCS and digestate, respectively, indicating that N was not limiting even at lower application rates. N removal was 6% higher in grass from DCS-amended plots than digestate, at 113 vs 106 g respectively. Furthermore, N losses via overland runoff pathways were 41 % and 94 % higher from DCS compared with digestate-amended plots following RS-24hr and RS-48hr respectively. Rapid soil incorporation would reduce NH_3 emissions (Nicholson et al., 2017), but may result in increased N_2O emissions (Webb et al., 2010) and is not typically applicable in grassland management.

When expressed as an emission factor (NH_3-N/kg N applied), NH_3 emissions from DCS were 6.3 ± 0.83 %, compared with 20.9 ± 1.2 % for digestate. The EFs observed for cattle slurry are in the lower range of those reported by Van Der Hoek (1998: 6 - 12.1%) and slightly lower than Bell et al. (2016: 8.2 - 18.6 %), and substantially lower than those reported by Nicholson et al. (2017: 24 - 31 %). As with this work, NH_3 EFs reported in the literature are regularly lower than the IPCC default EF of 20 %, highlighting the need for more accurate national inventories specific to climate and crop. Given that the cattle slurry EFs reported by Nicholson et al. (2017) are more than double those reported by Van Der Hoek (1998), some caution may be necessary with their reported EFs for digestate (38 - 42 %). Regardless of which end of the scale is accepted, NH_3 EFs do however highlight the likelihood of significant losses of valuable fertiliser due to volatilisation, and the consequent need for an effective treatment for organic fertilisers to optimise fertiliser performance. Finally, the lower NH_3 EFs observed in this study may be a result of N runoff and/or increased soil infiltration caused by heavy (~11 mm hr^{-1}) rainfall simulations.

6.3.3.1 Effect of rainfall on ammonia emissions

Ammonia emissions were significantly reduced by both rainfall simulations for all treatments (Figure 6.8). RS1 reduced NH_3 emissions from cattle slurry and digestate by 75.3 % and 82.6 %, respectively.

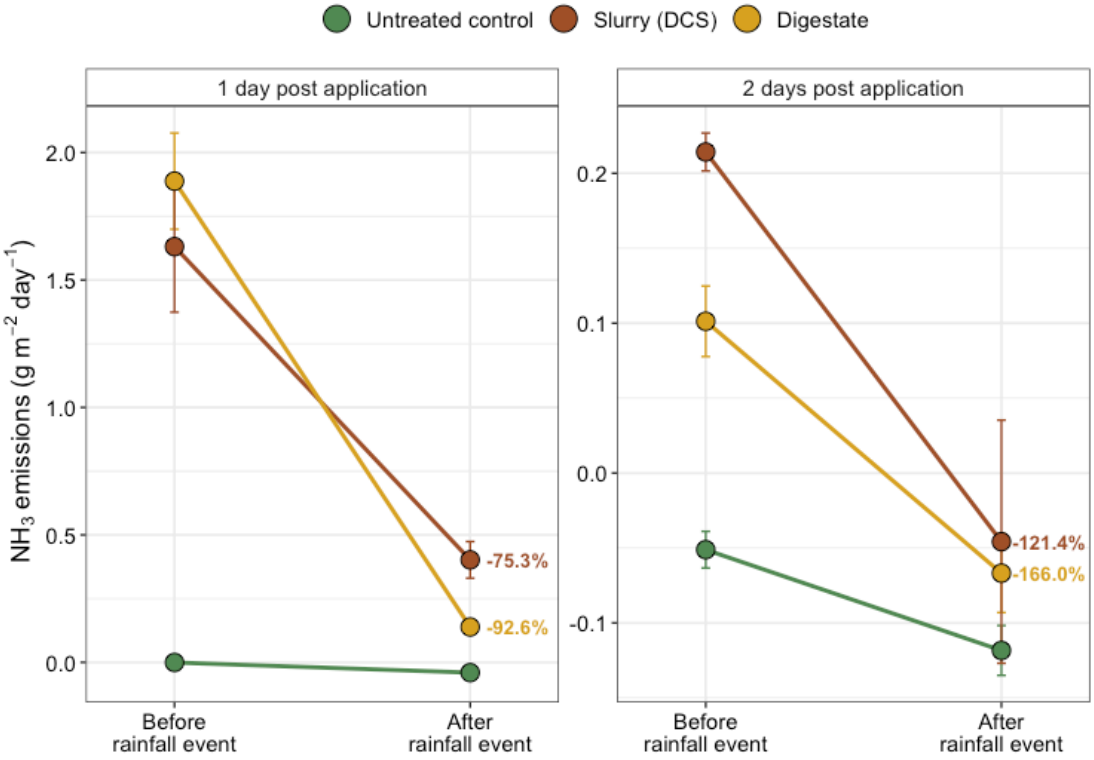


Figure 6.8: Effect of rainfall simulation on NH_3 emissions following from digestate and slurry amended plots (n = 3)

When expressed in percentage reduction RS-48hr had a more dramatic suppression effect than RS-24hr, but as NH_3 emissions had significantly dropped off by Day 2, the actual g NH_3 m^{-2} d^{-1} reduction was small. Misselbrook et al. (2005) observed rainfall simulation-associated NH_3 emission reductions of up to 65 % from cattle slurry applied to grassland. Their RS (5-10 mm hr^{-1}) was carried out immediately after application however, when emissions have been reported to be strongest (Section 6.3.2; Bell et al., 2016; Nicholson et al., 2017), and so a less effective reduction in emissions is to be expected. Rainfall in close proximity to landspreading essentially dilutes the organic fertiliser, which has been demonstrated as an effective means of reducing NH_3 volatilisation (Misselbrook et al., 2005), likely resulting from increased infiltration of total ammonia nitrogen (TAN) into the soil (Sommer and

Olesen, 2000). In addition to the likely improved infiltration however, some undesirable reduction through surface runoff is also a likely factor.

6.3.4 Nitrogen accumulation in soil and loss through runoff

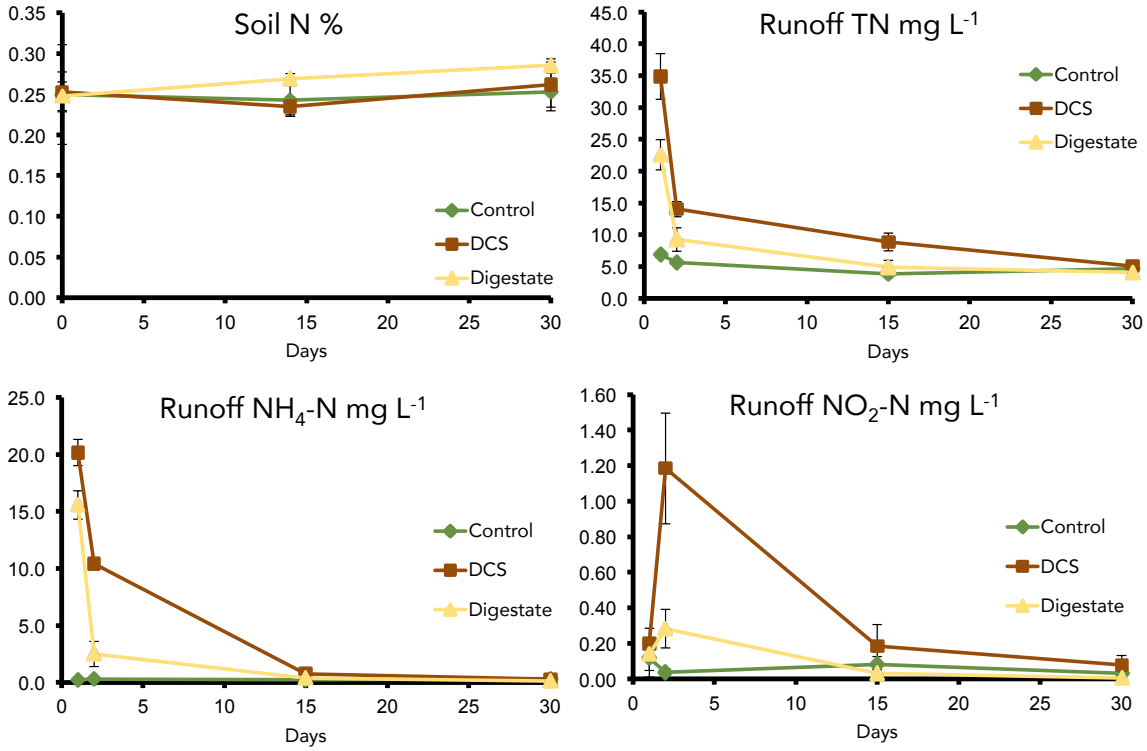


Figure 6.9: Nitrogen % in soil from three treatments (n=3) on Day 0, 15 and 30, and total nitrogen (TN), ammonium (NH_4-N) and nitrite (NO_2-N) in runoff from those plots on Day 1, 2, 15 and 30 (n = 3)

Soil samples were taken prior to treatment application, and on Day 15 and 30 for analysis of N concentration. Soil N % was similar across all plots on Day 0 and remained consistent in untreated controls (3 %). N % in plots treated with digestate rose progressively throughout the trial, up 8.6 % by Day 15 and 15.5 % by Day 30. Plots treated with DCS had slightly lower N % by Day 14, but increased by 3.6 % above starting levels by Day 30 (Figure 6.9).

Total N losses in runoff from rainfall simulations on plots amended with slurry were higher than those from digestate at each time point, with the most significant difference on the RS 15 days post-treatment (79 % higher; 8.9 ± 1.4 vs 4.9 ± 1.0 mg L^{-1}). Total N in runoff from digestate was higher than untreated controls on Day 1 and 2, but had returned to background levels by Day 15. Similarly to TN, ammonium levels in runoff from DCS were elevated compared with digestate and

untreated control plots (Figure 6.9), with the Day 2 rainfall simulation resulting in 4 times higher ammonium concentrations from DCS than digestate-treated plots (10.4 ± 0.18 vs 2.5 ± 1.1 mg L^{-1}).

There was no significant difference between treatments for ammonium concentration in runoff from RS on Day 15 or 30. Nitrite (NO_2-N) levels in runoff behaved differently to other N forms, initially lower in plots treated with slurry or digestate, but by RS-48hr were significantly higher in plots receiving organic amendment compared with untreated controls (Figure 6.9). Nitrite runoff from DCS-treated plots was particularly elevated compared with digestate during RS-48hr, 1.18 ± 0.31 vs 0.28 ± 0.11 mg L^{-1}).

6.3.5 Holistic comparison of DCS and digestate

A holistic toolbox has been developed which takes advantage of a broad range of techniques to characterise bio-based fertilisers and their impact on the environment and human health. For example, Nolan et al. (2020) reported the use of rainfall simulators and XRF analysis to assess runoff and accumulation in soil and grass of nutrients, metals and bacteria following land application of slurry and digestate, demonstrating that for each parameter tested, landspreading of digestate resulted in reduced pollution potential compared with unprocessed slurry. Another aspect of the toolbox utilises static chambers, gas-chromatography and photoacoustic spectrometry to assess GHG and ammonia emissions from organic fertilisers (Kavanagh et al., 2019; Krol et al., 2015). The present study has addressed a knowledge gap with respect to the land application of digestate from DCS co-digested with FOG. The final part of the toolbox, will take the field data and "model" various scenarios pertaining to incorporation of pollutants into the food or drinking water chain and examines risk to human health (Clarke et al., 2016).

6.4 Conclusions

This work examined gaseous emissions from grassland spreading of unprocessed slurry compared with anaerobically digested slurry under worst-case simulated rainfall conditions, whereby heavy rainfall events were simulated within 24 hours after landspreading. Anaerobic co-digestion of slurry with food processing waste addresses several EU Green Deal objectives, namely: reduction of greenhouse gas (GHG) emissions, while also improving air and water quality and maintaining a sustainable level of agricultural production (COM, 2019). AD is a particularly effective means of reducing (72 %) methane emissions from slurry application to grassland. Given the predominantly aerobic field environment, methane emissions following land application primarily result from release of trapped bubbles in the substrate, which may be exacerbated by rainfall droplet surface disturbance of the organic matrix.

As treatmenrs were applied as a function of P, more slurry was required, which resulted in extra N being applied in the slurry plots. This inevitably resulted in relatively higher N emissions from slurry plots, accounted for in the emission factors for each treatment. N_2O emissions from slurry peaked later than digestate, but over 117 days of the trial were 2x higher from slurry than digestate, while there was no significant difference between digestate-treated and untreated control plots. The effect of fungal growth on N_3O emissions, and possibilities for remediation requires attention.

No significant difference in CO_2 emissions was found between treatments, although the slurry treatment was slightly lower than untreated controls and digestate treated plots, perhaps as an artifact of the denser slurry "blocking" respiration. The higher NH_3 emissions from digestate immediately post-landspreading are in part attributable to unmitigated NH_3 emissions from slurry storage. Rainfall simulation increased CH_4 and N_2O emissions from slurry and digestate but suppressed NH_3 emissions for both. CO_2 emissions were higher post-RS for slurry, but lower for digestate, likely due to improved infiltration characteristics.

In light of the results obtained in the present work, the RED II e_{sca} bonus for slurry processed in AD appears to be a valid approach, particularly given the observed increased soil C in digestate amended plots relative to DCS treatment.

Anaerobic co-digestion of slurry with food processing waste is a viable means of reducing agricultural GHG emissions whilst maintaining fertiliser potential, thereby contributing significantly to development of a circular economy, but requires support that accounts for the emissions savings potential outlined in Annex VI of RED II. In the absence of an established AD infrastructure, there is an urgent need for safe and low capital expenditure treatment to reduce gaseous emissions from manure handling.

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Chapter 7: Conclusions and future research directions

7.1 Conclusions

The current work has taken a holistic approach to understanding the potential for mitigation of pollution from agriculture using anaerobic digestion, with a particular focus on reduction of pathogen load to the environment.

Miniature-scale trials were validated as proxies for investigation of FIB survival and biogas production where necessary for simultaneous examination of multiple variables. On that basis, 50 mL CSTR trials were established at different ratios of co-digestion feedstock, temperatures, retention times and loading rates. Response surface analysis was applied to model and optimise process parameters for different operational conditions. The model developed identified that with a combination of low organic loading and longer retention time, digestate sanitisation sufficient to satisfy EU standards is possible in AD at temperatures of 20 or 25°C, whilst also maintaining satisfactory methane production. Therefore as upgrading to biomethane becomes more prevalent, it may be possible to run AD systems at lower temperatures, thereby reducing the parasitic heat load.

The Irish AD industry predominantly utilises mesophilic CSTR of slurry co-digested with food production waste. Hence, the aim was to optimise sanitisation and biogas production under those conditions. By changing the feeding regime from daily to a three-day system, biogas yield per gram VS fed was increased by greater than 50 % and coliform and *E.coli* numbers were reduced below the EU pasteurisation standard. An initial examination of the metagenomic datasets demonstrated that semi-continuous feeding improves microbial community diversity and abundance compared with daily feeding. In particular, increased relative abundances of important fatty acid degrading bacteria (*Syntrophomonas*) and hydrogenotropic archaea (*Methanospirilium*) correlated closely with improved methane yield. A more comprehensive examination of this metagenomic dataset in conjunction with co-extracted RNA and proteins is required to examine microbial function in future work. Landspreading of unprocessed slurry presents risks of mobilisation during rainfall events thereby contributing to pathogen, nutrient and metal incidental losses. Field

trials carried out as part of this work demonstrated the reduced microbial load from application of digestate from slurry co-digestion to grassland and consequent reduced runoff compared with unprocessed slurry. Pasteurisation at two conditions further reduced microbial contamination. These results have been used by project partners in a risk analysis to demonstrate reduced risk to human and animal health from landspreading of pasteurised and unpasteurised digestate, compared with slurry. Metal and nutrient analysis of soil, grass and runoff also demonstrated reduced pollution potential from digestate compared with slurry.

Finally, a comparative examination of greenhouse gas and ammonia emissions following landspreading found 72 % and 50 % lower methane and N_2O emissions respectively from plots treated with digestate compared with slurry. NH_3 emissions were not significantly different between treatments but were higher than untreated controls, while CO_2 emissions were not significantly different between treatments and controls.

Taken holistically, this work highlights the efficacy of AD with or without pasteurisation as a means of reducing agricultural pollution. The benefits of AD for capturing methane emissions from slurry are well described in the literature. This work has demonstrated the further value of AD as a means of reducing pathogen load and further reducing GHG emissions when landspread. Where the requirement for pasteurisation is a prohibiting factor for development of agriculture-based AD, this work demonstrates the potential for optimisation of sanitisation through adjustment of operational parameters. In that scenario, processing of slurry with food production waste is a multi-beneficial solution for reducing the environmental impacts of unmitigated landspreading of animal slurry.

On that basis, and given the significant environmental effects associated with management of unprocessed manure slurries, policy encouraging or requiring processing through anaerobic digestion would be welcome.

7.2 Future research directions

The potential for optimisation of AD for improved methane yield through alteration of feeding regime has been demonstrated in laboratory-scale bioreactors in the present work. In the interim the author has had the opportunity to begin to apply these theories at full-scale (1.2 MWe) and the initial results look promising. The financial risk of AD system perturbation and potential failure is however, a significant deterrent to widespread implementation and hence, pilot-scale trials to further optimise the feeding regime and better understand the resulting system and microbial dynamics are required. Furthermore, the present work examined a relatively consistent feedstock mix (dairy cattle slurry with fats, oils and grease) while full-scale AD plants may utilise a more diverse mixture of feedstocks, or whatever is available. Thus, similar trials examining impact of feeding regime change on methane production from a range of feedstocks are required.

Although the present work focused on feeding regime manipulation as a mechanism for optimising biogas output from AD, there are several other parameters that may have equally significant impacts on biogas yield, and by extension, economic viability. The understandable reluctance to initiate system changes tends to extend to preparation of solid feedstocks, which is typically restricted to simple physical maceration or blending with liquid. Research into optimisation of biogas yield through alternative feedstock preparation mechanisms such as hydro-cavitation, microwave or sonication has progressed well at laboratory scale, but has yet to be scaled up. Pilot and demonstration-scale research is required to establish the (economic) validity of such pretreatment options, and should be coupled with trials of various operational parameter settings to optimise energy recovery.

Bacteriophage may play an important role in reducing *E. coli* in agricultural AD systems and their prevalence and potential for manipulation requires further attention. Bacteriophage of other potentially important pathogens appeared to increase in line with those relevant to *E. coli* during this work, and the possibility for further system manipulation for broad removal of pathogenic bacteria is worth exploring. There may also be some scope for dosing with bacteriophage as a process control agent to target specific pathogens, but given the relatively early stage of

bacteriophage research generally this would likely be prohibitively expensive in the short to medium term.

The present work has demonstrated several advantages of digestate utilisation as an organic fertiliser/soil improver, particularly when compared with slurry. However, dispatch of digestate to farmers is typically a significant net annual expenditure for AD plants, as farmers tend to be unwilling to pay for haulage or landspreading, sometimes due to skepticism about digestate fertiliser value. The benefits of digestate as an organic fertiliser/soil improver compound over time, as repeated application restores soil organic matter and recalcitrant N is released to the crop. Hence a knowledge transfer-oriented piece of comparative research is needed, whereby digestate, chemical fertiliser or slurry is annually applied to the same tracts of land on several crop and soil types near AD plants over the course of 2-3 years, either on the growing crop or directly prior to ploughing for soil incorporation. That research may serve to augment policy changes to encourage a shift toward slurry processing.

In the absence of such policy and farmer buy-in to the value of digestate, alternative uses for digestate are required to improve economic viability of digestate handling. These alternative uses may include precipitation of N to produce dry fertiliser, although the remaining liquor still requires haulage or transport off-site. In a cascading nutrient utilisation approach, research into the potential of digestate as a nutrient source for growing black soldier fly larvae or algae for animal feed is showing some potential, whereby waste heat from CHP units is utilised. The CO_2 by-product from biogas upgrading could also be used for growing algae, although only one AD plant in Ireland currently has an operational biomethane upgrading facility. There are a number of potential algae that have shown promise as a protein source for animal feed, including some that may have an effect on limiting methane production in ruminants.

The potential for AD as a biorefinery for producing high-value intermediates has been mentioned previously. Research is required into the persistence and fate of these intermediates in digestate when landspread. The use of volatile fatty acids from AD of food waste for production of bioplastics is gaining attention, but the fate of

those bioplastics in AD post-consumer usage is not yet understood. The potential for environmental contamination with partially degraded or persistent micro- or nano-plastics must be considered and addressed as bioplastics utilisation increases.

The metagenomic carried out in this work is a preliminary analysis, but presents an opportunity for high-resolution functional analysis of these dynamic systems. We plan to assemble novel genomes from the bioreactors and are working with a bacteriophage genomic specialist to better understand the role of phage in AD. The present work identified increased abundance of hydrogenotrophic archaea such as *Methanospirilium*, which also utilises CO_2, thereby contributing to improved biogas quality. Future research should work with full-scale operational biogas plants to examine the possibility of selecting for more dynamic and robust archaeal communities capable of producing higher quality and volumes of biogas.

Agriculture-based anaerobic digestion is a valuable mechanism for reducing agriculture-associated pollution. AD is a versatile system capable of capturing value from a multitude of organic wastes. There is significant potential for optimising AD, for efficiency of both sanitisation and biogas production, but also as a platform for a range of alternative high value outputs.