Microbial cycling at the farm - A systems approach to assess risks and opportunities.

Submitted by

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other university.

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Abstract

This thesis offers comprehensive characterisation of the farm microbe cycle. It tests the overall hypothesis that targeted stakeholder interventions may mitigate the generation and proliferation of microbial hazards on-farm and subsequently to the wider environment.

Grazed livestock production is the leading UK land use, a source of high quality human nutrition, a key component of the landscape and crucial environmental custodian. At its simplest it involves livestock rearing/producing upon their pasture food source, in landscapes not primarily suited to arable farming. This interaction between production animals, soil, forage and the wider environment (via water transport) poses unique challenges in managing disease and antimicrobial resistance (AMR) risk. Current controls focus upon veterinary interventions at animal scale and the resulting microbial hazard in water outflows external to farms.

This research identifies and defines specific increased disease potential and AMR hotspots within soil and grass at pasture on the North Wyke Farm Platform, a unique hydrologically isolated farm-system research facility. Microbiological risk was investigated alongside complementary soil chemistry and forage quality parameters. Field microbial hazard hotspots resulted through increased livestock interaction at locations due to traditional grazing management practices which do not take account of these risks. This increased animal activity resulted in higher faecal deposition rates, intensity of pathogen accumulation and transfer of AMR. The influence on bacterial population of these hotspots was greatest at 10 m proximal to location and did not extend beyond 20 m. Field hotspots role in subsequent hydrologic transfer was studied, with increased disease and AMR risk predicted by water outflow rate and hydrograph slope. Outflow was driven by precipitation events and field hotspots were disproportionate microbial hazard sources at a field catchment scale.

The negative impact of aerobic exposure upon silage nutritional and hygienic quality was found independently of soil contamination. The external depth of 0-30 cm in silage bales was found to present an increased *Escherichia coli* concentration and AMR prevalence. This increased microbial risk resulted through vulnerability to air ingress during silage storage due to sub-optimal production techniques. The impact on silage nutrition and health risks of prolonged feeding periods, extended aerobic exposure, was studied over 32 days. Correlation between increased *Listeria monocytogenes* pathogen concentration, decreased silage nutritional quality and increased temperature indicative of silage aerobic spoilage was found.

The conclusion of this multidisciplinary research found key temporal-spatial points exist within the farm microbial cycle where relatively simple stakeholder interventions, such as optimising pasture utilisation during grazing or ensuring aerobic exposure of silage is minimised, can generate mutual benefits to system productivity as well as reducing potential disease and AMR risk.

Dedications and Acknowledgments

My father was a farmer upon the Carrick border,
And carefully he bred me in decency and order.
He bade me act a manly part, though I had ne'er a farthing.
For without an honest manly heart, no man was worth regarding.

Robert Burns, 1784.

Dedications

Maw and Paw, you have always supported me and never stopped encouraging me. Your passion and belief in seeing me succeed is always with me, no matter how far from home I travel. Your values have gotten me where I am and I will forever be grateful.

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Author's declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Signed: Jamie McFadzean Date: 21 December 2018

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Glossary of terms

ADF – acid detergent fibre

ADL – acid detergent lignin

ADS – acid detergent solution

AMR – antimicrobial resistance

ASV – aerobic storage vessels

CAP – common agricultural policy

CFU – colony forming unit

CP – crude protein

DEFRA – Department for Environment, Food and Rural Affairs

DM – dry matter

EU – European Union

FIO – faecal indicator organism

GPS – global positioning system

HI – high income

ME- metabolisable energy

NDF – neutral detergent fibre

NDS – neutral detergent solution

NFC – non-fibre carbohydrates

NIRS – near-infrared spectroscopy

NVZ – nitrate vulnerable zones

NWFP – North Wyke farm platform

OECD - organization for economic cooperation and development

OM – organic matter

PCR – polymerase chain reaction

SOM – soil organic matter

UAA- utilised agricultural area

UK – United Kingdom

WFD – water framework directive

Chapter 1

Literature review: Challenges and opportunities posed by grassland based livestock farm microbial cycling

Summary Statement

Grassland based livestock is a production system of global economic, human food supply, land use and environmental importance. Often utilising agricultural land less well suited to arable farming, grazed livestock systems interact uniquely with their external environment through hydrological action. Grazing livestock existing upon their primary pastoral food source further connects this production system with the pasture, soil and wider landscape. Whilst often a low input extensive system the industry focus has developed significant gains in productivity. Similar gains to disease and antimicrobial resistance control have been limited to improvements in biosecurity and optimising veterinary drug usage. Whilst beneficial in reducing microbial risk they are primarily aimed at effecting change at the farm gate, by reducing incidence of disease or antimicrobial resistance development entering the farm at the animal scale and thus reducing the transmission of disease or antimicrobial resistance to the wider environment. Current surveillance strategies follow a similar methodology with focus upon testing at the animal scale during disease investigation and at water outflow external to the farm in regulatory or scanning surveillance. Few intervention or surveillance strategies target the on-farm microbial cycle at points beyond the animal or water outflow scale. Implementing surveillance or recommending microbial hazard control strategies at key points requires an improved understanding of the dynamics of farm microbial cycling. Effective mitigation strategies to reduce disease and antimicrobial resistance risk on-farm should ensure producer engagement by presenting mutual benefits to health and productivity. This can only be achieved through a multidisciplinary research approach combining agricultural management techniques with culture based microbiology and next generation microbial sequencing.

1.1. Grassland based livestock production

Livestock production accounts for approximately a third of global non-tundra land use (Steinfeld et al, 2006), generating a value chain in excess of £1.1 trillion (Thornton, 2010). Of the total land under agricultural use, grassland based livestock systems predominate (Gerssen-Gondelach et al, 2017, Prochnow et al., 2009). This is especially true in temperate climates (FAO, 1995) with OECD, other high income (HI), Central and South American countries representing more than two thirds of global grassland based livestock production (FAO, 1995). Approximately 35 % of Europe's utilised agricultural area (UAA) is categorised as grassland (Smit et al, 2008). Grazing delivers over 70 % of total ruminant nutritional requirements in North Western European countries (Mayne et al, 2000). Grazing livestock productions land-use dominance results from its adaptability as a farming system in various topographies, altitudes and climates (DEFRA, 2018) (Thornton, 2010). The principal forage type may occur naturally or can be adapted to best utilise regional conditions and performance targets (Rook et al, 2004).

Grassland based livestock production in the UK represent over 66 % of the UAA, grazing is the predominant feed source for ruminants (Qi et al, 2018, DEFRA, 2016, Smit et al, 2008). England has the lowest % of UAA classified as grassland at approximately 50 % (DEFRA, 2017) compared to Scotland, Wales and Northern Ireland whose grassland area each totals more than 80 % of their UAA (Smit et al, 2008). Pasture grazing livestock diets are supplemented with preserved forage such as silage during periods of pasture growth dormancy, annualy totalling over 34,000,000 tonnes FM in the UK (Wilkinson & Toivonen, 2003). The geographic variation in grazing livestock production across the UK is significant (DEFRA, 2016) and demonstrates the adaptability of livestock and forage species. UK grazing livestock systems dominate land unsuitable for arable farming, either due to topography (Nadal-Romero et al, 2016, Zervas et al,

1998), soil type (Cox & Amador, 2018, Pulido et al, 2016) and/or climate (Derner et al, 2018, Sloat et al, 2018). Active grazing remains the principal application in UK grassland production unlike other countries (Qi et al, 2018, Orr et al, 2016) where cut and fed forage systems are increasing. The importance of grazing systems as a human food source, economic contributor and landscape stewards are recognised by their continued support at government level (Ciaian et al, 2010, MacDonald et al, 2000).

1.1.1. Interaction of livestock and grazed pasture

Grazing systems demonstrate a unique farming environment within livestock production techniques. Livestock are afforded certain independence in decisions of their time spent, dependent on grazing strategies employed (Kaufmann et al, 2013, Galt et al, 2000). Grazing, relative to feed-lot or zero grazing production, enables livestock to exhibit preferences in their behaviours towards feeding, ruminating, resting and socialising (Handcock et al, 2009, Rutter, 2006, Robinson & Oddy, 2004). Particularly this supports independent livestock behaviour in locations within permitted grazing areas (Petherick, 2005). As for most of the UK production season grazed livestock are at pasture, this provides the ability to derive changes in their farmed environment driven by their behaviours. The situation of grazed production within natural or semi-natural landscapes also results in pressures upon the wider environment (Eccard et al, 2000, Milchunas & Lauenroth, 1993). The production relationship between livestock and pasture is often characterised as beneficial in supporting biodiversity (Marty, 2005, Fuhlendorf & Engle, 2001, Olff & Ritchie 1998), welfare (Petherick, 2005, Hovi et al, 2003), the direct return of nutrients to soil and forage (Saleem, 1998, Haynes & Williams, 1993). There are also negatives associated, primarily with the environmental impact of emissions (Garnett, 2009, Lassey, 2007). Grazing livestock welfare can

be reduced in terms of access to veterinary health provision if inadequately managed (Nieuwhof & Bishop, 2005, Silanikove, 2000). The nutrient management of housed livestock production systems are also more efficient than grazing in terms of nutrient use efficiency as feed can be rationed to live weight yield (Wilkinson & Lee, 2018). The direct interaction of livestock production with the natural landscape is increasingly highlighted as a negative route for environmental contamination through livestock excretion on pasture and the managed application of manures (Hedley & Grace, 2008, Jones, 2001), particularly given the potential for widespread transfer of chemical (Hedley & Grace, 2008, Kemper, 2008) and biological (Tian et al, 2002, Mawdsley et al, 1995) isolates by hydrological action from farmland.

The application of grazing strategies is principally used to optimise pasture and livestock productivity. This theory was demonstrated by Chapman et al (2006) who compared a set-stocking system (where livestock are given unrestricted access to total pasture area) with a rotational managed grazing system (where livestock are given access to a restricted portion of pasture before being moved onto the next portion). This study, and others (Hoden et al, 2009, Walton et al., 1981), indicated rotational managed grazing resulted in increased total pasture productivity and thus greater potential for live-weight gains. If applied correctly, grazing strategies enable the maximal gain in animal live weight whilst maintaining pasture growth (Chapman et al, 2006, Walton et al., 1981). Grassland production is maximized through utilisation, yield and management input required. Grazing strategies effectively influence livestock behaviours by restricting access to pasture and controlling when stock are provided with access (Handcock et al, 2009, Petherick, 2005). This results in more uniform grazing behavior, more uniform nutrient return and pasture regrowth (Oates et al, 2010, Chapman et al, 2006). This partitioning of grassland requires additional boundaries, water sources and management. However, the necessary outlays benefits

through increased pasture production with optimised land use and improved live weight gain of stock.

1.1.2. Rothamsted Research North Wyke Farm Platform

In response to the dominance and importance of grazed livestock production within the UK and global agricultural sector the Rothamsted Research North Wyke Farm Platform (NWFP) was established in 2010 (Takahashi et al, 2018, Orr et al, 2016). The national capability site was designed as a system scale experiment to investigate management influences on grazed livestock economic, environmental and social sustainability. Located in Devon, South West England, UK (50º46'10" N, 3º54'05" W) the site is representative of many common industry features of grazed livestock production throughout the UK. Situated on an undulating ridge with elevation ranging from 120-180 m, the NWFP is bordered by two natural water courses and receives annual rainfall averaging 1044 mm. The sites dominant soil type of heavy/medium clay sub-soils are not conducive to significant sub-surface vertical water flow, delivering outflow from fields as surface and sub-surface horizontal flow. This soil type, topography and climate is demonstrable of conditions which would be more challenging for arable production. The site was divided into three sub-farms, of 21 ha each. Each sub-farm was allocated five outflow field-catchments that gave equal representation of conditions. Catchments were defined by topography and engineered infrastructure of deep sub-surface drains. This process ensured catchment outflow was hydrologically isolated and could be individually assessed at the single outflow point. The sub-farms were designated under three differing pasture management strategies: Permanent pasture - perennial ryegrass (*Lolium perenne* L.) improved pasture with inorganic fertilizer application, Increased use of legumes - mixed high sugar perennial ryegrass and white clover (*Trifolium repens*

L.) pasture with zero inorganic fertilizer application and Planned reseeding - regular renewed pasture, presently high sugar and deep rooting grasses (Orr et al, 2016). All three sub-farms are supplied with cattle and sheep from the same primary herd/flock and are managed to industry representative standards of best practice and assessed to evaluate sustainability (Takahashi et al, 2018).

1.2. Risk of disease and AMR from grazed livestock systems

The unique interaction of grazing livestock with pasture and the wider natural environment, as previously described, poses challenges for the successful control of disease and antimicrobial resistance (AMR) development. The occurrence of disease in livestock can result from environmental acquirement (Gilbert et al, 2006, Morgan et al, 2006, Waller, 1999) or through animal to animal transfer (Durand et al, 2004, D'Allaire et al, 1987). Similarly, AMR may be acquired from the environment (Allen et al, 2010, Kummerer, 2004), through the close relationship with pasture during grazing, directly from other livestock (Carty et al, 2003) or driven by veterinary drug use (McEwan and Fedorka-Cray, 2002, Teuber, 2001).

There is also a risk of livestock acquired disease, colonisation or infection with AMR bacteria in the agricultural industry workforce (Graveland et al, 2011, Bender & Shulman, 2004). This relates to specific zoonotic pathogens such as *Listeria monocytogenes* (Nightingale et al, 2004) or resistance patterns such as livestock associated methicillin resistant *Staphylococcus aureus* (Graveland et al, 2011). Generally, these instances of directly livestock acquired disease or AMR have limited onward human transmission (Holmes et al, 2016, Kock et al, 2013, Graveland et al, 2011). Their control depends on strict observance or biosecurity and personal hygiene (Graveland et al, 2010). However, the dissemination of disease of livestock origin and AMR to the wider

environment poses a larger scale risk to public health (Craft, 2015, Woolhouse et al, 2015). This route principally occurs through passive carriage by hydrological action (Sayah et al, 2005, Hooda et al, 2000), spreading pathogens and AMR bacteria from grazed fields to wider natural water courses and beyond. This not only increases the likelihood of human contact with zoonotic diseases but also the spread of resistance from one sphere of use to many others (Baquero et al, 2008). Other external transmission routes of farm microbial hazards exist beyond waterborne transfer. There is a limited potential of farm to human microbe transfer directly through the food chain (Newell et al, 2010) (this should be negligible if appropriate food preparation and hygiene is applied). There is also notable impact of wild animals (Allen et al, 2010, Bengis et al, 2002) (particularly wild bird species) who have potential to contact multiple microbial hazard environments, as well as farms, and deliver pathogens and AMR bacteria to naive locations.

Livestock production is recognised as a pathogen and AMR source (Woolhouse et al, 2015, Patz et al, 2004), which has the potential to significantly impact public health. However, there is undoubtedly a significant impact on human disease rates and AMR prevalence originating from livestock production (Craft, 2015, Woolhouse et al, 2015).

1.2.1. Current control strategy for farm microbe risk

The current strategy for mitigating microbial hazard in livestock relies on a 'farm gate' control policy. Meaning that infection and the generation of AMR are treated and managed at perceived point of entry to the farming system. In the case of disease control strategies these relate to external introduction, either through new livestock entering the farm or from wildlife spread (Daniels et al, 2003, Smith et al, 1998). This relies on strict stakeholder implementation of biosecurity measures, reducing opportunities for external disease risk to enter the farm, such as required quarantine for

new stock (Gunn et al, 2008, Little et al, 1992) and/or usage of therapeutic treatments (LeBlanc et al, 2006). The process of reducing the rate of AMR development is again managed at perceived point of entry, being principally veterinary drug usage (LeBlanc et al, 2006, Aarestrup, 2005). This has resulted in the widespread success of the 'responsible use' strategy (Anthony et al, 2001), where appropriate use is dictated by demonstrable threat of disease.

Similarly, the control of external transfer from livestock to the wider environment is implemented at the point of exit from the farming system. Appropriate application of biosecurity measures can mitigate opportunity for wildlife transmission (Smith et al, 1998) (Daniels et al, 2003). However, the impact of passive waterborne transfer of microbial hazard from grazed and manured fields to natural water courses relies fully on 'up-stream' measures (Woolhouse et al, 2015, Sayah et al., 2005, Hooda et al, 2000). The pathogen and AMR hazard exiting livestock farming systems is proportional to the successful application of management strategies to reduce incidence of disease or AMR development in livestock (Craft, 2015, LeBlanc et al, 2006). The development of effective microbial hazard control steps after animal scale interventions requires improved characterisation of how microbial disease and AMR are transferred on-farm. Only through identifying demonstrable key points in the farm microbe cycle can a holistic control strategy, beyond the linear route of microbial hazard entering and leaving the farm, be developed.

1.2.2. Better characterising farm microbe risk

Disease and AMR both on-farm, and following transmission, externally exists as a dynamic temporal and spatial risk (Daniels et al, 2003). This is not reflected by our control strategies which rely on animal scale or system input interventions. The reliance on landscape measures of agricultural microbe transmission inaccurately generalises potential risk (Woolhouse et al, 2015,

Keeling et al, 2001). This also results in ‘catch all’ control strategies which lack the tailored resolution to effect optimum reductions in risk (Martinez, 2009). To improve the accuracy of disease and AMR risk determination within grazing production requires system specific characterisation of the microbial cycle. This approach of developing a multiple point intervention strategy to disrupt on-farm risk cycles has long been recognised within parasitology (Stear et al, 2007, Waller, 1999). Gaining an understanding of specific key points in parasite life cycle has enabled stakeholder mitigation strategies to be developed beyond the animal scale, involving field and land management (Stear et al, 2007). While potential pathogenic and AMR bacteria do not exist within similar life cycles as parasites their accumulation and transfer on-farm is not considered to be linear, but cyclical in their accumulation and transfer within agricultural systems (Paull et al, 2011, Lindsay et al, 2009). Identifying the key temporal spatial points at which microbial hazard on-farm is increased can inform targeted intervention strategies to affect their reduction and potential for external transmission.

Zoonotic diseases and emerging patterns of AMR are recognised as critical and mutual threats to human and animal health globally. The necessary resources to operate effective veterinary surveillance within the livestock sector are under increasing pressure and a pragmatic approach of targeted surveillance to highest risk hazards offers the greatest cost-benefit potential (DEFRA, 2011, Thornton, 2004). Targeted surveillance was first described in bovine spongiform encephalopathy surveillance, focusing sampling resources to cattle populations of higher disease prevalence (Doherr et al, 2001). The definition of risk in epidemiology is the probability an individual will develop disease over a given time interval (Rothman and Greenland, 1998) varies with risk as defined in risk analysis as the likelihood of an unwanted event occurring and its subsequent cost (International Animal Health Code, Stark et al, 2006). The concept of risk based

surveillance has been widely adopted. However, identification of the highest risk hazard sources within grazed livestock production lacks a robust evidence base. The strategy of risk based disease and AMR surveillance relies on identifying sampling points which poses the greatest potential for early hazard detection and subsequent management of hazard risks (Stark et al, 2006). This risk based surveillance with targeted sampling of high-risk populations at a field and farm scale remains underutilised in livestock production systems (Salman et al, 2003). The traditional surveillance model operated in grazing livestock systems relies on the principle external human and animal exposure pathway of hydrological transfer. This is a logical surveillance target given that 20% of all UK waste water originates from agricultural sources, with 57% (271million tonnes) of that defined as non-controlled waste such as slurry, farmyard manure and silage effluent all sources of multiple hazards (Rushton, 2003). Catchment scale water sampling does not meet the thresholds of risk based surveillance, namely enabling early hazard detection, as sampling occurs after microbial hazards have been generated and only identifies them once they have reached sufficient concentrations to be recorded in waterborne dissemination at a landscape scale. This information remains extremely useful in monitoring the external risk of agricultural systems through principle exposure pathways and enables some broad mitigation strategies to be implemented which inform regulatory policy (McGonigle et al, 2012, Souchere et al, 2003, Sudol & Ambrose, 2002). Catchment scale surveillance does not sufficiently enable management of hazard risk at point of source as true risk based surveillance does.

Risk based surveillance or targeted sampling at identified key microbial hazard sources on-farm within grazing livestock systems could provide crucial data on potential risk potential of pathogens and AMR patterns (Stark et al, 2006). These points should provide potential to identify emerging hazards early (prior to their dissemination at a catchment scale) and be points were

effective management strategies can be directly applied to achieve a reduction in risk (Stark et al, 2006). The logical more focused surveillance target within grazing systems after catchment scale sampling, accepting that hydrological transfer is the principal exposure pathway to the wider environment, humans and animals, is at field scale. Beyond the field scale, specific environments within fields and production cycle where livestock origin microbial hazards can accumulate should be identified as part of a risk based surveillance strategy (Brundl et al, 2009). This sampling model acknowledges the key role of livestock in supply of pathogens and AMR hazard within grazing systems and through a principally hydrographical exposure pathway to wider human and animal populations (Brundl et al, 2009). This targeted sampling data has the potential to inform on-farm risk assessments such as those widely adopted in the food production industry (Todd, 2003). Developing microbial criterions for key targeted sampling points within grazing systems would enable thresholds of risk to be set and management interventions applied where necessary (ICMSF, 2002). Similar to the food production industry, utilising measures of concentration for indirect indicators of pathogens such as generic *E. coli* or multi-AMR and the absence/presence for pathogens such as *L. monocytogenes* in targeted sampling of grazing livestock systems can inform on-farm risk assessments (FAO, 2001). The food industry principally utilises the hazard analysis critical control point (HACCP) approach in risk assessment with the public perceiving a zero tolerance to foodborne pathogens (Todd, 2003). This approach would not be practical or constructive if applied to on-farm microbial risk assessments, in part as mitigation strategies applied at the critical control point of hydrological dissemination do not effect a reduced risk for on-farm hazard accumulation, Adapting the described microbial criterions for use within an Acceptable Level of Protection (ALOP) strategy has far greater practical potential (Office of Disease Prevention and Health Promotion, 2000). The ALOP approach to risk assessment enables

direct correlations to be made between hazard reduction and the nessecary interventions required alongside defined microbial tests or criterions (Todd, 2003). Achievable thresholds for microbial criterions can be set beyond baseline and subsequently reduced as management strategies improve. Pursuing this risk based surveillance strategy at key points of the greatest livestock pathogen/AMR concentration within grazing systems would enable risk assessments that not only effect reductions in onward risk potential through the hydrographical exposure pathway but also a reduction in microbial hazard accumulation on-farm.

1.3. Surveillance of agricultural microbe risk

As described the determination of disease and AMR on-farm currently focusses on points of system entry and exit. Microbial disease, and to a lesser extent AMR, surveillance is primarily undertaken during infection investigation of livestock (Woolhouse et al, 2001). Current scanning surveillance undertaken by active testing of animals or environmental sampling is limited (Stark et al, 2006). Surveillance is designed to identify causative pathogens (Bennett & l'Jpelaar, 2005) or identify resistance relating to treatment failures (Woolhouse et al, 2001) but again this can only inform general control strategies at system entry. Thus, the perceived risk of disease and AMR resulting from livestock is primarily characterised by animal testing and landscape scale environmental sampling. This surveillance is crucial in identifying disease and AMR trends circulating in livestock or transferred to the environment but cannot inform additional stakeholder mitigation strategies on-farm as it does not characterise the field scale hazard sources (Stark et al, 2006, Bennett & l'Jpelaar, 2005). A multi-disciplinary approach combining agricultural management techniques with culture based microbiology and next generation microbial sequencing is required to identify key points for on-farm microbial risk surveillance. The

utilisation of a culture and sequence based approach to determine microbial hazard alongside associated production parameters will aid in the development of effective on-farm control strategies with mutual benefits to health and production.

1.3.1. Faecal indicator organisms

The use of faecal indicator organisms (FIO) as quantifiable markers for faecal contamination in various environments is well established (Fenlon et al, 2012, Crowther et al, 2002, Lau & Ingham, 2001). Their application in determining an increased risk to public health is supported throughout policy legislation (Souchere et al, 2003), such as the EU water framework directive (WFD) (McGonigle et al, 2012) and the clean water act in the USA (Sudol & Ambrose, 2002). The use of FIO as a surveillance methodology for quantifying potential disease hazard resulting from faecal contamination has been applied within water (Crowther et al, 2002), soil (Lau & Ingham, 2001) and plant (Fenlon et al, 2012) environments. The microbial species currently defined as FIO are *Escherichia coli* and intestinal enterococci (Standing Committee of Analysts, 2018). Their demonstrable association to faecal concentration and environmental hardiness permit the inference of disease hazard resulting from faecal contamination (Standing Committee of Analysts, 2018). While FIO are widely used in different research fields their application in regulation primarily focusses on waterborne risk (McGonigle et al, 2012, Crowther et al, 2002). This application of FIO analysis enables the benchmarking of faecal contamination at a landscape and catchment scale but not point of source, particularly in reference to aquaculture, drinking and bathing water (Wilkinson et al, 2006). The policy application of FIO such as within the WFD, the associated requirements relating to CAP subsidy (Souchere et al, 2003) and the expressed commitment of DEFRA (DEFRA, 2011) deliver legal requirements for agriculture to reduce the

prevalence of livestock origin disease within the wider natural environment. The established metrics within nitrate vulnerable zones (NVZ) to control nitrogen water pollution by fertilisers and manure (Worral et al, 2009, Jordan & Smith, 2005) indicate the potential for future on-farm regulations relating to FIO. To develop similarly strategies or regulatory requirements as exemplified by NVZ, FIO prevalence dynamics on-farm must be better understood to enable regulations which reduce the sources of these hazards.

1.3.1.1. Escherichia coli

The application of *E. coli* as a marker for faecal contamination across various environments results from its ubiquitous occurrence at high concentrations in all mammal faeces (Edberg et al, 2012). While *E. coli* exhibits significant environmental hardiness and survival abilities it is not considered to achieve significant environmental proliferation (Edberg et al, 2012, Anderson et al, 2005). This interrelatedness of *E. coli's* wide environmental survival potential and reduced ability for environmental multiplication make it a key marker for measuring livestock origin disease hazard on-farm (Hodgson et al, 2009, Oliver et al, 2005).

While potentially pathogenic as a species, increased environmental *E. coli* concentrations also indicate the potential for other zoonotic diseases (Wyer et al, 2012). This can inform judgements based on perceived disease risk to be applied in response to determined *E. coli* concentrations. The use of *E. coli* as a preferential indicator species for AMR is also related to its capacity for environmental survival but also ability to exchange and accumulate resistance encoding genes (Reinthaler et al, 2003). This enables the use of *E. coli* as a sentinel microbial model species, indicative of AMR patterns within specific environments (Sayah et al, 2005, Reinthaler et al, 2003). The application of culture based AMR determination facilitates the identification of

potential expressed resistance patterns within microbial communities. The analysis of *E. coli* concentrations can therefore be directly related to faecal contamination of environments, indicating increased disease risk and determination of its expressed resistance can suggest trends in environmental AMR risk.

1.3.1.2. Intestinal enterococci

The use of intestinal enterococci species as faecal contamination markers is similarly related to *E. coli*. Intestinal enterococci are present at high concentrations within all mammal faeces (Pourcher et al, 1991) and are considered to exhibit significant environmental survival potential but, as with *E. coli*, limited environmental proliferation (Anderson et al, 2005). The culturing of intestinal enterococci as a FIO requires the differentiation from environmental enterococci species by expressed kanamycin resistance (Nowakiewicz et al, 2017). Kanamycin resistance is associated with high expression in *Enterococcus faecium* and *Enterococcus faecalis* (Tiwari et al, 2018) with aesculin hydrolysis required for intestinal enterococci confirmation (Tiwari et al, 2018). The concurrent sampling of intestinal enterococci and *E. coli* is a requirement of most FIO policy applications (McGonigle et al, 2012, Sudol & Ambrose, 2002). Given the proposed use of FIO sampling in more poorly characterised environments on-farm, beyond water sampling, it is considered prudent to similarly apply concurrent sampling. This is primarily to previous study findings indicating variations in intestinal enterococci and *E. coli* decay rates in specific environmental conditions (Anderson et al, 2005) but also to ensure results have the widest external relevance to the current application of FIO measures (McGonigle et al, 2012, Sudol & Ambrose, 2002).

1.3.2. Listeria monocytogenes

The use of *Listeria monocytogenes* culturing is not widely prevalent within scanning surveillance (Grif et al, 2006). It is a critical feature in animal and human disease (Grif et al, 2006, Piffaretti et al., 1989) investigations in the identification of causative organisms. Its application within more comprehensive on-farm microbial hazard sampling is due to its relevance and recognition both within the agricultural industry (Nightingale et al, 2004, Piffaretti et al, 1989) and human healthcare (Grif et al, 2006, Piffaretti et al, 1989). Its association as a livestock pathogen with significant production implications is well understood and its significant public health impacts have been extensively studied (McLauchlin et al, 2004, Piffaretti et al, 1989). *L. monocytogenes* inclusion within on-farm sampling provides a well understood and demonstrable disease risk that is likely to increase potential stakeholder uptake of any resulting recommendations. This specific pathogen has known associations with livestock disease origination from contaminated forage and this is considered a key point in investigating on-farm microbial cycling.

1.3.3. Antimicrobial Resistance in the environment

Addressing the threat posed by increasing AMR is recognised as a priority for the continued safeguarding of human and animal health (Singer et al, 2016, Berendonk et al, 2015). Crucially identifying and monitoring AMR patterns in the environment is necessary if surveillance is to indicate effective mitigation strategies (O'Neill, 2016, Bush et al, 2011). The major driver in acquisition of clinically relevant resistance patterns is recognised to result from treatment application within clinical settings, dissemination of these genes and their interaction with other external microbial populations has generated a significant environmental risk (Allen et al, 2010,

Canton, 2009). The proliferation of AMR within the environment is driven principally by horizontal gene transfer of AMR genes, selective pressures imposed on microbial populations, mutation and recombination of genes (Wright, 2010, Baquero et al, 2008). Beyond clinical settings there exists specific locations associated with high microbial deposition/concentration of human or animal origin microbes (Berendonk et al, 2015, Wright, 2010). Livestock production systems can be characterised as such a location, potentially contributing sub-therapeutic concentrations of antibiotics and high concentration of AMR microbes to their environment (Berendonk et al, 2015). This continual process of introduction of AMR drivers or genes to otherwise naive environmental microbe populations is considered to be a potentially significant exposure pathway in the proliferation of clinically relevant AMR bacteria (Carattoli et al, 2012, De Gelder et al, 2008). This is particularly relevant within grazing livestock systems where deposition of animal origin and potentially AMR bacteria occurs annually at a landscape scale. The boundary between grazing livestock with the wider natural environment to human and animal populations beyond is transected by hydrographical transfer (Sayah et al, 2005, Hooda et al, 2000). This process enables selective pressures or genetic exchange influencing AMR patterns to be disseminated beyond the direct environment of livestock pasture and their localised microbe populations (Kyselkova et al, 2015, Su et al, 2015). There remains insufficient knowledge surrounding the factors which result in mobilisation, selection and transmission of these AMR bacteria from the environment and into human and animal populations (Bush et al, 2011). Contextualising the prevalence and patterns of AMR, even in a comparatively low therapeutic input system, is crucial if the wider environmental risks are to be assessed and impacts on clinical treatments be mitigated.

1.3.4. Next generation sequencing

The application of sequencing based microbial surveillance has resulted in significant advancement in the understanding of microbe population dynamics on-farm (Garbeva et al, 2004, Lawlor et al, 2000). The potential for species specific sample characterisation can indicate underlying pressures on microbial communities (Lawlor et al, 2000, Bossio et al, 1998). The targeted application of this approach can demonstrate environmental variations in microbial community structures driven by agricultural management practices (Calloway et al, 2010, Lawlor et al, 2000) (Bossio et al, 1998). Within a multi-disciplinary study sequencing based results can provide additional higher resolution descriptions of the drivers of microbial population differences. Combining sequencing and culture based approaches can provide more comprehensive descriptions of the factors influencing increased disease or AMR risk at identified key points on-farm (Goddard et al, 2012, Hugenholtz et al, 1998).

1.4. Farm microbe cycle

The farm microbial hazard as described is not a linear process of disease or AMR entry and exit but a dynamic cycle within which several environments are key determinants (Figure 1) (Paull et al, 2011, Lindsay et al, 2009). The relationship between grazed livestock and pasture is critical in the ultimate external transmission of farm origin microbial hazard (Craft, 2015, Woolhouse et al, 2015, Patz et al, 2004). This interaction involves the deposition of faecal material by livestock and its managed application as fertilizer but also the consumption of pasture or harvested forage by livestock. Determining the dynamics of microbial hazard deposited on field soil and grass is critical, as is characterising the potential circulation risk through grass and harvested forage

consumption. A requirement also exists to quantify the external transmission risk of disease and AMR from farm to contextualise the wider environmental impact of identified risks.

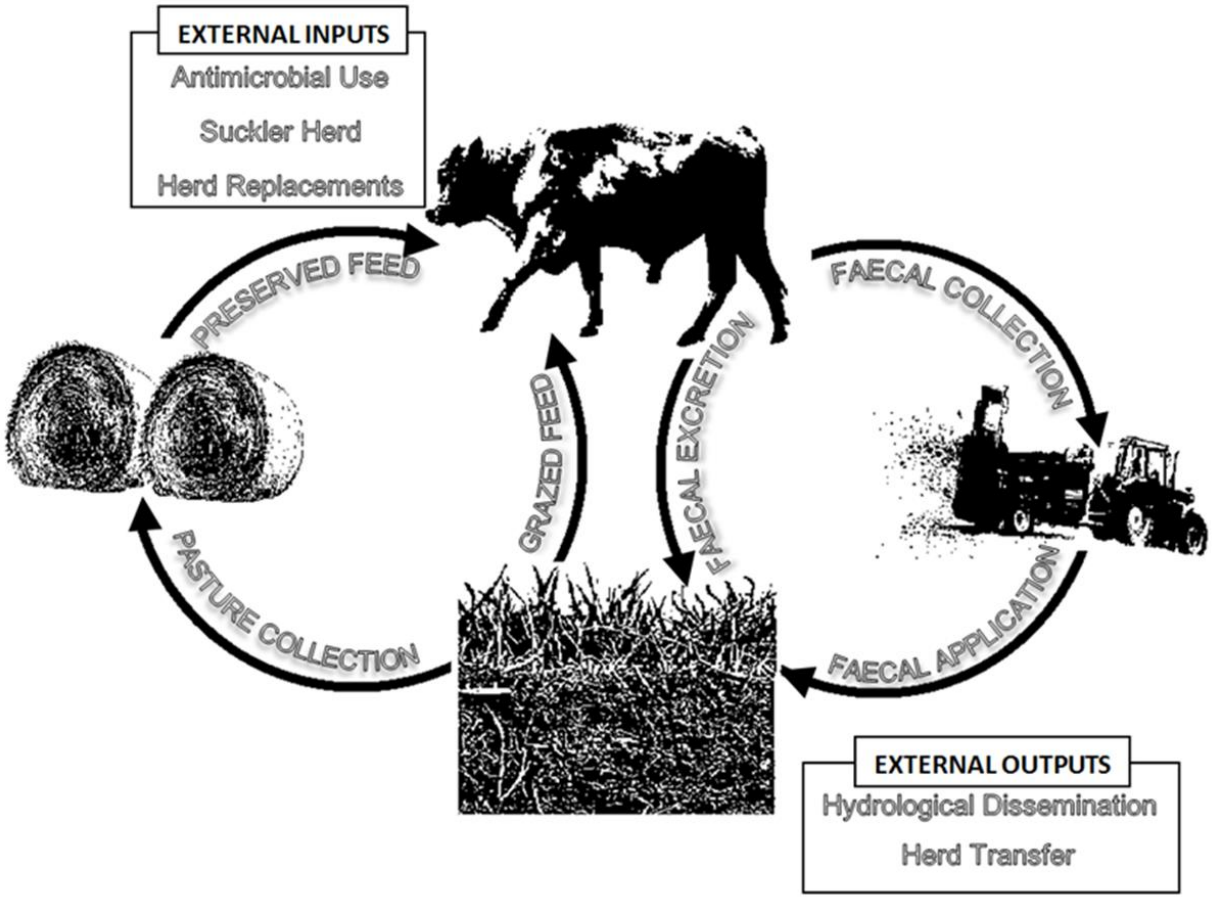

Figure 1. Hypothesised on-farm microbial cycle.
External drivers of disease and AMR hazard are presented informing the suggested on-farm microbial cycle and its resulting external outputs.

1.5. Conclusion

The established dominance of grazed livestock farming as the principal ruminant production system across the globe has resulted in its presence across whole landscapes. The intrinsic relationship between livestock and the environment they graze produces both challenges and opportunities for the mitigation of disease and AMR. The development of improved control strategies requires a more comprehensive characterisation of microbial cycle dynamics on-farm.

To achieve this a multi-disciplinary, multi-scalar surveillance approach is needed. The demonstration that key points in farm microbial cycling exist temporally and spatially within grazing systems will enable effective recommendations to be developed. These mitigation strategies should endeavor to provide the greatest reduction in disease/AMR risk alongside production benefits. There is a need to provide stakeholders with on-farm interventions that disrupt the cycling of microbial hazard on-farm beyond point of entry and exit to the system.

This thesis overall hypothesis is that microbial risk on-farm increases significantly in specific temporal/spatial points and that the impact of key points in disease and AMR cycling can be mitigated through modified stakeholder practice eg. grazing practices and silage preservation management. This hypothesis is tested using a multi-disciplinary approach combining agricultural management techniques with culture based microbiology and next generation microbial sequencing. Analysis undertaken ranges from individual animal to farm scale, investigating associations between health and production parameters,

Chapter 2

Lysimeter study: investigating the transfer of faecal microbes to soil and leachate

Summary

The soil environment is a key stage in on-farm microbial cycling. In grazed livestock production soil, and the associated microbial populations, directly interact with the animals through incorporation of faecal material. The principally hydrological process by which farm origin microbes are disseminated to the wider environment is directly influenced by the characteristics of the soil environment. This preliminary laboratory scale study investigated faecal indicator organism concentration and microbial population sequencing of cattle faeces from permenant pasture, high sugar perennial ryegrass and white clover, and monoculture high sugar ryegrass diets. Analysis was conducted on faecal treated and untreated soil lysimeter leachate following a simulated storm event. For each treatment, the faecal indicator organism colony forming units, the microbial diversity and abundance was analysed. This experiment indicated a 1 - 2 log reduction in *Escherichia coli* concentration in leachate derived from livestock faeces on soil under action of lateral transfer by rainfall, relative to original faecal deposit. The concentration of faecal indicator organisms in faecal treated soil leachate was higher than untreated soil. The 16S sequencing analysis indicated microbial diversity varied across all treatments. The permanent pasture diet was found to possess the greatest microbial diversity and the monoculture high sugar ryegrass the least. Differences in leachate microbe populations were found to be influenced by diet as determined by forage water soluble carbohydrate content. Leachate microbial composition differences were primarily accounted for by soil devived microbial variation. The combined culture and sequencing based approach of this study indicates the retaining potential of the soil matrix and the influence which faecal deposits on pasture elicit on the wider watercourse. Chapters 3 and 5 will benefit from targeting these spatial and temporal sampling points, of greatest faecal deposition and microbial accumulation, eliciting the greatest effects.

2.1. Introduction

Currently lacking from disease transfer knowledge is a fundamental understanding of the role of the environment and potential for microbial cycling within production systems. There exists an understanding of the negative effects from livestock production to the wider environment at a landscape scale by hydrological transfer, disseminating animal origin contaminants and pathogens (Kay et al, 2009, Hooda et al, 2000, Khaleel et al, 1980). The direct interaction which faecal material and soil undergo on pasture is less well characterised (Avery et al, 2004, Tyrrel & Quinton, 2003, Jamieson et al, 2002). The additive effect of faecal material on the soil microbial community is considered significant (Fermaux et al, 2008, Jamieson et al, 2002). However, the focus is often on the results of transport outside of the system boundary (Soller et al, 2010, Kay et al, 2007, Collins et al, 2007). Understanding this process is key to developing effective strategies to study microbial cycling at a larger scale.

The influence of soil type and characteristics should not be underestimated as one of the principal factors influencing microbial cycling (Van Der Heijden et al, 2007, Desmarais et al, 2002). The role of soil both in the hydrological passage of microbes outside of individual farm boundaries (Tallon et al, 2005, Byappanahalli & Fujioka, 2004) and in the retainment and proliferation of populations within fields (Rogers et al, 2011, Anderson et al, 2005) is crucial for human (Soller et al, 2010, Ishii, 2008, Ashbolt, 2004) and animal health (Pell, 1997). However, this influence is extremely regionally specific and larger landscape studies may rely on assumptions which are not valid for particular soil types or characteristics (Crowther et al, 2002, Andersson et al, 2001, Crowther et al, 2001). To develop effective field studies a site-specific understanding of soil properties is required, combining existing data or undertaking preliminary experiments can achieve this (Di & Cameron, 2007, Vereecken, 2005, Ogden et al, 2001). As

described in Chapter 1 a significant understanding of the soil characteristics on the North Wyke Farm Platform exists (Orr et al, 2016). This knowledge facilitates the development of farm representative laboratory scale experiments, allowing for field scale theories to be tested and methodologies trialed (Takahashi et al, 2018).

The use of lysimeters is a well-established technique in controlled condition experiments (Di & Cameron, 2007, Vereecken, 2005, Ogden et al, 2001). Replicating accurate knowledge of soil characteristics within well designed lysimeters can provide accurate, externally relevant, predictions of a variety of metrics in given conditions (Gupta et al, 2017, Pang et al, 2008). This methodology is often employed in modelling the role of water in lateral transmission of associated factors, both chemical (Di & Cameron, 2007, Vereecken, 2005) and biological (Vereecken, 2005, Ogden et al, 2001). Effective lysimeter design can indicate the capacity of a given medium to bind, or release in throughflow, specific factors. Lysimeters, and the resulting leachate following application of water, can suggest specific processes which may warrant investigation by larger subsequent studies (Shaheen et al, 2014, Bastissnssen et al, 2005, Youcai et al, 2002). This preliminary study approach can guide a more targeted approach of field scale sampling and optimise experimental resources. The limitations of controlled condition lysimeter based methods should also be reconised when developing wider theories (Vereecken, 2005). The field scale relevance of lysimeter findings are derived from the accuracy of the soil conditions modelled and require robust datasets of field characteristics (Bergstrom et al, 2010). The physical design of the lysimeters should also be considered as influencing results and must be applied to minimise artifacts deriving from the engineering (Corwin, 2000, Cameron et al, 1992).

The disease potential and antimicrobial resistant properties derived from agricultural shed microbes are not limited by individual species survival characteristics. The environmental

dissemination of livestock source microbes from faeces which possess antimicrobial resistance or disease causing factors is well documented (Kummerer, 2003). The passage of microbes from livestock, where the potential for antimicrobial drug resistance and disease exists, onto pasture is spread by a variety of mechanisms, such as transmission by carriers (Olsen et al, 2006, Yu et al, 2006) but also directly on to pasture and watercourses by hydrological action (Martinez, 2009, Martinez, 2008). The introduction of antimicrobial resistant and potentially pathogenic livestock origin microbes with external environmental microbe populations can increase the prevalence of disease and antimicrobial resistance (Oliver et al, 2009, Ochman et al, 2000). Of the UKs total land 70% is deemed Utilisable Agricultural Area with merit for crop or livestock production, of this area approximately 1/3 minimally supports seasonal livestock grazing (Westbury et al, 2011, Davey, 2010, Stoate, 2009). The sizable area of land supporting livestock through pasture based production systems dominates in regions of climates with high rainfall. The potential for microbe transmission by action of rainwater as surface runoff and by lateral transport through pasture into the wider watercourse is considered significant (Kay et al, 2009, Collins et al, 2007). As discussed in Chapter 1 the interaction and potential horizontal gene transfer by potentially antimicrobial resistant bacteria and diseases originating from livestock effluent with populations such as soil micro flora (Bennett, 2008, Martinez, 2008, Ochman et al, 2000) has the capacity to self-perpetuate as a source for increasing levels of antimicrobial resistance and infection (Radhouani, 2012, Guenther, 2010, Poeta, 2008). Crucially it is the dissemination of antimicrobial resistance amplified from drug use within intensive livestock productions that is perceived to be a significant threat to wide scale antimicrobial resistance development and the corresponding impacts upon food security and public health (Zhang et al, 2009, Chee-Sanford et al, 2001).

This research project applied accurate knowledge of the North Wyke Farm Platform soil characteristics to controlled environment lysimeters and modelled the effect of simulated rainfall event upon microbe populations in resulting leachate. The study utilised cattle faeces from herds with three different diets, soil originating from North Wyke Farm Platform and representative artificial rainfall event. This experiment will aid in the development of farm scale hypotheses on microbial cycling, inform subsequent field studies design and methodology.

2.1.1. Research aims

The following hypothesis were tested as the main aims of the study:

Hypothesis 1 - Soil has the capacity to both capture potentially pathogenic microbes of livestock origin and also to transfer them under hydrological action into the wider watercourse.

Hypothesis 2 - Differences in cattle forage diet type can drive corresponding variations in microbe populations of leachate from faecally contaminated land through selective pressures in the gut.

2.2. Materials and Methods

2.2.1. Forage diet analysis

Differences in forage diet were assessed by nutritional quality analyses. Forage samples (n=11) from each of the three production systems (permanent pasture (PP), high sugar perennial ryegrass and white clover (HSC), and monoculture high sugar ryegrass(HS)) were first prepared for analysis by oven drying to a constant weight at 80℃. Samples were then finely ground (<2 mm).

Sample OM, and conversely ash content, were measured by weight loss at 505℃ for 12 hours (Thermolyne F6000, Thermo Scientific, Waltham, Massachusetts, USA), of 1 g FW samples in pre-furnaced and desiccated crucibles. The remaining sample was determined as ash and weight lost as OM. Fibre content was determined by FOSS wet (FT 121 Fibertec) and cold (Fibertec 8000) extraction protocol as recognized by EN ISO 16472 (as fully described in Chapter 4). The fibre components neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL) and modified acid detergent lignin (MADF) were all assessed by this method. The predicted metabolisable energy (ME) content was determined by calculation using Clancy and Wilson (1966) conversion factor ME (MJ/Kg DM) = 16.20 - 0.0185 (MADF). The total water soluble carbohydrate of the different forage diets was assessed via high performance liquid chromatography (HPLC) (Arrizon et al, 2010) by Rothamsted Research laboratory staff (method described in Appendix 7.1).

2.2.2. Soil sampling and preparation

Soil samples were selected to be representative of the North Wyke Farm Platform predominating Hallsworth Series (Pelo-Stagnogley) and were designed to reduce the impact that field or sample site level differences would have on resulting microbial analysis. All soil samples

were GIS mapped and random samples taken from constituent fields of all three production systems. The 60 intact soil cores were obtained from the topsoil layer (Frouz, 2005, Schoumans & Groenendijk, 2000) from sites randomly dispersed over the farm platform. These individual soil samples were homogenized by 2 mm sieving (Thomson et al, 2010, Quevauviller et al, 2002) and pooled to make an artificial medium, representative of the whole farm platform with large organic and inorganic debris removed. Thus, removing soil core differences as experimental variance (Zul et al, 2007, Matlou & Haynes, 2006). Soils were air dried to mean moisture content of 25.1 % (SD-0.44), to optimise system release kinetics (Blackwell et al, 2009, Skopp et al, 1990), this was assessed by 15 subsamples (Wu & Brookes, 2005, Sparling & Ross, 1988). Final moisture content was measured by heating subsamples at 105 °C until stable weights were obtained (Storer, 2008, Verstraeten, 2008).

2.2.3. Faecal sampling

Faecal material was collected from three separate weaned beef herds as described in Chapter 1 (Takahashi et al, 2018, Orr et al, 2016). Housed cattle were fed on preserved forage based diets of: PP, HSC and HS. This facilitated the modelling of microbial flow from faeces on pasture of three separate beef production systems with comparable genetics, veterinary interventions and management practices. At the time of external sampling from cattle all were housed and being fed high quality (pH 3.9) clamp produced silage cut from their respective pasture. All three system's herds are composed of both Belgium Blue cross and Charolaise cross breeds, with the initial intention being to examine breed and production system differences. This was not possible due to the production required concentrate supplementation to the more energy demanding Belgium Blue cross breeds (Fiems et al, 2010, Webb & Casey, 2010) and the consequential disturbance of

microbial populations (Callaway et al, 2009, Huhtanen, 1988). Focussing on sampling from the Charolaise cross breeds the study group was restricted to cattle of marketable or slaughter age, removing those calves under a year of age due to influence of parents (Donnet-Hughes, 2010, Eadie, 1962, Smith & Crabb, 1961) (Table 1).

Table 1. Populations from the three diets yearling beef cattle and faecal sample collected.

Diet type	EU UK identification	Sex	Faecal sample (g)
HS	101327	Steer	1207.9
	201335	Steer	587.4
	301301	Heifer	607.1
	401316	Steer	1165.2
	501324	Steer	1191.1
HSC	201342	Steer	1120.3
	401323	Steer	1173.6
	501345	Heifer	892.2
	601290	Heifer	1086.9
	701347	Heifer	720.7
PP	101292	Heifer	594.6
	101334	Steer	1083.7
	601181	Heifer	1129.5
	601325	Steer	1134.8
	701312	Heifer	1156.2

Faecal sampling was conducted during winter housing period, with all cattle fed on silage produced from the respective pasture system. Sample populations were matched in age.

Faecal sampling was undertaken of the livestock groups (Table 1), during morning ration provision of the respective systems clamp silage. The individual faeces were taken by external grab sampling, stored separately before initial weight for each was obtained (Table 1). A subsample of 500 g from each individual animal were divided within the different production systems to facilitate production of replicates, population representative sample for each of the three systems Charolaise cross breed herds faeces. From these samples (n=4) all subsequent direct faecal analysis for Dry Matter (DM), pH and Faecal Indicator Organisms (FIO) Colony Forming Units (CFU) completed for the three pasture systems. Each production systems faecal composite samples

DM was analysed by subsampling 100 g to undergo heating at 65 °C for a period until the weight measured was stable. The pH of the three systems separate composite faeces was determined by four 1 g subsamples from each being diluted in sterile de-ionised water and calculated separately by means of a portable pH meter (Piccolo, Hanna Instruments).

2.2.4. Lysimeter design and construction

To facilitate sufficient replication 15 lysimeters were designed and constructed. A stable base unit was created, with 110 cm diameter bores. Polypropylene pipe closed end cap, modified to allow 2 cm bore polythene collection pipe through centre, was attached below each bore. Polypropylene pipe coupling was fitted flush into the modified closed end cap and through the bore. Polyvinyl Chloride filter funnel was then attached internally to the coupling thread using innate adhesive, microbial neutral Loctite SF 770 primer and Loctite 403, and connected to the collection pipe. Cut sections of 250 micron nylon filter mesh was then sealed on top of the filter funnel at thread and coupling joint with innate sealant, microbial neutral silicone Loctite SI 5331.

Sections of polypropylene piping of bore circumference 110 cm were cut to 15 cm lengths. All 15 sections were then packed with the homogenised composite soil to a height of 7.5 cm, to minimise anaerobic microbe conditions (Veeh et al, 1996), and bulk density (BD) of 0.8, representative of the North Wyke Farm Platform soil (Orr et al, 2016, Logsdon & Karlen, 2004) calculated at packing using a custom statistical programme (Loick, 2014). The design, including soil type, origin, depth and BD will provide a representative indication of the Farm Platform in-field process (Blackwell et al, 2009). The significant variation sources of growing pasture and non-soil material associated with intact cores were removed to reduce compounding factors in this

preliminary experiment. The soil packed sections of piping were then suspended on the filter mesh and funnel within the coupling (Figure 1).

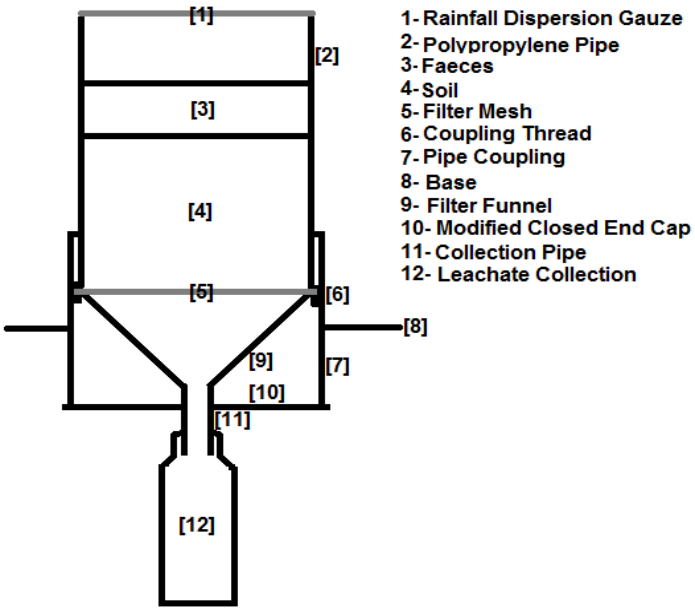

Figure 1. Individual lysimeter design.
All lysimeters were packed with soil to depth of 7.5 cm and BD 0.8. Non-control lysimeter soil was topped with HS, HSC or PPcomposite faeces to depth of 2 cm and DM 50.0 g.

Each of the three production systems composite herd faecal sample was divided on top of soil cores of four lysimeters to depth of 2 cm and equal total DM g of dung of 50.0 g. Three control lysimeters were maintained only packed with soil. This total depth of soil medium with uniform packed BD and faeces was maintained to minimise the potential for sidewall flow within the lysimeters (Corwin, 2000, Cameron et al, 1992) upon application of artificial rainwater. All lysimeters were covered by 500 micron gauze to disperse the rainwater when applied, minimising the heterogeneous disturbance of contents.

2.2.5. *Artificial rainwater composition and application*

The generation of artificial rainwater comparable to that analysed on North Wyke Farm Platform was produced by dissolving identified salts in deionized water to equivalent chemical constituent and pH 5.76. The composition (g l^{-1}): $CaCl_2$, 2.465; $MgCl_2$, 1.919; $FeCl_2$, 0.0445; $N_2H_4O_3$, 0.430; K_2SO_4, 0.617; NaCl, 3.317. The mean pH was calculated from each of the eight litres, required in total for all lysimeters with uniform application as 5.76 (SD- 0.04). The artificial rainwater underwent standard liquid autoclave sterilisation (15 min at 121 °C). Using a sterilised automated peristaltic pump dispenser 20 doses of 19 ml were added at low pressure through the dispersal gauze to all 15 lysimeters in six minute intervals totalling two hours. This resulted in each lysimeter model system undergoing 380 ml of simulated rainfall equivalent to 24 mm hr^{-1}, comparable to regular rain events recorded on North Wyke Farm Platform (Blackwell et al, 2009). The collection of leachate through 2 cm collection piping connected to the filter funnel was sealed to the collection vessel with sterile polythene to prevent environmental contamination. After two-hour application process of artificial rainwater the lysimeters were allowed to stand for 12 hours replicating release periods at field scale following similar rainfall events. All lysimeters were maintained in controlled environment conditions throughout the study at 15.5 (±0.25 °C). The resulting leachate was collected and underwent immediate analysis.

2.2.6. Faecal indicator organism culturing

The FIO concentration of each production systems composite herd sample was then completed by direct sampling from the faeces and from all lysimeters resulting leachate. Direct from the three composite faecal samples 1 g and from the 15 lysimeter leachates 1 ml was aseptically transferred to 9 ml of sterile Ringers solution (Thermo Scientific, Waltham, Massachusetts, USA). Serial

dilutions were made to enable standard membrane filtration determination of bacterial concentrations as described by Hodgson et al (2016). Samples were washed through the filtration manifold unit with 20 ml of sterile Ringer's solution. Membrane filters of 0.45 μm (Sartorius Stedim Biotech, Goettingen, Lower Saxony, Germany) were placed onto species selective sterile agars for FIO culturing. *E. coli* growth was targeted on Membrane Lactose Glucuronide Agar (Oxoid, Basingstoke, Hampshire, UK) and incubated for 18-24 h at 44.5 °C (±0.20 °C) (Hodgson et al, 2016). All samples were processed in triplicate throughout culturing. The initial FIO counts were transformed to Log_{10} colony forming units (CFU) g^{-1} DM.

2.2.7. Microbial DNA extractions and sequencing preparation

Total DNA was isolated from 100 ml leachate samples washed through filtration manifold unit with 0.22 μm membrane (Sartorius Stedim Biotech, Goettingen, Lower Saxony, Germany) using PowerWater DNA Isolation methodology (MoBio Laboratories INC., Solana Beach, California, USA) (Buse et al, 2014, Wilcox, 2013).

The manufacturer's recommended protocol was followed, with specified exceptions: 1) At step 16 after addition of high salt solution PW3 500 μl of supernatant was centrifuged as attempting at the 650 μl recommended resulted in residue. 2) At step 20 after addition of product cleanser ethanol solution PW5 to remove the alcohol based solution PW4, centrifuging by 1, 1, and 2 minutes (totalling 4 minutes) was completed as the 2 minutes recommended resulted in significant solution PW5 residue in spin filter basket which would inhibit proposed downstream DNA applications such as PCR and gel electrophoresis.

Upon release from high salt binding to spin filter basket, by the 10 mM Tris DNA sterile elution buffer, the extracted DNA was stored at -20 °C in preparation for downstream applications.

Extraction success of DNA was qualified by gel electrophoresis by removal of 50 µl template DNA and adding 5 µl loading buffer. Extracted DNA quality was assessed visually on agarose gel and if poor quality was indicated extraction was reattempted.

Standard PCR reactions were completed on extracted template DNA in an Agilent Technologies Sure Cycler 8800 PCR system using GoTaq® G2 Flexi DNA polymerase (Promega Corp., Madison, Wisconsin, USA) for each sample. Each individual reaction contained the following components: 50 ng of template DNA, 0.25 µl GoTaq® G2 Flexi DNA polymerase, 5 x Green Flexi Buffer, 3 µl $MgCl_2$, 1 µl of each dNTP, 1 µl of a 1 µM stock solution for each forward and reverse primers and Nuclease-Free sterile H_2O (Sigma-Aldrich Co., St. Louis, Missouri, USA) to 50 µl. Existing primers were selected for the PCR amplification of microbial marker gene sequence 16S as 27 forward and 1492 reverse (Kim et al, 2011, Frank et al, 2008, Lee et al, 2003) (Table 2).

Table 2. Primer Sequences and PCR conditions.

Primer set	Product	Sequence length (bp)	Primer Sequence	Annealing Temperature	Extension Time
27f—1492r	16S	1458	5′-AGA GTT TGA TCM TGG CTC AG 5′-TAC GGY TAC CTT GTT ACG ACT T	57 °C	1.5 min

Primer sequences and PCR conditions for amplification of target microbial marker gene sequence 16S.

The PCR was completed on the 50 µl solutions from all 15 lysimeter leachates under the following cycling protocol after initial extended denaturation of 94 °C for 2 min, 30 cycles of: 94 °C for 30 s, 57 °C 45 s, 72 °C for 1.5 min; 72 °C for 7 minutes, hold at 4 °C. Each sample had 5 µl PCR product and 1 µl of loading buffer added in preparation for gel electrophoresis. Gel electrophoresis allowed for further quality assessment and visualisation of potential downstream contaminates. Specific sizes of distinct banding or large association at the bottom within the gel,

were assessed for impact on subsequent sequence quality and extractions or PCR reattempted when deemed necessary.

Template DNA and 16S PCR product was analysed using gel electrophoresis (Idota et al, 2018, Maldonato et al, 2018, Boon et al, 2002) (BioRad PowerPAC-300, Hercules, California, USA) in 0.8% (w/v) agarose gel matrices in 1x Tris-acetic EDTA buffer (TAE; 4.84 g Tris base, 11.42 ml acetic acid, 0.01 M EDTA, pH 8.3), containing ethidium bromide (0.3 µl) to visualise the DNA. A 1-kb plus ladder (Invitrogen, Thermo Scientific) was used to qualify DNA fragment size. Gels were run at 100 V for 2 h and imaged with an Omega Fluor Thermal Imaging system (Aplegen, San Francisco, California, USA) and associated computer documentation programme.

2.2.8. DNA sequencing and analysis

Following initial quality check of successful DNA extraction by gel electrophoresis, DNA quantities were assessed by a NanoDrop UV/Vis spectrophotometer (ND-1000 Spectrophotometer, Wilmington, Delaware, USA). The calculated 260/280 nm absorbance ratio, of values 1.8–2.0 was considered indicative of good quality DNA. Analysis of sample quantification by Qubit (Qubit 2.0 Fluorometer, Life Technologies Ltd., Thermo Scientific) and subsequent steps was then completed in collaboration with the Exeter Sequencing Service, University of Exeter. Qubit dsDNA HS Assay Kit (Life Technologies Ltd) was utilised as samples were deemed to not exceed 120 ng/µl. The working solution was mixed as 19.9 ml of Qubit HS buffer and 100 µl of Qubit HS Reagent. Aliquots of 190 µl working solution were vortexed with 10 µl of Qubit HS Standard 1 and 10 µl of Qubit HS Standard 2. Following selection of dsDNA HS programme and calibration by standards, 199 µl of the working solution was added to the Qubit tubes with 1 µl of the corresponding sample. DNA concentrations were then analysed and

recorded. Library preparation followed as initial 16S rRNA gene amplification by PCR. The PCR utilised reagent NEBNext high-fidelity PCR master mix (New England Biolabs) and was undertaken (Table 3).

Table 3. PCR reaction preparation per sample.

Component	25 µl Reaction	50 µl Reaction	Final Concentration
NEBNext PCR mix	12.5 µl	25 µl	x1
1 µM F Primer	5.0 µl	10 µl	0.2 µM
1 µM R Primer	5.0 µl	10 µl	0.2 µM
Template DNA	2.5 µl	5 µl	2.5-5 ng

PCR reaction preparation 4 x 25 µl (50 µl) per sample

A PCR clean-up was completed using Agencourt AMPure XP beads (Beckman Coulter) to purify the 16S V1 and V3 amplicons away from free primers and primer dimer species. A supplementary quality assessment of PCR recovery was undertaken by Tapestation D1000 high sensitivity screen tape. The second PCR amplification utilising the Nextera XT Index Kit (Illumina, San Diego, California, USA) was completed. Each well combining 15 µl of purified product DNA, 5 µl Nextera XT Index Primer 1 (N7XX), 5 µl Nextera XT Index Primer 2 (S5XX) and 25 µl 2x NEBNext PCR reaction mix. Primers employed (Table 4). A second PCR clean-up was completed using Agencourt AMPure XP beads (Beckman Coulter, Brea, California, USA).

Table 4. Sequencing primer sequences and PCR condition.

Primer set	Product	Sequence length (bp)	Primer Sequence	Annealing Temperature	Extension Time
341f—785r	16S	300	5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G	55 °C	0.5 min

Primer sequences and PCR conditions for amplification of target microbial marker gene sequence 16S.

Validation of libraries using 1 µl of the final library was completed by dilution as required a Tapestation D1000 high sensitivity screentape. Verifying expected fragment size (~630 bp) and concentration as presented in Figure 2 and Table 10, respectively, determined by a 2100 Bioanalyzer (Agilent Technologies). DNA concentration (nM) was calculated from this by:

(concentration in ng/µl) / (660 g/mol × average library size) × 106 = concentration in nM

Final libraries' concentration were then diluted to 4 nM using 10 mM Tris pH 8.5. Libraries were pooled by mixing of a 5 µl aliquot of diluted DNA from each library. Removal of dimers was confirmed by 2100 Bioanalyzer (Agilent Technologies).

Sequence data clean-up was completed through modified pipeline design in Mothur as developed by Uli Klumper (European Centre for Environment and Human Health, University of Exeter) in collaboration.

Sequencing data was analysed using R statistical software (R Core Team, 2015) in collaboration with Joanna L'Heureux leading (Department of Sport and Health Sciences, University of Exeter). Alpha diversity analyses were used to calculate Rarefaction and Shannon Diversity Index to interpret species richness and diversity. Homogeneity of group samples were visualised using non-multidimensional scaling (NMDS) and multidimensional scaling (MDS) plots (Benjamino et al, 2018). Differences in microbe composition across lysimeter groups were assessed by visualisation in bar graphs and heatmaps. Percentage median relative abundance in microbes was calculated to identify the most relative abundant genera across groups. Multiple t-tests comparing median microbe abundances, normalised with log transformation, between each lysimeter group were completed and presented in a differential heat tree (Foster et al, 2017, Wickham, 2009)

2.2.9. Statistical analysis

All culture and initial sequencing based statistical analysis was completed in Genstat (16th Edition, 2013). The variation of FIO CFU g^{-1}, pH, DM and forage quality parameters was calculated by general ANOVA.

The ANOVA model used was:

Y variate: FIO CFU g^{-1}, pH, DM, forage quality parameters

Treatment: Group

Where: Group = HS, HSC, PP or Control.

Significance was accepted if $P \leq 0.05$.

Statistically significant differences ($P \leq 0.01$) were assessed between HS, HSC, PP and Control groups by Multiple Response Permutation Procedure. Multiple t-tests comparing median microbe abundances across groups was completed by Wilcoxon signed rank test. Correction for false discovery rate controlled multiple testing between lysimeter groups HS, PP, HSC and Control.

2.3. Results

2.3.1. Analysis of forage diet nutritional differences

Significant differences found between forage diets for water soluble carbohydrate (WSC) content. The highest WSC content was found in the HS forage diet relative to the PP. The WSC content of the HSC forage diet was found to be intermediary to HS and PP and not significantly different to either (Table 5).

Table 5. Variations in measured forage nutritional quality parameters across diet type groups.

Strata	Mean OM (g kg^{-1} DM)	Mean NDF (g kg^{-1} DM)	Mean ADF (g kg^{-1} DM)	Mean ADL (g kg^{-1} DM)	Mean ME (MJ kg^{-1} DM	Mean WSC (g kg^{-1} DM)
Group						
HS	910	406	211	37.1	15.7	167^{b}
sd	10.9	44.6	32.2	11.69	0.06	31.7
PP	915	397	205	34.3	15.7	124^{a}
sd	8.8	52.4	29.5	9.01	0.06	23.2
HSC	909	368	190	34.4	15.7	142^{ab}
sd	5.2	43.2	22.7	8.43	0.04	28.7
P value	0.229	0.151	0.212	0.765	0.469	0.003

Mean values of HS, PP and HSC diet group forage samples OM, NDF, ADF, ADL, ME and WSC. General ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters

2.3.2. Direct analysis of faeces and lysimeter operation

No significant differences were indicated for DM content between composite faecal samples for HS, HSC and PP groups. pH varied between groups, with lower pH in HS compared to PP and HSC composite faecal samples (Table 6).

Table 6. Variations in pH and DM content of composite faecal samples across groups.

Strata	Mean pH (kg^{-1} DM)	sd. pH (kg^{-1} DM)	Mean DM (g^{-1} FM)	sd. DM (g^{-1} FM)
Group				
HS	6.63^{a}	0.402	49.6	2.67
PP	7.63^{b}	0.358	49.2	3.08
HSC	7.43^{b}	0.330	48.2	2.48
P value	0.008		0.773	

Mean values of HS, PP and HSC group composite faecal samples pH and DM content. General ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters.

No significant differences were indicated for *E. coli* concentration between composite faecal samples for HS, PP or HSC groups (Table 7).

Table 7. Variations in faecal indicator organism concentration (log_{10} $CFUg^{-1}$) of composite faecal samples across groups.

Strata	Mean *E. coli*	sd. *E. coli*
Group		
HS	5.90	0.362
PP	5.90	0.328
HSC	5.90	0.403
P value	0.952	

Mean values of HS, PP and HSC group composite faecal samples E. coli concentration. General ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters.

No significant differences were indicated for leachate volume between lysimeters for HS, PP, HSC or Control groups. The control group produced the most consistent mean leachate volumes (Table 8).

Table 8. Variations in leachate volume (ml) of lysimeters across groups.

Strata	Mean volume	sd. volume
Group		
HS	131	21.2
PP	108	14.1
HSC	130	7.5
Control	124	2.4
P value	0.853	

Mean values of HS, PP and HSC group composite faecal samples E. coli concentration. General ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters.

2.3.3. Culture based analysis of FIO in leachate

Significant differences were indicated for leachate *E. coli* concentration between lysimeters for HS, PP or HSC groups. The Control lysimeters produced leachate of zero FIO CFU, significantly lower in concentration compared to HS, PP or HSC groups (Table 9).

Table 9. Variations in leachate faecal indicator organism concentration (log_{10} $CFUg^{-1}$) of lysimeters across groups.

Strata	Mean *E. coli*	sd. *E. coli*
Group		
HS	3.81^{a}	0.197
PP	3.77^{a}	0.262
HSC	4.15^{a}	0.131
Control	0.00^{b}	0.000
P value	0.001	

Mean values of lysimeters HS, PP, HSC and Control group leachate E. coli concentration. General ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters.

2.3.4. Sequencing based analysis of microbe populations in leachate

Verification of fragment size was completed to validate the final library. Completed as described on Tapestation D1000 high sensitivity screen tape (Figure 2). Alongside the Ladder (Lad.) is the fragment size of each labelled sample with upper and lower markers included at 1500 and 25 bp, respectively. Final sample concentrations in ng μl^{-1} are presented in Table 10. This determined subsequent volumes for pooling of validated libraries. Analysis was conducted across all quality checked sequences. Producing sequences for diversity analysis totalling 5400096 (Table 10). Randomised subsampling at 150000 was performed for each experimental grouping.

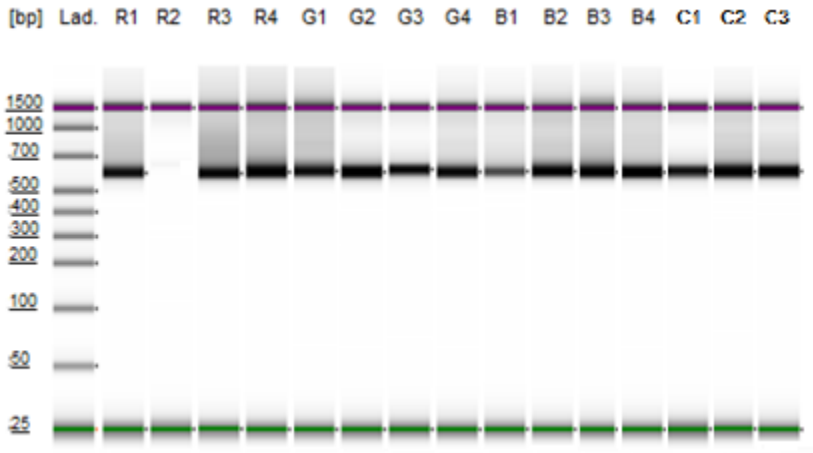

Figure 2. Electrophoresis gel of individual leachate sample libraries, scaled to view larger molecular weight range.
Sample DNA library fragment sizes. Groups are indicated by letters corresponding to R - HS, G – PP, B – HSC and C – Control. Replicates are indicated by associated numbers. Ladder is labelled as - Lad. with associated fragment banding size.

Table 10. Recorded sample concentration (ng/µl) and quality checked sequences of individual leachate samples final validated libraries.

Sample name	Concentration (ng/µl)	Quality checked sequences
Lad.	20.0	-
R1	10.8	423168
R2	0.194	159821
R3	12.9	475820
R4	15.6	273664
G1	10.5	511980
G2	17.6	309254
G3	11.5	292503
G4	11.9	297508
B1	6.15	355259
B2	14.0	494033
B3	13.5	344834
B4	16.5	490677
C1	9.75	454563
C2	14.8	217997
C3	16.1	299015

Sample DNA library concentration. Groups are indicated by letters corresponding to R - HS, G – PP, B – HSC and C – Control. Replicates are indicated by associated numbers. Ladder is labelled as - Lad.

2.3.3.1. Analysis of microbial richness and diversity

The quality checked and subsampled sequences were clustered, representing operational taxonomic units (OTUs) of 3% nucleotide dissimilarity. Rarefaction curves were completed on randomised subsample of 15,000 sequences to calculate OTU numbers in each sample (Figure 3). The results indicate potential increased microbial richness of samples R4, R1 and B4 whereas the richness of other samples was relatively decreased. Rarefaction curves appear to approach saturation plateau, indicating sequencing was sufficiently in-depth to accurately estimate microbe population composition.

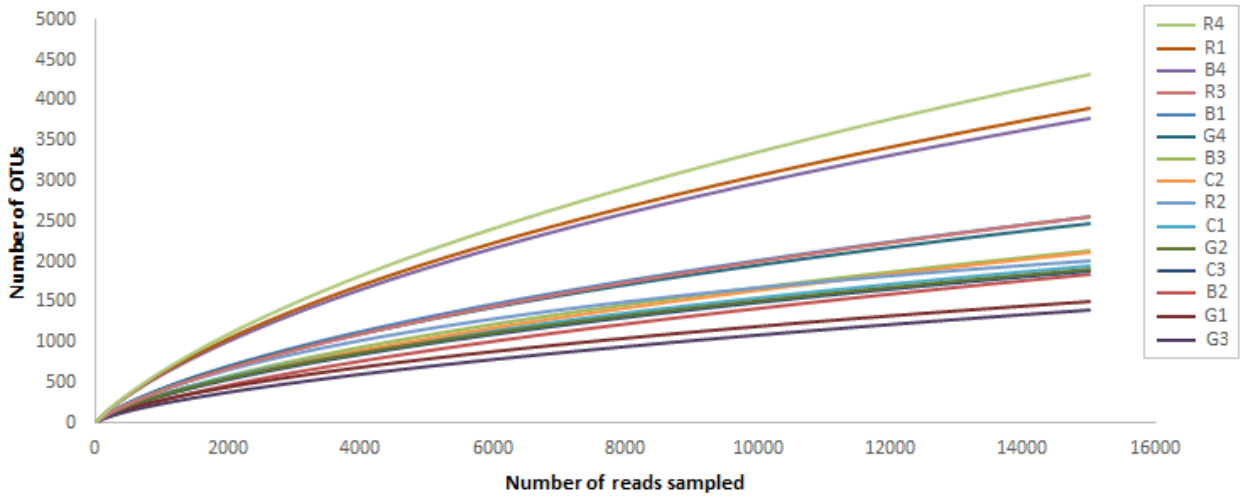

Figure 3. Rarefaction curves based on individual leachate samples subsampling of 15,000 sequences clustered as operational taxonomic units.
Sample subsampled 15,000 sequences clustered as operational taxonomic units (OTUs). OTUs were defined at 0.03 distances Groups are indicated by letters corresponding to R - HS, G – PP, B – HSC and C – Control. Replicates are indicated by associated numbers.

Grouped leachate microbial diversity was analysed by Shannon diversity index, combining richness and evenness, indicated significant differences between groups (Figure 4). While limited by individual sample number, increased species diversity was found in PP group leachate relative to HS (MRPP $p < 0.05$).

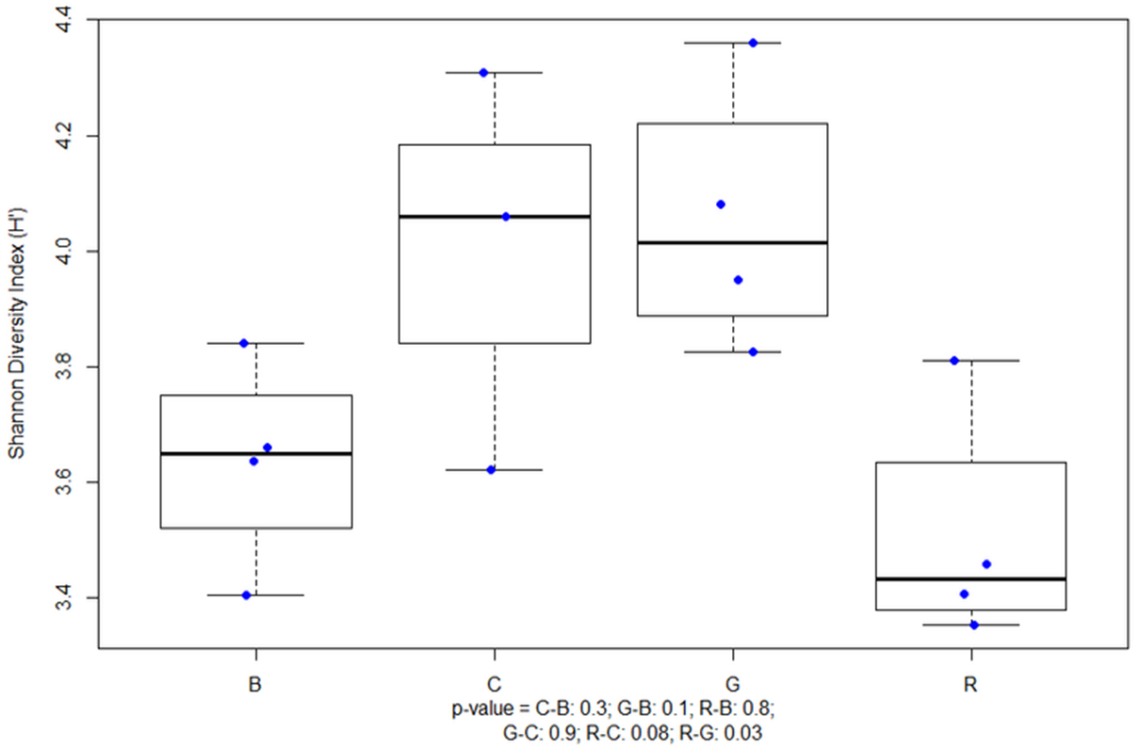

Figure 4. Microbial diversity as determined by Shannon diversity index across group leachate.
Significant differences between PP and HS group leachate was found in Shannon diversity index analyses (MRPP $p < 0.05$). Groups are indicated by letters corresponding to R - HS, G – PP, B – HSC and C – Control. Replicates are indicated by Scatter plots and box plots represent the mean and standard deviation for Shannon Diversity Index.

2.3.3.2. Visualisation of microbial sample similarities

Non-Multidimensional Scaling (NMDS) plot indicated closer clustering of microbial populations in leachate of HS and HSC groups relative to PP and Control groups. Variability between groups also indicated differences in HS and HSC compared to PP and Control Groups.

The significance of clustering was computed using ADONIS (PERMANOVA) ($p < 0.01$). Paired analysis indicated no statistical differences between microbial populations of groups (Figure 5). This was supported by Multidimensional Scaling (MDS) plot (Figure 6), indicating high intra group variability with only limited visual clustering of some HS group samples.

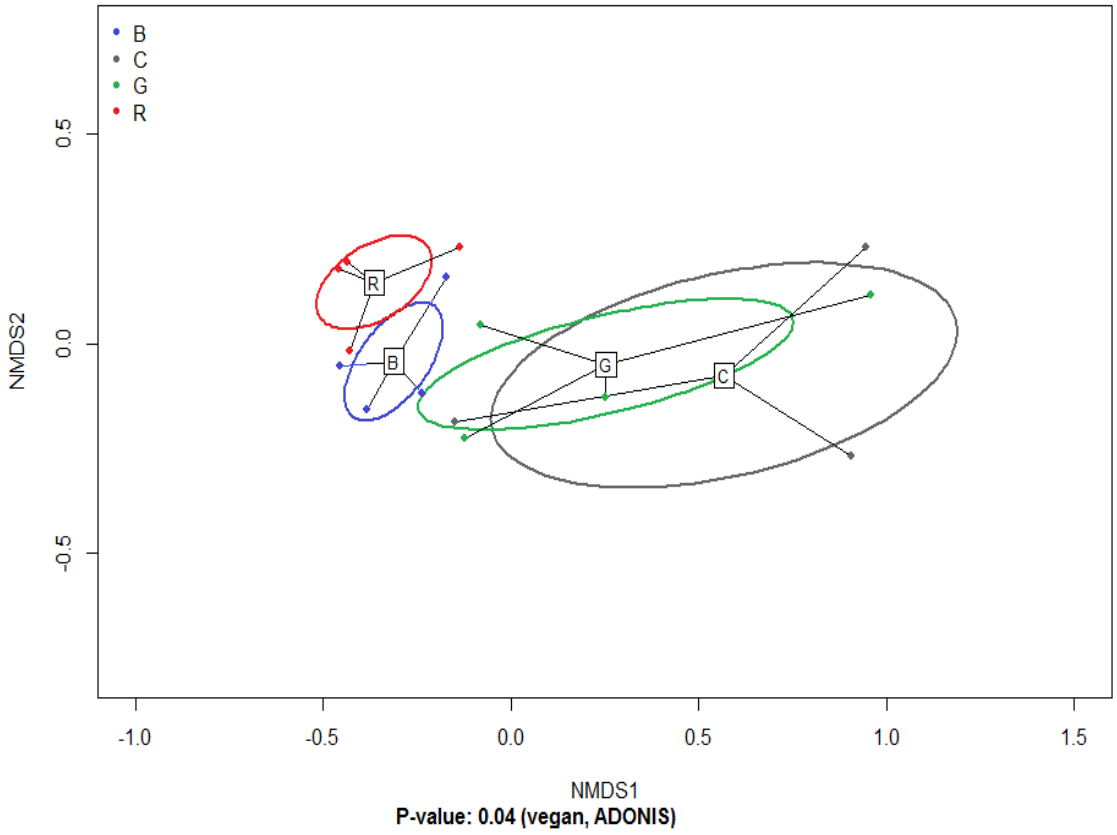

Figure 5. Visualisation of microbial population similarity of group leachate by Non-Multidimensional Scaling plot.
No significant differences ($p < 0.01$) between microbial populations of group leachate was found in paired analysis by ADONIS. Groups are indicated by letters corresponding to R - HS, G – PP, B – HSC and C – Control. Replicates are indicated by associated individual points.

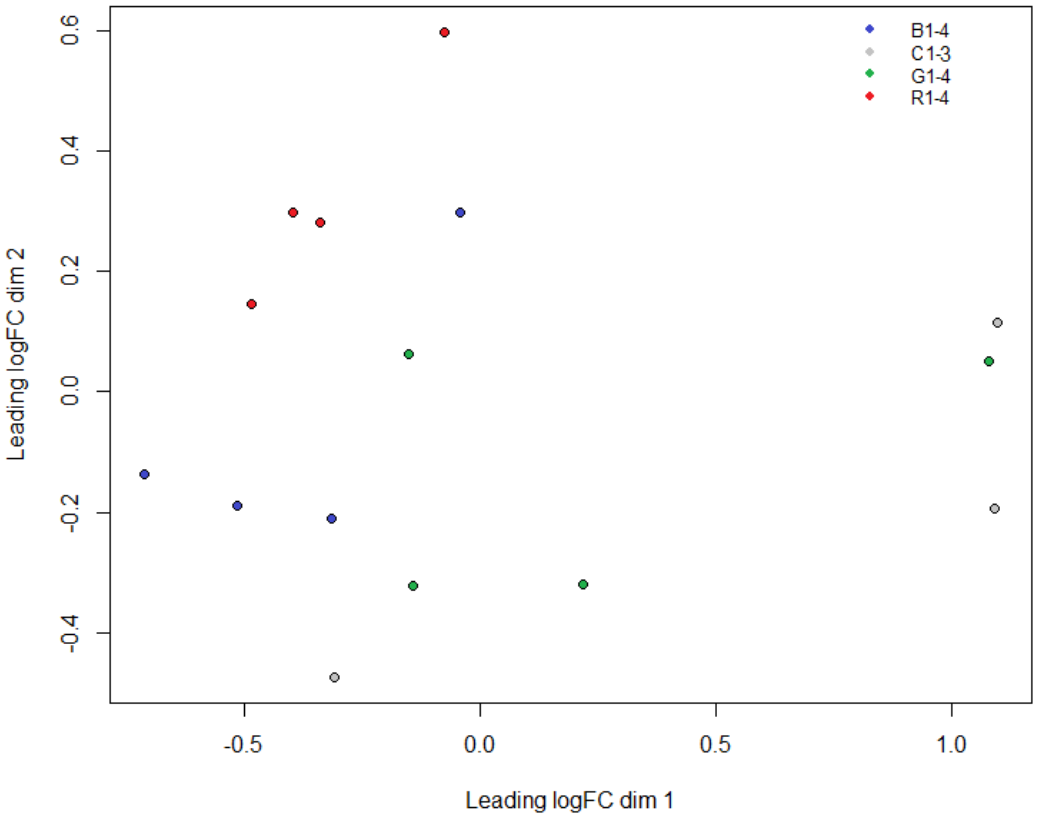

Figure 6. Visualisation of microbial population similarity of group leachate by Multidimensional Scaling plot.
Groups are indicated by letters corresponding to R - HS, G – PP, B – HSC and C – Control. Replicates are indicated by associated individual points.

2.3.3.3. Analysis of groups microbial composition

The microbe population composition of each groups leachate was analysed to genus taxonomic level. Percentage relative abundance of all phyla indicate Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria predominate across HS, PP, HSC and Control groups (Figure 7). Proteobacteria was the most abundant (percentage average across samples) in Control and PP groups, Bacteroidetes in HSC and Firmicutes in HS. Lentisphaerae abundance was increased in HSC and HS groups. The differences in sample leachate microbial composition by genera (>0.01 % relative abundance) indicate particular dominance within four most abundant phyla by *Legionella, Oscillibacter, Alistipes* and *Corynebacterium* respectively (Figure 8).

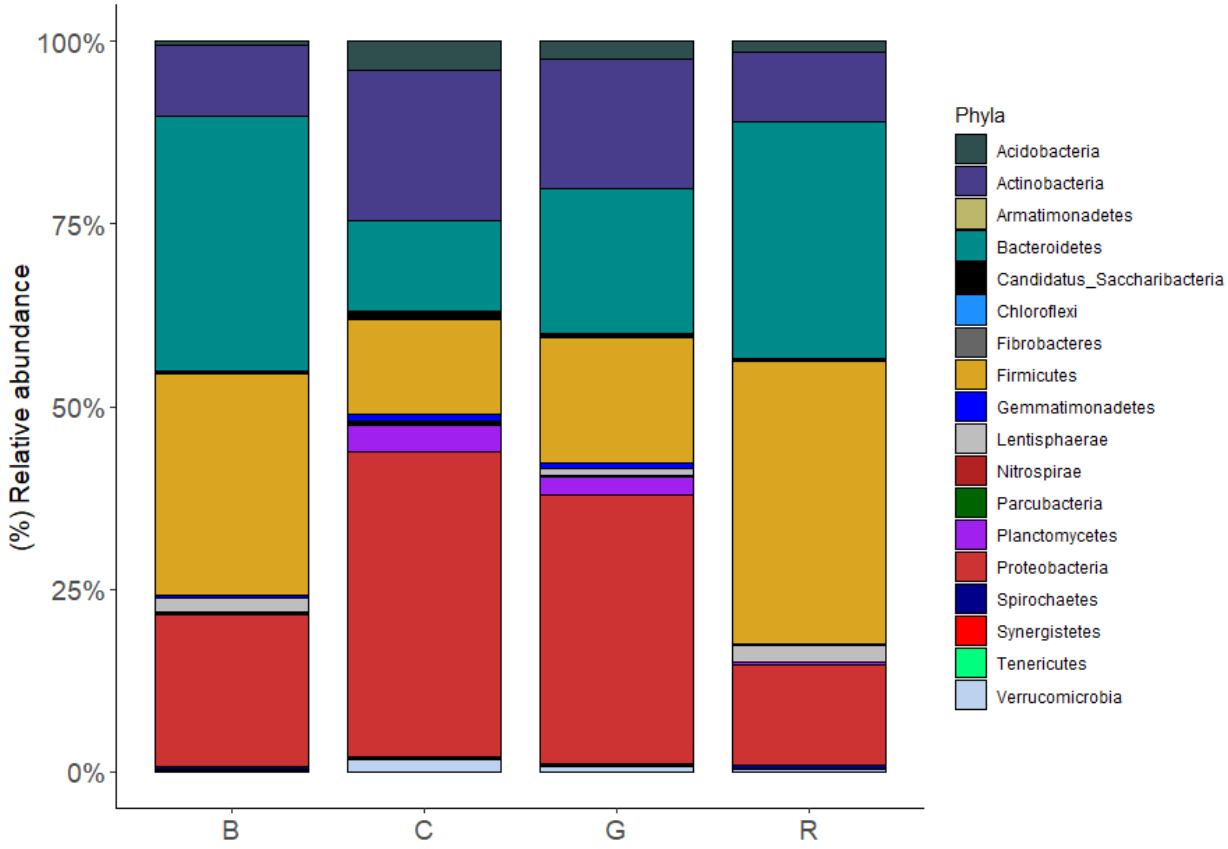

Figure 7. Microbial population composition, phyla percentage relative abundance, of group leachate by stacked bar graph.

Bar graph of normalised sequence data for percentage relative abundance by phyla. Groups are indicated by letters corresponding to R - HS, G – PP, B – HSC and C – Control.

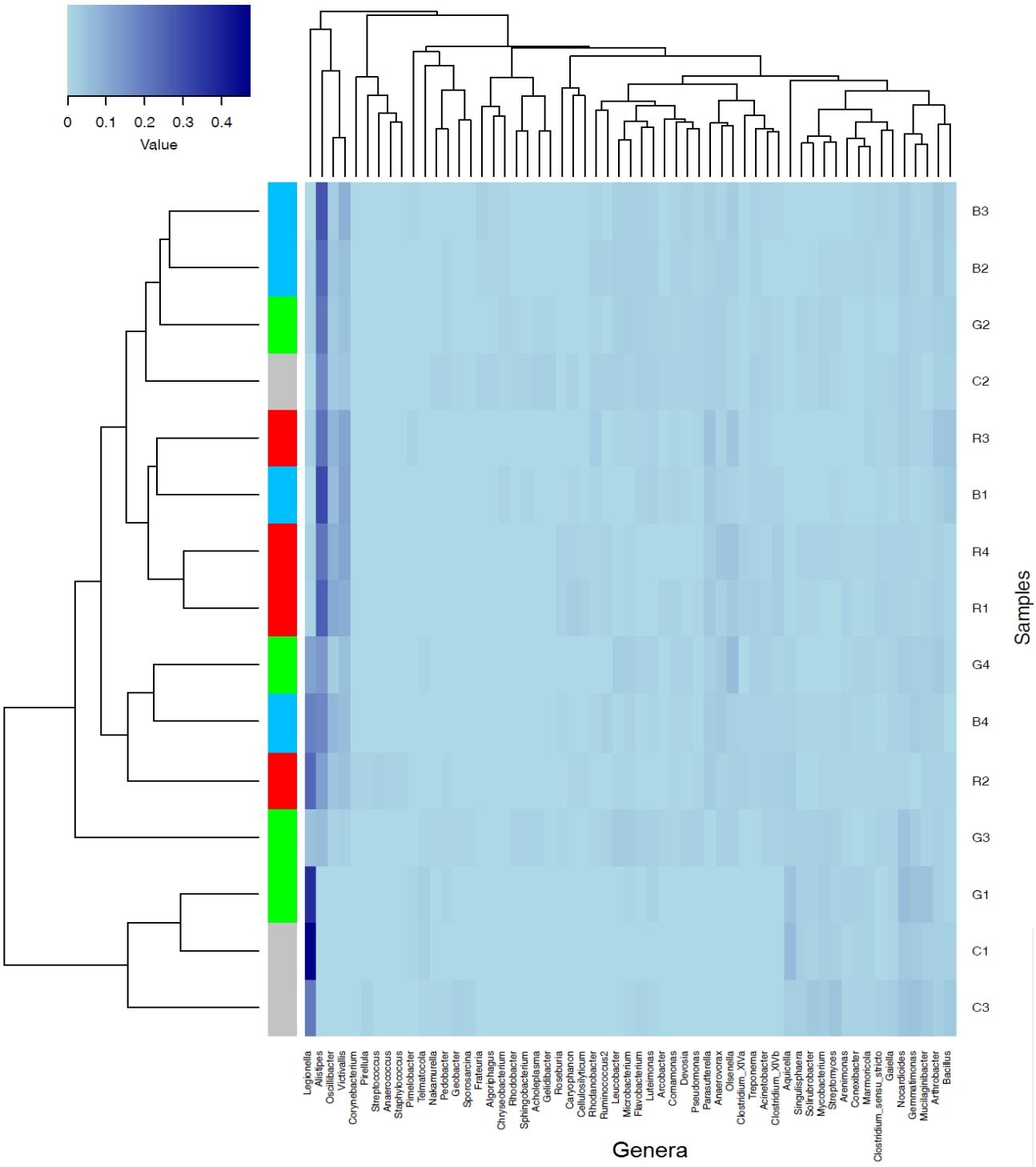

Figure 8. Microbial population composition, genera percentage relative abundance, of individual lysimeter leachate by heatmap.

Heatmap presenting the percentage relative abundances of microbe genera across individual samples. Groups are indicated by letters corresponding to R - HS, G – PP, B – HSC and C – Control. Replicates are indicated by associated numbers.

2.3.3.3. Differences in groups microbial composition

The microbe population composition of HS, PP, HSC and Control lysimeter group leachate was analysed across taxons from phylum to genus level. Significant differences in pairwise analysis of group by group variation are presented alongside the microbe phylogenetic tree of samples. The tree presents overall sample microbe composition with branch and node width indicating median OTU abundance at each taxon level. Statistically significant differences in microbe proportion ($p < 0.01$) between sample groups are indicated by highlighted colour on the comparison tree. Colouring indicates higher prevalence of microbe in matching coloured group for each comparison tree. Significant differences between group leachate microbe composition were found in phylum through genus taxon levels (Figure 9).

Analysis between PP and Control group indicated no significant differences in microbe composition. Sample groups PP and HSC indicated the fewest number of identified statistical differences. At family taxon level Chitinophagaceae microbes were found to be increased in PP, HSC and Control relative to HS group leachate. Similarly, all but two microbes found to have multiple differences across groups were indicated as increased relative to HS group. The genus *Ochrobactrum* and *Aeromicrobium* were increased in PP and HSC relative to HS group. The genus *Kaistia* and *Parvibaculum* were increased in PP and Control relative to HS group. However, the genus *Olsenella* and *Victivallis* were increased in HS and HSC relative to Control group.

The highest number of repeated differences in microbe composition in one group compared to others was found in HS group. The genus *Alloprevotella*, *Parasutterella*, and Oscillibacter were increased in HS relative to PP and Control group. The genus *Enterorhabdus* was also increased in HS relative to HSC and Control groups. The Control group was found to present only one difference common to more than one other group, increased proportion of the genus *Nitrospira*

relative to HS and HSC groups. Significant differences between microbe compositions that were not present in comparisons between more than two individual groups are listed in Table 11. The PP group leachate had the greatest incidence of increased microbe composition, HS and Control group had the fewest.

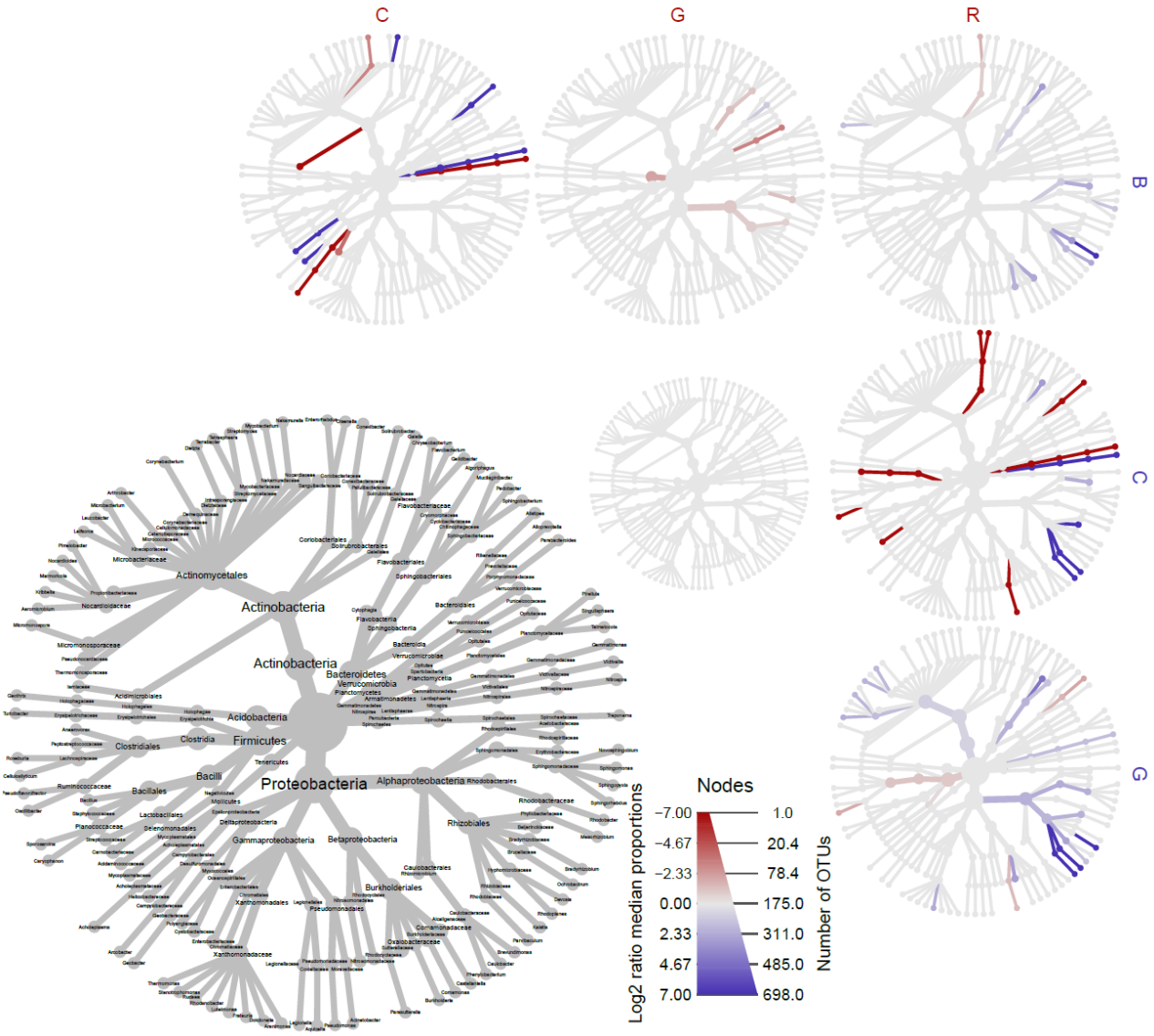

Figure 9. Phylogenetic tree of all sample microbes and abundance, statistically significant increased proportion of microbes by pairwise comparison is highlighted by group associated colour.

Phylogenetic tree branch and node width indicate median OTU abundance at each taxon level. Coloured statistically significant (p <0.01) increased microbe proportions are associated to matching coloured group identifier in each pairwise comparison. Groups are indicated by letters corresponding to R - HS, G – PP, B – HSC and C – Control.

Table 11. Significant differences between median microbe composition that were not present in comparisons between more than two individual groups.

Microbe name	Taxonomic rank	Increased group proportion	Decreased group proportion
Gemmatimonas	Genus	PP	HS
Leifsonia	Genus	PP	HS
Luteimonas	Genus	PP	HS
Microbacterium	Genus	PP	HS
Nocardioides	Genus	PP	HS
Rhodoplanes	Genus	PP	HS
Alloprevotella	Genus	HSC	Control
Devosia	Genus	HSC	HS
Sphingopyxis	Genus	HSC	HS
Geobacter	Genus	Control	HSC
Nakamurella	Genus	Control	HSC

Significant differences in median microbe proportion of leachate indicated between only two groups. Taxonomic level and corresponding name is presented alongside group of increased and decreased proportion.

2.4. Discussion

This study modelled the influence of livestock faecal deposits and soil upon the transfer of microbes under hydrological action. This preliminary controlled environment experiment indicates the requirement for high resolution sampling in field scale studies. As the influence of faeces (Fermaux et al, 2008, Jamieson et al, 2002) and soil (Van Der Heijden et al, 2007, Desmarais et al, 2002) characteristics is key in on-farm microbial cycling the degree of spatial variation at field scale must be accounted for in future studies. As indicated by both culture and next generation sequencing based methodologies in this study the capacity of soil to capture faecal origin microbes is significant. The cumulative effect of this process, involving active binding and the influence of 'dead end' pores, on regions of pasture under high faecal deposition rates should be assessed in subsequent field scale studies. The influence of livestock forage diet nutritional quality differences upon microbe population was evident in lysimeter leachate and is similarly theorised to occur at a field and farm scale.

2.4.1. Inter group faecal and diet variation

The direct analysis of the DM content for HS, PP and HSC group faecal samples was primarily undertaken to ensure homogeneity between lysimeter preparation. The selection of comparable individual animals for the study grouping contributed to similarity in faecal DM (Table 6). Cattle included in all groups were of similar age and positive health status, both influencers of faecal DM content (Medrano-Galarza et al, 2018, Grandl et al, 2017, Donnet-Hughes, 2010). While diet is a principle driver of differences in faecal DM content (Appuhamy et al, 2018, Chaney et al, 2018), all study diets were high quality forage based and the effect lessened. No differences in the faecal DM content between groups was found with minimal processing required to achieve uniform DM content of all faecal packed lysimeters. The impact at a field scale of these study population

controlled factors should be considered. There is increased potential transfer of microbes into soil from low DM content faeces by calves during initial grazing periods and from diseased livestock (Medrano-Galarza et al, 2018, Grandl et al, 2017, Donnet-Hughes, 2010). Unlike the controlled conditions in this study, faecal deposits DM content varies as does the potential for transfer of potentially pathogenic or antimicrobial resistant bacteria from faeces to pasture. Identifying the impact in-field of such differences may enable the disproportionate effect on microbial transfer at pasture to be better managed (Howell et al, 2018, Wang et al, 2017).

The pH analysis obtained directly from faecal samples was considered as a marker in characterising differences of the faecal environment resulting from the three forage based diets. Variations in diet are known to effect livestock GI and faecal conditions, such as pH, with resulting impacts on microbial population (Pitta et al, 2018, Zhou et al, 2018). The significantly lower pH found in HS compared to HSC and PP group faeces (Table 6) corresponds to previous literature (Fox et al, 2007, Klieve, 2003, Bezkorovainy, 2001) and the effect of high sugar grasses. A related effect upon microbe population is also indicated (Fox et al, 2007, Berg et al, 2005). The similarity between HS and HSC forage, monoculture high sugar ryegrass and high sugar perennial ryegrass and white clover respectively, may have been considered to result in comparable faecal pH. However, the addition of white clover, particularly during ensiling, as demonstrated by Winters et al (2004) results in elevation of pH. This may indicate the difference between the HS and HSC group faecal pH.

The equal FIO concentration cultured directly from the faecal sample of all three groups (Table 7) indicates the comparable selection of study animals was successful. The inclusion of faeces from animals of similar age and disease status (Medrano-Galarza et al, 2018, Grandl et al, 2017, Donnet-Hughes, 2010) ensured the study was representative of the principal grazing population. This ensured

that when culture based results in leachate were compared any differences were not driven by outlying individual animals and, so far as possible, were group representative. The low variation observed confirmed this.

The similarity in all forage nutritional quality parameters excluding WSC indicated that sugar content was the principal dietary variable in this study. The increased WSC content of the HS grass is not surprising given its genetic selection for this characteristic (Merry et al, 2006) The relative decreased WSC content in the PP perennial ryegrass (*Lolium perenne L*) diet but comparable other nutritional parameters indicated its status a contemporary species to the HS diet, varying only in sugar content. This was further indicated by the intermediary WSC content of the HSC indicating the dilution effect on sugar content of the high sugar clover mixed diet.

2.4.2. Inter group lysimeter leachate variation

2.4.2.1. Volume and culture based variation of leachate

Controlling for the influence of lysimeter microenvironments was crucial in the study design (Corwin, 2000, Cameron et al, 1992). The accuracy of soil packing throughout all lysimeters would significantly influence results as has been previously indicated (Dokoohaki et al, 2018). Soil packing, whilst representative of the Farm Platform as a whole, does not represent the significant in-field variation and the influence of individual sites compaction on whole catchments leachate microbial profile. No differences were found in the volume of leachate produced by lysimeters following the equal application of water, modelling a rain event (Table 8). While comparable in volume of leachate produced, the degree of variation did differ within groups as indicated by standard deviation. Low variation was observed between the soil only Control lysimeters with greatest intra group differences between the HS group lysimeters. These non-significant results may indicate potential biological

significance within the limited parameters of this controlled environment study. The closer association of Control lysimeter leachate volume supports the accuracy in soil packing replication in the study operation.

A comparison of the results obtained for FIO concentration directly from the three systems faecal samples (Table 7) and from leachate (Table 9) indicated the significant loss of viable FIO from the modelled pasture by lateral transport under action of rainwater (Soller et al, 2010, Kay et al, 2007). The three systems FIO concentration in leachate was not found to differ between groups but was significantly increased to the zero-concentration found in Control lysimeter leachate (Table 9). A reduction in FIO concentration within leachate compared to direct from faeces was similarly found in all non-Control groups (FIO $\log_{10}$ $CFUg^{-1}$ reduction in leachate from faeces: HS -2.09, PP -2.13, HSC -1.75). This experimental result is comparable to that found by Kay et al 2018, in comparable environments. This demonstrates the ability of soil to enable water transport of viable FIO but also to accumulate a significant portion (Stott et al, 2017, Mosaddeghi et al, 2009, Oliver et al, 2005). This preliminary result highlights the important role of soil in the wider transport or capture of livestock origin microbes. This will inform future field scale studies of the importance of regions of high faecal deposition rates and the potential cumulative risk of potentially pathogenic or antimicrobial resistant bacteria. The use of FIO CFU methodology is valued as a prevalent industry accepted measure of faecal contamination and results present wide external validity (Gronewold & Wolpert, 2008, Wade, 2006, Noble et al, 2003). The accuracy, economy and rapidity of this method allows for larger scale study design. Applying FIO CFU technique in field scale studies enables valuable comparisons to be made across systems.

2.4.2.2. Sequencing based variation of leachate

Next generation sequencing was utilised in this preliminary study to investigate specific differences arising in faecal, subsequent soil and water microbe populations across the three differing pasture systems at Rothamsted Research North Wyke Farm Platform (Takahashi et al, 2018, Orr et al, 2016). This was intended to inform the design of future field scale studies and ensure subsequent conclusions possessed sufficient external validity. Identifying differences in the microbial composition of leachate derived from HS, PP and HSC group faeces as well as soil only Controls highlights potential pasture system specific variation.

The microbial composition presented in this study is consistent with similar field based (Kuramae et al, 2012) findings. The most dominant phyla across all samples were found to be Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria (Figure 7) (Janssen, 2006). This composition trend continued in the most abundant genera corresponding to these phyla in *Legionella*, *Oscillibacter*, *Alistipes* and *Corynebacterium* respectively.

An assessment of microbe alpha diversity of all samples in each groups leachate by Shannon Diversity Index using MRPP ($p < 0.05$) indicated increased diversity in PP relative to HS. The highest median diversity was found in Control and PP groups (Figure 4). This supports existing knowledge, particularly indicating the diversity of soil microbe communities generally (Luo et al, 2017) and microbe richness within permanent pasture systems (Schmid et al, 2018). The lowest median diversity was the HS group, possibly indicating the negative influence on GI and faecal diversity which the high sugar ryegrass monoculture diet causes (Neubauer et al, 2018, Schmid et al, 2018). While no statistical significance was found between the increased microbe diversity of PP compared to HS group leachate, variation may be of biological significance. The greatest intra

group variability in microbe diversity of leachate samples was found in the Control group, indicating that some lysimeter specific influence remains.

Initial differences between groups leachate microbe composition indicated by alpha diversity analysis were visualized by NMDS (Figure 5) and MDS (Figure 6) plots (Benjamino et al, 2018). These indicated high intra group variation, particularly within PP and Control groups. Relatively increased clustering of samples within HS and HSC group were indicated. This may again result from constrictive effect on microbial population diversity which a high sugar ryegrass diet causes through altered GI and faecal environment (Neubauer et al, 2018). This is supported by the WSC findings of forage diet analysis, indicating increased WSC in the HS group diet. Analysis by ADONIS (PERMANOVA) indicated no significant differences ($p < 0.01$) between groups. However, future higher resolution sampling may support the suggested difference in HS and HSC relative to PP and Control groups.

The median proportions of microbes in all groups leachate were assessed by pairwise comparison with FDR correction (Figure 9). Differences between groups were indicated at phyla through genus taxon level. family taxon level. The family Chitinophagaceae microbes, with principally oxidative metabolism (Ke et al, 2018, Pérez-Jaramillo et al, 2018), were found to be increased in PP, HSC and Control relative to HS group leachate. Potentially indicating biological significance driven by diet.

All genera increased in HS were found relative to PP or Control group leachate. The genus *Parasutterella*, *Alloprevotella* and *Oscillibacter* were all found at increased proportions in HS relative to PP and Control group leachate. These genera are commonly found in the digestive tract and exhibit fermentative metabolism (Espinoza et al, 2018, Noble et al, 2017, Iino et al, 2007). *Parasutterella* has been experimentally associated with high sugar diets (Noble et al, 2017) and

this may explain why no difference was found between HS and HSC group leachate. Similarly, *Oscillibacter* has demonstrated preferential growth in high sugar conditions (Iino et al, 2007) as well as potential pathogenicity (Sydenham et al, 2014).

The common gut microbe genus *Alloprevotella* was found at increased proportion in HSC compared to Control group leachate and is considered to be of faecal origin. However, as this genus was not found to be of similarly increased proportion in HS or PP compared to Control group lysimeters its dominance may derive from diet specific differences (Zhou et al, 2018). Interestingly considering the similarity in high sugar ryegrass diets, specific genus were found to be of increased proportion in HSC relative to HS group leachate. These included *Sphingopyxis* and *Devosia*. The increased proportion of genus *Devosia* is suggested to result from its presence in soil and potential role in Nitrogen fixing (Yoon et al, 2007, Rivas et al, 2002). Laranjo et al 2014, presented *Devosia* as part of legume rhizobia population and in this study, may result from the white clover component of the HSC group diet.

The genus *Victivallis* and *Olsenella* were found at increased proportion in HS and HSC compared to Control group leachate. Both genus are fermenters and commonly found in the digestive tract (Dewhirst and Wade, 2015), *Victivallis* also demonstrates preferential growth at relatively lower pH 6.5 (Zoetendal et al, 2003). This may correspond with the decreased HS group faecal pH (Table 6). These increased proportions relative to Control group leachate suggest the influence of livestock diet from faeces, through soil and into modelled watercourse.

All genus at increased proportion in PP were found relative to HS group leachate, suggesting an association to diet differences. The pasture and environmental associated genus *Gemmatimonas* (Takaichi et al, 2010), *Leifsonia* (Evtushenko et al, 2000) and *Rhodoplanes* (Wang et al, 2016) were all found to at increased proportions in PP compared to HS group leachate. The digestive

tract associated genus *Luteimonas* (Yi-Wei et al, 2016), *Microbacterium* (Gneiding et al, 2008) and *Nocardioides* were also found to be proportionally increased in PP compared to HS group leachate. The environmental genus *Aeromicrobium* (Kim et al, 2008) and *Ochrobactrum* (Bathe et al, 2006) were found at increased proportions in PP and HSC compared to HS group leachate.

The Control indicated specific differences in microbial proportion compared to both HS and HSC group leachate. The oxidising genus *Nitrospira* was found at higher proportions in Control compared to both HS and HSC group leachate. Studies have found *Nitrospira* inhibited in low pH soil and water environments (Daims et al, 2001). The soil associated genus *Geobacter* (Zhou et al, 2014) and *Nakamurella* (Nouioui et al, 2017) were found at increased proportion relative to HSC group leachate. The environmental genus *Kaistia* (Jin et al, 2012) and *Parvibaculum* (Schleheck et al, 2004) were found at increased proportion in Control and PP compared to HS group leachate.

Substantial variation in leachate microbial population derives from soil microbial populations, the importance of this must be considered in future field scale studies and utilise a comparable sequencing approach as undertaken in this preliminary study. The PP group leachate presented the greatest microbial diversity and the HS the least. The similarities in high sugar ryegrass diets between HS and HSC group leachate indicates the whole system influence which diet can elicit from faeces to soil and watercourse. The diversity in HS group leachate compared to others was relatively decreased, this is potentially a function of diet resulting in dominance of particular species at the expense of diversity.

2.5. Conclusion

This preliminary study provides valuable information on the interface between livestock faeces and soil, particularly under simulated storm rainfall hydrological action. This experiment demonstrated the critical role of soil in both retaining and releasing microbes from the farming system. The comparable culture based FIO CFU findings of all three faecal based lysimeter leachates indicates the capacity of soil to bind substantial portion of faecal origin microbes. This was supported with the findings of next generation sequencing which highlighted the influence of soil microbial composition in resulting leachate. Sequencing results supported the forage nutritional analysis, indicating the impact of livestock diets on microbial populations was present from faeces through soil and into modelled watercourse. Differences in microbe genus proportions of leachate suggest considerable diversity exists within the permanent pasture (PP) this variation was decreased in the culture high sugar ryegrass pasture (HS) were diet adapted digestive tract microbes predominated. The variation in microbial population derived solely from soil was also noted, reinforcing the importance of a high-resolution understanding of this environment within future field studies. Chapters 3 and 4 field scale study design and methodology will further develop multi-disciplinary approach. Additional chemical analyses will be applied in future Chapters 3, 4 and 5 to present a more complete understanding of the microbial environment. A subsequent focus will be applied to the most industry applicable, permanent pasture, system at Rothamsted Research North Wyke Farm Platform to present microbial cycling findings of the widest external relevance. Chapter 3 will examine the role of soil in field scale microbial hazard.

Chapter 3

The role of soil in microbial cycling on-farm: Higher risk zones of potentially pathogenic microbes and antimicrobial resistance in fields.

Summary

Grazed livestock production is a significant human food source and is of critical economic importance. The land and soil subject to this management system contributes a large portion to the total agricultural land used in Norther Europe and particularly the UK. The impacts of livestock grazing upon soil microbe populations have been investigated but greater emphasis has been placed on the microbe composition of faeces and subsequent composition of water outflow from agricultural land. This study was undertaken to investigate the impacts of grazed livestock production on microbial hazard (as suggested by faecal indicator organisms (FIO) concentration) and antimicrobial resistance (AMR) patterns. This study determined the variation in these factors at a field scale alongside soil chemistry parameters, prior to high resolution testing of soil microbial differences at identified in-field hotspots. At a field scale, increased FIO concentration were associated with specific points in fields, but did not correlate to soil nitrogen or SOM. Subsequent soil hotspot sampling identified the extent of the spatial influence of these in-field features in increased prevalence of potentially pathogenic and AMR bacteria relative to surrounding field soils. The persistence of these soil hotspots within the landscape regardless of current management use for grazing or forage production was also demonstrated. This work suggests that potential grazing management strategies could reduce the development of these high microbial risk hotspots within fields and minimise their disproportionate impact to on-farm and external health.

3.1. Introduction

In Chapter 2 the capacity of soil to leach faecal indicator organisms (FIO) such as *Escherichia coli* was demonstrated. The influence of soil and faecal deposition upon microbial composition was also illustrated. This preliminary study utilised a combination of culture based and next generation sequencing approaches to produce results with wider application potential. This chapter will further investigate the role of soil in maintaining potentially pathogenic and AMR bacteria populations at a field scale.

The importance of soil as the principal microbial environment within agricultural land has been previously characterised (Goddard et al, 2012, Lawlor et al, 2000, Bossio et al, 1998). The importance of soil characteristics in supporting specific microbial populations within livestock production systems has been studied across various farming systems indicating the crucial role in providing environmental niches (Garbeva et al, 2004, Lawlor et al, 2000). Soil microbes role as part of more extensive grazed animal production has been previously identified, with studies principally focusing on its impact at the wider landscape scale accumulation and dissemination (Gilbert et al, 2006, Morgan et al, 2006, Waller, 1999). The direct transfer of FIO via faecal deposition of grazing livestock to soil, has been suggested as a high-risk route for the dissemination of potentially pathogenic and AMR bacteria to the wider environment (Allen et al, 2010, Sayah et al, 2005, Hooda et al, 2000). Soil, as a matrix, can retain and maintain FIO which pose a significant risk to the preservation of human (Woolhouse et al, 2015, Patz et al, 2004) and animal (Durand et al, 2004, D'Allaire et al, 1987) health. The capture and sustainment of potentially pathogenic and AMR bacteria originating from livestock facilitates the increased genetic exchange of virulence factors and resistance between populations (Sayah et al, 2005, Reinthaler et al, 2003). The accumulation within agricultural soils of higher risk microbes and the genetic exchange with soil

origin microbe populations this enables, has been indicated as a factor in the increased dissemination of agricultural origin pathogens (Lau & Ingham, 2001) and AMR (Allen et al, 2010, Kummerer, 2004). Most previous literature highlights the transfer of this soil accumulated microbial hazard by hydrological action to the wider environment (Sayah et al., 2005, Hooda et al., 2000). The release dynamics of these soil populations by hydrological action will be examined more fully in Chapter 5. The impact of these soil accumulated populations within the on-farm microbial cycle is less clearly defined. This Chapter and Chapter 4 aim to identify the impact of high concentration livestock origin microbes on pasture accumulation at a field and farm scale.

3.1.1. Landscape scale and importance

Previous studies of microbial contamination resulting from grazing systems have highlighted the impact at a landscape scale, principally in natural watercourses (Sayah et al, 2005, Hooda et al, 2000) and in soil (Hodgson et al, 2009, Oliver et al, 2005). These studies clearly present the impact which extensive livestock production can cause upon the soil micro-environment. The alteration of soil microbe populations has been found throughout various grazing livestock systems, topography and climates (Calloway et al, 2010, Lawlor et al, 2000, Bossio et al, 1998). However, the degree of impact which different production systems, topography or soil types exert upon resulting microbial hazard is not consistent. Previous results primarily focus on the impact of livestock origin microbe accumulation within soils and any subsequent hydrological transfer at the landscape scale, there are fewer farm scale targeted studies. This scale informs enforcement policies on pollution or contamination (McGonigle et al, 2012, Sudol & Ambrose, 2002). It also provides broad indications, within the specific study landscape, of negative influences on microbial risk (Wilkinson et al, 2006). These wider scale studies also inform the design of higher

resolution sampling. The impact of soil type (Van Der Heijden et al, 2007, Desmarais et al, 2002) and topography (Crowther et al, 2003) in the accumulation and survival of FIO in agricultural soil are noted as key factors.

3.1.2. Field scale: land use, topography and soil type

The application of higher resolution soil microbial sampling is crucial in accurately quantifying the microbial risk, of pathogenic and AMR bacteria, and effectively targeting any on-farm interventions. Only by identifying the differences generated in microbial soil populations as a point or field scale can meaningful mitigation strategies be implemented. Previous studies have employed various strategies to identify in-field microbial risk variation such as grid (Franklin & Mills, 2003) or site targeted sampling (Buckley & Schmidt, 2003, Pereira-Neto & Smith, 1986). The initial application of grid sampling ensures the most comprehensive survey area and increases the likelihood of field representative results. This is particularly important given the NWFP variation in landscape identified soil microbe key factors of soil type and topography as indicated by Figure 1.

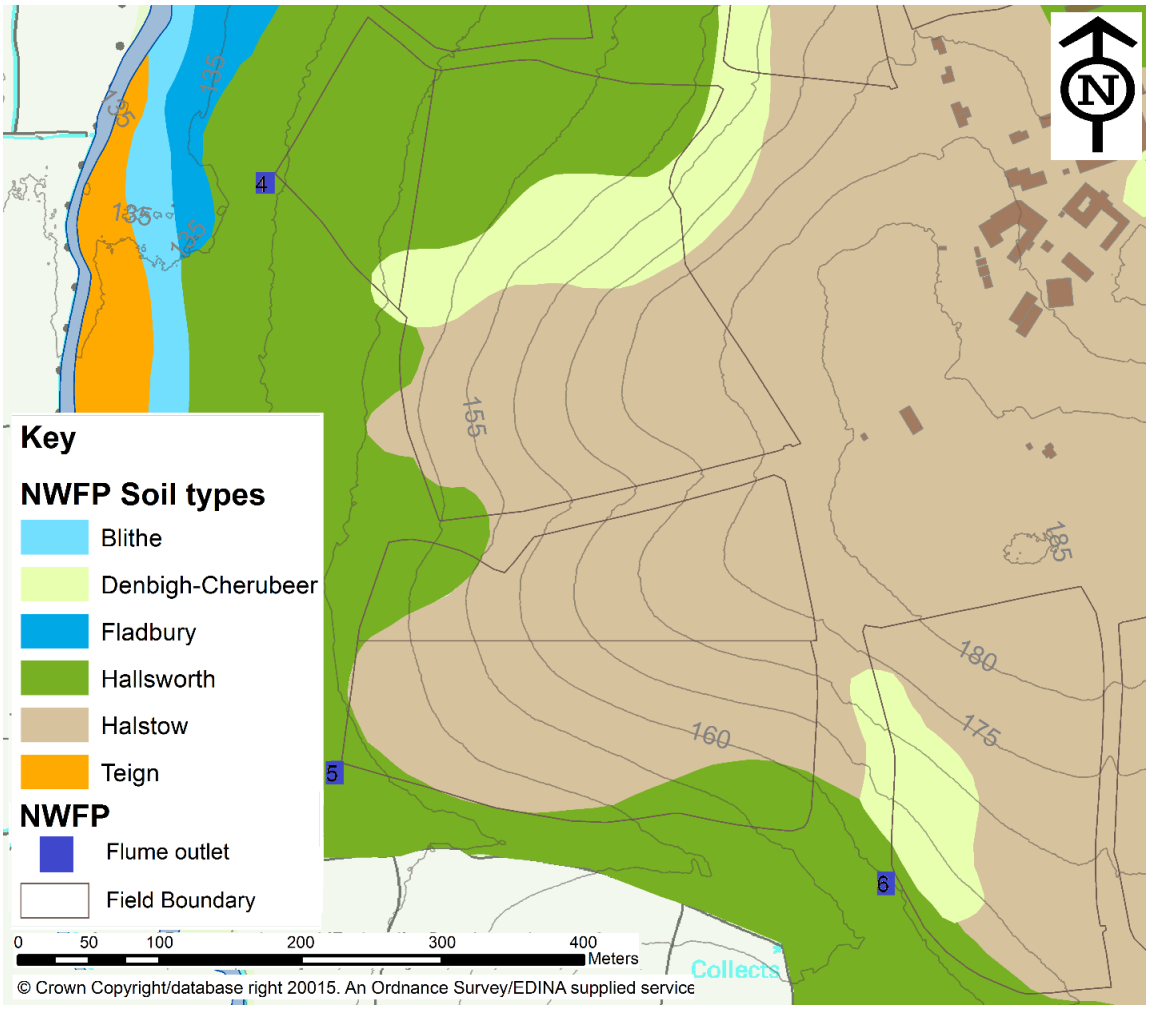

Figure 1. Soil type and elevation map of the permanent pasture catchments 4, 5 and 6 of Rothamsted Research North Wyke Farm Platform (NWFP).
Soil sampling site and variations in soil type and field topography.

Given that previous field scale research has identified specific high risk land use areas for faecal deposition (Betteridge et al, 2010, Oudshoorn et al, 2008, White et al, 2001) it is considered prudent to include these as supplemental points in addition to grid sampling.

Across the NWFP the dominant soil type is clay and thin subsidiary sandstone layers. The properties of these different soil types are described in Table 1 and their spatial variation given in Figure 1. The distribution of heavy and intermediary clay soils throughout the UK gives this soil type particular importance when investigating its capacity to retain and support FIO

populations (Conboy & Goss, 2000, Cannell et al, 1984). These soils share comparable properties in available oxygen pore space (Douglas et al, 1981), water retention (Ravina & Magier, 1984) and elemental accumulation, such as Carbon and Nitrogen (Batjes, 1996, Lugo et al, 1986). The distribution of these soil types within fields may still generate microbial differences.

Table 1. NWFP catchments 4, 5 and 6 soil type and characteristics.

Soil series	Lithology	Characteristics
Denbigh-Cherubeer	Fine loamy typical brown earth in head from clay shale.	Clay loam, brown colouring throughout, shaley at depth.
Hallsworth	Clayey pelostagnogley soil in head from clay shale.	Clayey throughout, grey colouring, mottled throughout.
Halstow	Clayey noncalcareous pelosol in head from clay shale.	Clayey throughout, Brow colouring, mottled below 40cm.

Description of soil type characteristics (36) of the soil sampling sites.

Field scale differences in site specific microbial populations have been previously identified within grazing production systems. These are principally driven by site specific variation in topography or animal interaction (Calloway et al, 2010, Lawlor et al, 2000, Bossio et al, 1998). Extensive studies have indicated in-field variation in animal behaviours resulting in differing rates of faecal deposition but also in the soil properties (Betteridge et al, 2010, Oudshoorn et al, 2008, White et al, 2001). This site specific intensive influence by grazing animals has been found to alter soil density, moisture and organic matter (Beylich et al, 2010, Lipiec & Hatano, 2003) principally by compaction through locomotion. These site-specific variations are critically important at a field scale as they have the potential to generate significantly different soil FIO concentrations and soil properties. These specific zones determined by hypothesised animal interaction and differences in topography (influencing water movement) should be included within grid sampling to properly

represent field scale microbial risk variation. The impact of these zones upon microbial hazard will be further investigated in grass at pasture and subsequently in preserved forage in Chapter 4. Their influence upon field water outflow microbial populations will be examined in Chapter 5,

3.1.3. Research aims

The characteristics of soil are the foundation of productivity in grassland based livestock production. Effective soil management also plays an important role in the farm microbial cycle and animal health. In this chapter the North Wyke Farm Platform permanent pasture is utilised to test the overall hypothesis that: soil of livestock based pasture systems is a significant source of potentially pathogenic and AMR bacteria.

Hypothesis 1 - There are specific zones of increased pathogen and AMR bacteria in soil at field scale which pose a greater hazard to health.

Hypothesis 2 - The extent that pathogen concentration and percentage AMR isolates is increased relative to specific zones in soil can be measured.

3.2. Materials and methods

3.2.1. Experiment 1: Field scale soil sampling- Hypothesis 1.

The initial field scale survey of soil sampling was completed across catchments 4, 5 and 6 of the North Wyke Farm Platform (Orr et al, 2016) as shown in Figure 2 (constituting fields: Bottom Burrows and Burrows, Orchard Dean North and Orchard Dean South, Golden Rove). These catchments represent the principal basis of the North Wyke Farm Platform permanent pasture functional 'farmlet' and have the widest external stakeholder relevance as the most common grassland system within the UK (Qi et al, 2018, DEFRA, 2016, Smit et al, 2008). As described in Chapter 4 the 'semi-rotational continuous (variable)' grazing strategy utilised in this study results in multifunctionality of fields within catchments. Catchment 4 and 5 (Orchard Dean North only) were under grazing rotation of the beef herd and sheep flock, respectively. At the time of sampling catchment 6 and 5 (Orchard Dean South only) were not grazed and were under silage production.

The application of a regular 50 m grid soil sampling pattern enabled maximal field coverage and greatest potential for accurately characterising any spatial variation (Franklin & Mills, 2003). This resulted in n=70 regular grid sampling points and following statistical advice n=22 random supplementary sampling points. In addition to these sample points n=9 preferential supplementary points were allocated 3 to each catchment at points of increased livestock activity and hypothesized increased microbial risk (Betteridge et al, 2010, Oudshoorn et al, 2008, White et al, 2001). These included water troughs, in-field features such as weather stations and trees and field position immediately proximal to catchment flume outlet (Sayah et al, 2005, Hooda et al., 2000). In total n=101 soil survey sampling points across catchments 4, 5 and 6 were completed. As the predictive spatial influence of soil chemistry parameters is well characterised the addition

of supplemental points, such as necessitated in the microbial sampling, was not required for statistical analysis. GIS positioning located each individual sampling point in-field and replicate sampling was conducted in triplicate. Sampling procedure required using a soil corer, with samples taken to a uniform depth of 20 cm below surface. Sterility was maintained between sampling points by washing soil corer with ethanol. Each individual sample point replicates were mixed and apportioned 25 g for soil chemistry analysis and the remainder for faecal indicator organisms (FIO) analysis. Samples were sealed in sterile bags and immediately transported for laboratory analysis. Field scale soil sampling results were mapped in ArcGIS 10.4th edition (ESRI, Redlands, California, USA) by inverse distance weighting extrapolation (IDW).

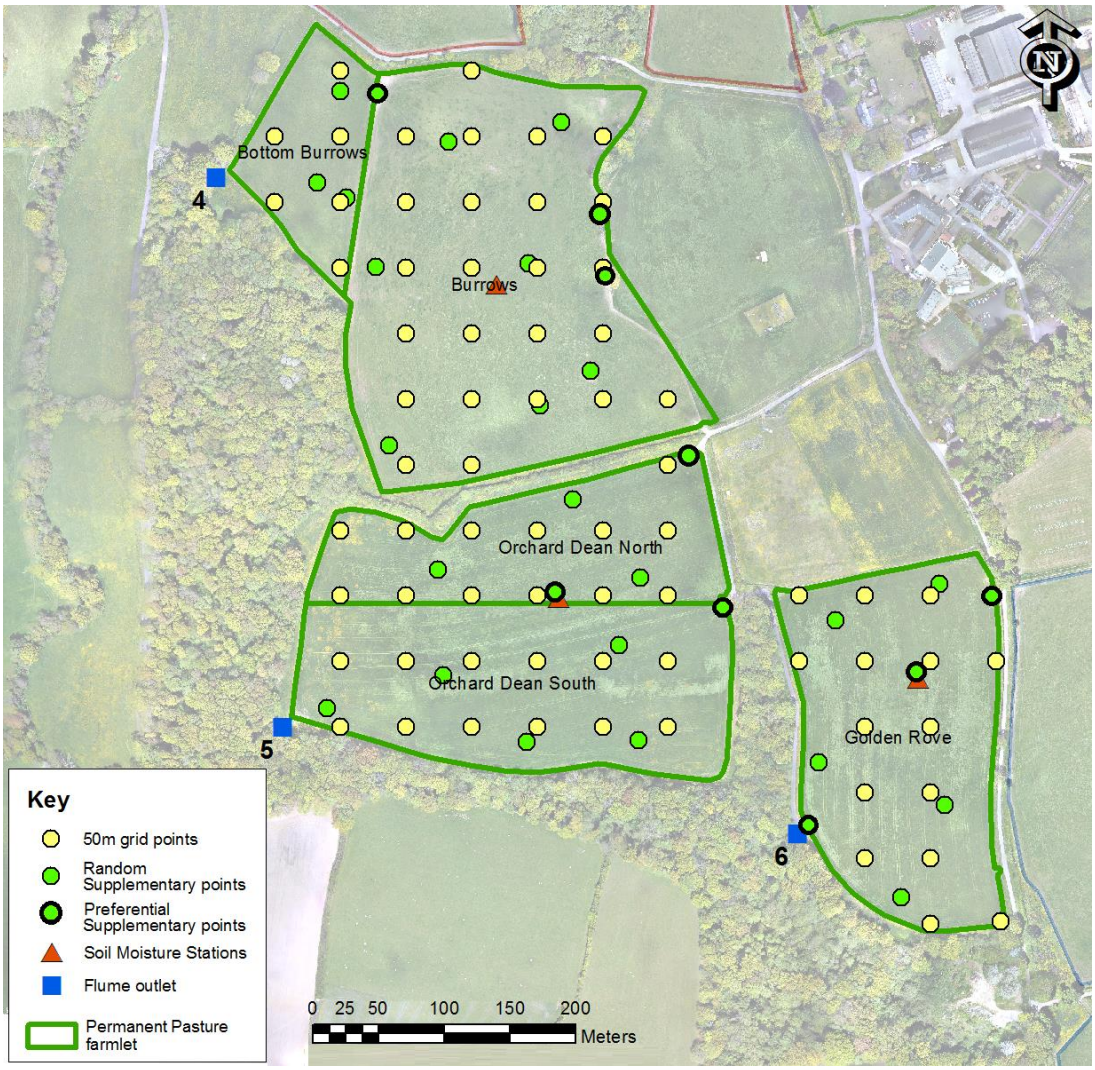

Figure 2. Field scale soil survey sampling conducted on NWFP.
Each point was sampled in triplicate and combined totaling n=101 samples for analysis. Management field boundaries are indicated within catchments 4, 5 and 6.

3.2.2. Experiment 2: Soil 'hotspot' sampling- Hypothesis 2.

Soil sampling at considered 'hotspots' was completed similarly to the protocol undertaken in the field scale soil survey in experiment 1 and is presented in Figure 3. Following identification of higher microbial risk zones associated with specific in-field features (Oudshoorn et al, 2008, White et al, 2001) in experiment 1 sites were chosen with commonality across each of the three catchments to enable replication. The 'semi-rotational continuous (variable)' grazing strategy

utilised in this study results in multifunctionality of fields within catchments. As described in experiment 1, Catchment 4 and 5 (Orchard Dean North only) were under grazing rotation of the beef herd and sheep flock, respectively. At the time of sampling catchment 6 and 5 (Orchard Dean South only) were not grazed and were under silage production. Sampling was conducted across grazed and ungrazed field use to enable analysis of any differences in FIO concentration and % AMR resulting from temporal differences between periods since livestock contact. Ungrazed field use had not had livestock contact for over 6 months, since the previous production season. Grazed field use had livestock present for over 3 months prior and during sampling but had no contact in the previous production season. The hotspots were designated in classification as Trough (principal water trough in each field), Station (soil moisture/weather station on each catchment) and Flume (boundary of catchment immediately adjacent to catchment flume outlet). The hypothesized hotspots were identified as deriving from direct animal interaction (Trough and Station) or driven by field scale hydrology (Flume). Sample points within the 10, 20 and 30 m regions were randomly allocated using GIS mapping. Within in each region n=3 sample points were allocated and soil sampling in triplicate as described for non-hotspot areas was completed. Thus, individual hotspots were sampled n=9 times in triplicate, with n=3 hotspots considered on each catchment and n=3 of each hotspot classification across all catchments.

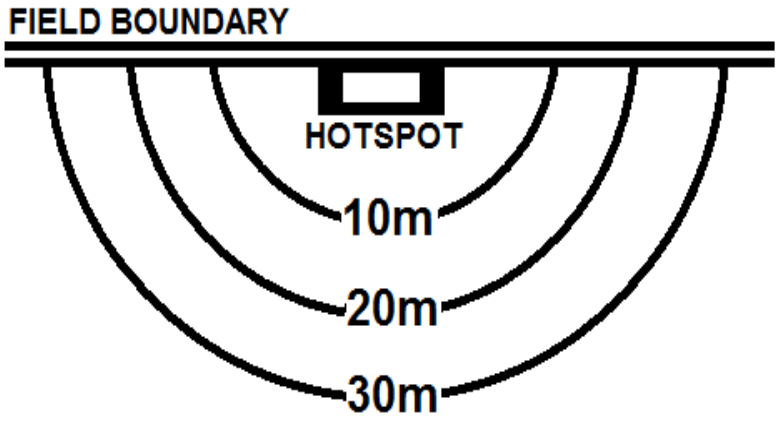

Figure 3. Hotspot spatial sampling.
Radiating sampling regions of <10, <20 and <30 m, Trough and Flume hotspots were situated at field boundaries as presented but Station hotspots were situated within fields. Three sample points were taken within each region.

In addition to soil hotspot sampling n=22 randomly allocated non-hotspot sample points were mapped across catchments 4, 5 and 6 to enable comparison with hotspot results (Figure 4). Each individual sample point replicates were mixed and apportioned 5 g for DNA sequencing, with the remainder for faecal indicator organisms (FIO) and subsequent antimicrobial resistance (AMR) analysis. Samples were sealed in sterile bags and immediately transported for analysis.

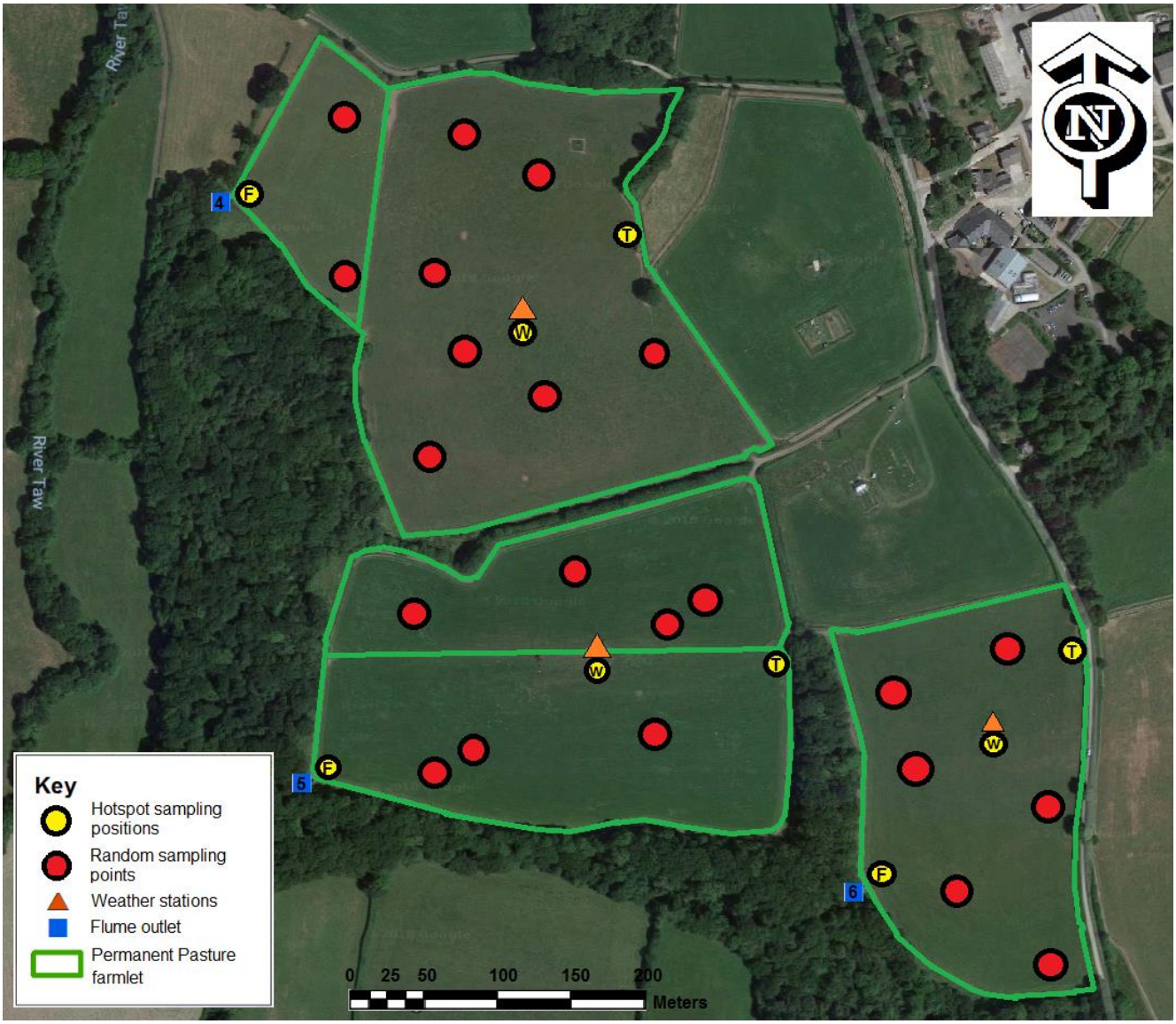

Figure 4. Hotspot soil sampling conducted on NWFP.
Each random point was sampled in triplicate and combined, each hotspot was sampled n=9 times in triplicate, totaling n=103 samples for analysis. Management field boundaries are indicated within catchments 4, 5 and 6. Hotspot classification is denoted by T- Trough, W- Station and F- Flume.

3.2.3. Faecal indicator organism culturing- Experiment 1 and 2

The determination of FIOs of each 5 g sub sample, from field scale or hotspot soil sampling, was serially diluted to enable standard membrane filtration as described by Hodgson et al (2016). Samples were washed through the filtration manifold unit with 20 ml of sterile Ringer's solution.

Membrane filters of 0.45µm (Sartorius Stedim Biotech, Goettingen, Lower Saxony, Germany) were placed onto species selective sterile agars for FIO culturing. *E. coli* growth was targeted on Membrane Lactose Glucuronide Agar (Oxoid, Basingstoke, Hampshire, UK) and incubated for 18-24 h at 44.5 °C (±0.20 °C) (Hodgson et al, 2016). Intestinal enterococci growth was targeted on Slanetz and Bartley agar (Oxoid) and incubated for 44-48 h at 37.5 °C (±0.20 °C) (Hodgson et al, 2016). Confirmation of intestinal enterococci concentrations were cultured on Kanamycin aesculin azide agar (Oxoid) plates pre-warmed to room temperature, and incubated at 37.5 °C (±0.20 °C) for 4 h (±5 minutes). All samples were processed in triplicate throughout culturing. The initial FIO counts were transformed to Log_{10} colony forming units (CFU) g^{-1} DM.

3.2.4. Antimicrobial resistance analysis- Experiment 2

Assessment of AMR prevalence, from field scale or hotspot soil sampling, was tested by Kirby-Bauer disc diffusion test (Bauer et al, 1959). *E. coli* colonies deemed representative were isolated in triplicate from each experimental unit on non-selective, Mueller-Hinton agar (Oxoid), incubated for 24 h at 35.0 °C (±0.20 °C) (Tenover et al, 2001). Cells were then suspended in phosphate-buffered saline (PBS) to a concentration of 1.5×10^8 CFU ml^{-1}, visually assessed against a 0.5 McFarland standard (Wiegand et al, 2008). Mueller-Hinton agar plates were immediately inoculated by spread plating prior to the application of 6 antimicrobial susceptibility testing disks (Oxoid) and incubated for 18 h at 35.0 °C (±0.20 °C) (Jorgensen and Turnidge, 2007). Antimicrobials used and their amounts were: amoxicillin/clavulanate (30 µg), cefpodoxime (10 µg), cephalexin (30 µg), doripenem (10 µg), enrofloxacin (5 µg), erythromycin (15 µg), florfenicol (30 µg), neomycin (30 µg), sulphisoxazole (300 µg) tetracycline (30 µg). These were selected to best capture veterinary and human antimicrobials, and resistance patterns of critical or high

importance (WHO AGISAR, 2017). The panel was correlated against farm specific history. Each antimicrobial's zone of inhibition was measured to the nearest millimetre. Inhibition zones were assessed against EUCAST (http://www.eucast.org), BSAC (http://www.bsac.org.uk) (Andrews et al, 2001) and CLSI (https://clsi.org) agreed clinical breakpoints, indicated in Table 2.

Table 2. Antimicrobial breakpoint assessments for Kirby-Bauer disc diffusion test used in experiments 1 and 2.

Antimicrobial	Resistance breakpoint (mm)	Source
amoxicillin /clavulanate	19.0	EUCAST[a]
cefpodoxime	19.0	BSAC[b]
cephalexin	14.0	EUCAST
Doripenem	18.0	BSAC
enrofloxacin	16.0	CLSI[c]
erythromycin	13.0	CLSI
Florfenicol	32.0	EUCAST
Neomycin	13.0	CLSI
sulphisoxazole	12.0	CLSI
tetracycline	14.0	CLSI

Resistance was defined as visible isolate growth measured to be equal to or less than the specified breakpoint radiating from antimicrobial inoculated disk. a- European Committee on Antimicrobial Susceptibility Testing (EUCAST), b- British Society for Antimicrobial Chemotherapy (BSAC), c- Clinical and Laboratory Standards Institute (CLSI).

3.2.5. Microbial DNA extractions, sequencing and analysis- Experiment 2

Total DNA was isolated from 1.00 g soil samples using PowerSoil DNA Isolation methodology (MoBio Laboratories INC., Solana Beach, California, USA) (Buse et al, 2014) (Wilcox, 2013). The manufacturer's recommended protocol was followed, with specified exceptions: 1) At step 16 after addition of high salt solution PW3 500 µl of supernatant was centrifuged as attempting at the 650 µl recommended resulted in residue. 3) At step 20 after addition of product cleanser ethanol solution PW5 to remove the alcohol based solution PW4,

centrifuging by 1, 1, and 2 minutes (totalling 4 minutes) was completed as the 2 minutes recommended resulted in significant solution PW5 residue in spin filter basket which would inhibit proposed downstream DNA applications such as PCR and gel electrophoresis.

Upon release from high salt binding to spin filter basket, by the 10 mM Tris DNA sterile elution buffer, the extracted DNA was stored at -20 °C in preparation for downstream applications. Extraction success of DNA was qualified by gel electrophoresis by removal of 5 µl template DNA and adding 1 µl loading buffer. Extracted DNA quality was assessed visually on agarose gel and if poor quality was indicated extraction was reattempted. Standard PCR reactions were completed on extracted template DNA in an Agilent Technologies Sure Cycler 8800 PCR system using GoTaq® G2 Flexi DNA polymerase (Promega Corp., Madison, Wisconsin, USA) for each sample. Each individual reaction contained the following components: 50 ng of template DNA, 0.25 µl GoTaq® G2 Flexi DNA polymerase, 5 x Green Flexi Buffer, 3 µl $MgCl_2$, 1 µl of each dNTP, 1 µl of a 1 µM stock solution for each forward and reverse primers and Nuclease-Free sterile H_2O (Sigma-Aldrich Co., St. Louis, Missouri, USA) to 50 µl. Existing primers were selected for the PCR amplification of microbial marker gene sequence 16S as 27 forward and 1492 reverse (Kim et al, 2011, Frank et al, 2008, Lee et al, 2003) (Table 3).

Table 3. Primer Sequences and PCR conditions.

Primer set	Product	Sequence length (bp)	Primer Sequence	Annealing Temperature	Extension Time
27f—1492r	16S	1458	5′-AGA GTT TGA TCM TGG CTC AG 5′-TAC GGY TAC CTT GTT ACG ACT T	57 °C	1.5 min

Primer sequences and PCR conditions for amplification of target microbial marker gene sequence 16S.

The PCR was completed on the 50 µl solutions from all 31 soil hotspot and random samples under the following cycling protocol after initial extended denaturation of 94 °C for 2 min, 30

cycles of: 94 °C for 30 s, 57 °C 45 s, 72 °C for 1.5 min; 72 °C for 7 minutes, hold at 4 °C. Each sample had 5 µl PCR product and 1 µl of loading buffer added in preparation for gel electrophoresis. Gel electrophoresis allowed for further quality assessment and visualisation of potential downstream contaminates. Specific sizes of distinct banding or large association at the bottom within the gel, were assessed for impact on subsequent sequence quality and extractions or PCR reattempted when deemed necessary.

Template DNA and 16S PCR product was analysed using gel electrophoresis (Idota et al, 2018, Maldonato et al, 2028, Boon et al, 2002) (BioRad PowerPAC-300, Hercules, California, USA) in 0.8% (w/v) agarose gel matrices in 1x Tris-acetic EDTA buffer (TAE; 4.84 g Tris base, 11.42 ml acetic acid, 0.01 M EDTA, pH 8.3), containing ethidium bromide (0.3 µl) to visualise the DNA. A 1-kb plus ladder (Invitrogen, Thermo Scientific) was used to qualify DNA fragment size. Gels were run at 100 V for 2 h and imaged with an Omega Fluor Thermal Imaging system (Aplegen, San Francisco, California, USA) and associated computer documentation programme.

Following initial quality check of successful DNA extraction by gel electrophoresis, DNA concentrations were assessed by a Qubit fluorometer (Qubit 3 Fluorometer, Invitrogen, Thermo Scientific). Samples were transported by refrigeration on dry ice and were received in 72 hrs. Subsequent steps were then completed by Novogene Technology Co. Ltd, Hong Kong (Dang et al, 2015). Upon receipt of samples DNA concentration and purity was monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1ng/µL using sterile water. 16S rRNA genes of distinct regions 16S V4-V5 were amplified using primers 515f-907r with barcode (Table 4).

Table 4. Sequencing primer sequences and PCR condition.

Primer set	Product	Sequence length (bp)	Primer Sequence	Annealing Temperature	Extension Time
515f—907r	16S	393	5′-GTG CCA GCM GCC GCG GTA A 5′-CCG TCA ATT CCT TTG AGT TT	55 °C	0.5 min

Primer sequences and PCR conditions for amplification of target microbial marker gene sequence 16S.

All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Equal volume of 1X loading buffer (contained SYB green) was mixed with PCR products and electrophoresis on 2% agarose gel was completed. Samples of high intensity between 400-450bp were used in subsequent steps. PCR products were mixed in equidensity ratios. PCR product mix was purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using NEBNext Ultra DNA Library Pre® Kit for Illumina, following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit 2.0

Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The library was sequenced on the Illumina platform and 250 bp paired-end reads were generated. Sequence data clean-up was completed through standard Novogene 16S processing pipeline.

Sequencing data was analysed using QIIME software (Version 1.7.0) and displayed with R software (Version 2.15.3). Alpha diversity analyses were used to calculate Rarefaction and Shannon Diversity Index to interpret species richness and diversity. Homogeneity of group samples were visualised using non-multidimensional scaling (NMDS) (Benjamino et al, 2018). Differences in microbe composition across groups were assessed by visualisation in bar graphs and heatmaps. Percentage median relative abundance in microbes was calculated to identify the most relative abundant genera across groups. Multiple t-tests comparing median microbe

abundances, normalised with log transformation, between each group were completed (Foster et al, 2017, Wickham, 2009).

3.2.6. Soil chemistry analysis- Experiment 1

In laboratory conditions, each individual 25 g sample was mixed thoroughly. Any visible organic matter such as earth worms or surface vegetation as well as stones were removed. Samples were oven dried for 72-120 h at 40.0 °C (±0.25 °C) until soil moisture was fully evaporated. Samples were then sieved and ground by automated mortar (RM 200, Retsch GmbH, North Rhine-Westphalia, Germany) prior to final oven drying for 16 h at 105.0 °C (±0.25 °C). Dried soil samples were stored in sealed containers prior to individual chemical analysis.

3.2.6.1. Total Nitrogen analysis

Sub-samples were taken and weighed to 17 mg (±4.00 mg) by microbalance (MX5, Mettler-Toledo Ltd., Leicestershire, UK). Samples were analysed using a Carlo Erba NA2000 analyser (CE Instruments, Greater Manchester, UK) and a SerCon 20-22 isotope ratio mass spectrometer (SerCon Ltd, Crewe, UK) for total Nitrogen (Ohlsson & Wallmark, 1999). Analysis was conducted alongside known standards to ensure result accuracy.

3.2.6.2. Soil organic matter analysis

Ceramic sample dishes were cleaned then furnaced for 2 h at 400.0 °C (±2.00 °C) to ensure removal of residue and were weighed. A 20 g (±0.50 g) sub-sample of sieved, ground and dried soil was then added. Combined weight noted and samples were furnaced for 24 h at 400.0 °C (±2.00 °C). The remaining weight was noted as the soil ash (inorganic component) and the

difference from original sub-sample weight the soil organic matter component (Hoogsteen et al, 2018).

3.2.7. Statistical analysis

All statistical analysis was completed in Genstat (16th Edition, 2013). Correlation of field scale FIO CFU g^{-1} analysis to total Nitrogen and SOM was by linear mixed modelling. The soil hotspot variation of FIO CFU g^{-1} and percentage AMR isolates was calculated by blocked general ANOVA.

3.2.7.1. Experiment 1: Field scale soil statistical analysis

The linear mixed model used was:

Y variate: FIO CFU g^{-1}

Fixed model: Nitrogen or SOM

Block: Soil type/Field

Where: Nitrogen = Total Nitrogen mg N g^{-1}; SOM = Soil Organic Matter percentage of dry matter; Soil type = Hallsworth, Halstow or Denbigh-Cherubeer; Field = management field number (1 to 5).

Significance was accepted if $P \leq 0.05$.

3.2.7.2. Experiment 2: Soil 'hotspot' culture statistical analysis

The ANOVA model used was:

Y variate: FIO CFU g^{-1} and AMR

Treatment: Class*Distance

Block: Catchment

Where: Class = non-hotspot, flume, station or trough, Distance = non-hotspot, <10 m, <20 m or <30 m, Catchment = number (4 to 6).

Significance was accepted if $P \leq 0.05$.

3.2.7.3. Experiment 2: Soil 'hotspot' sequencing statistical analysis

Statistically significant differences ($P \leq 0.01$) were assessed between random and hotspot associate soil sample groups by Multiple Response Permutation Procedure. Multiple t-tests comparing median microbe abundances across groups was completed by Wilcoxon signed rank test. Correction for false discovery rate controlled multiple testing between groups.

3.3. Results

3.3.1. Soil field survey

Following individual inverse distance weighting extrapolation (IDW) of *E. coli* (log_{10} $CFUg^{-1}$), Nitrogen (mg N g^{-1}) and soil organic matter (% DM) some potential point associations were visually suggested (Figure 5). Any correlations between soil chemistry and *E. coli* concentration were analysed statistically by linear mixed modelling, see Figure 6 and Table 5. The field scale survey of *E. coli* concentration was not found to be significantly correlated to soil organic matter ($P = 0.451$, $r^2 = 0.90$) but indicated a weak correlation with total nitrogen ($P = 0.043$, $r^2 = 26.2$).

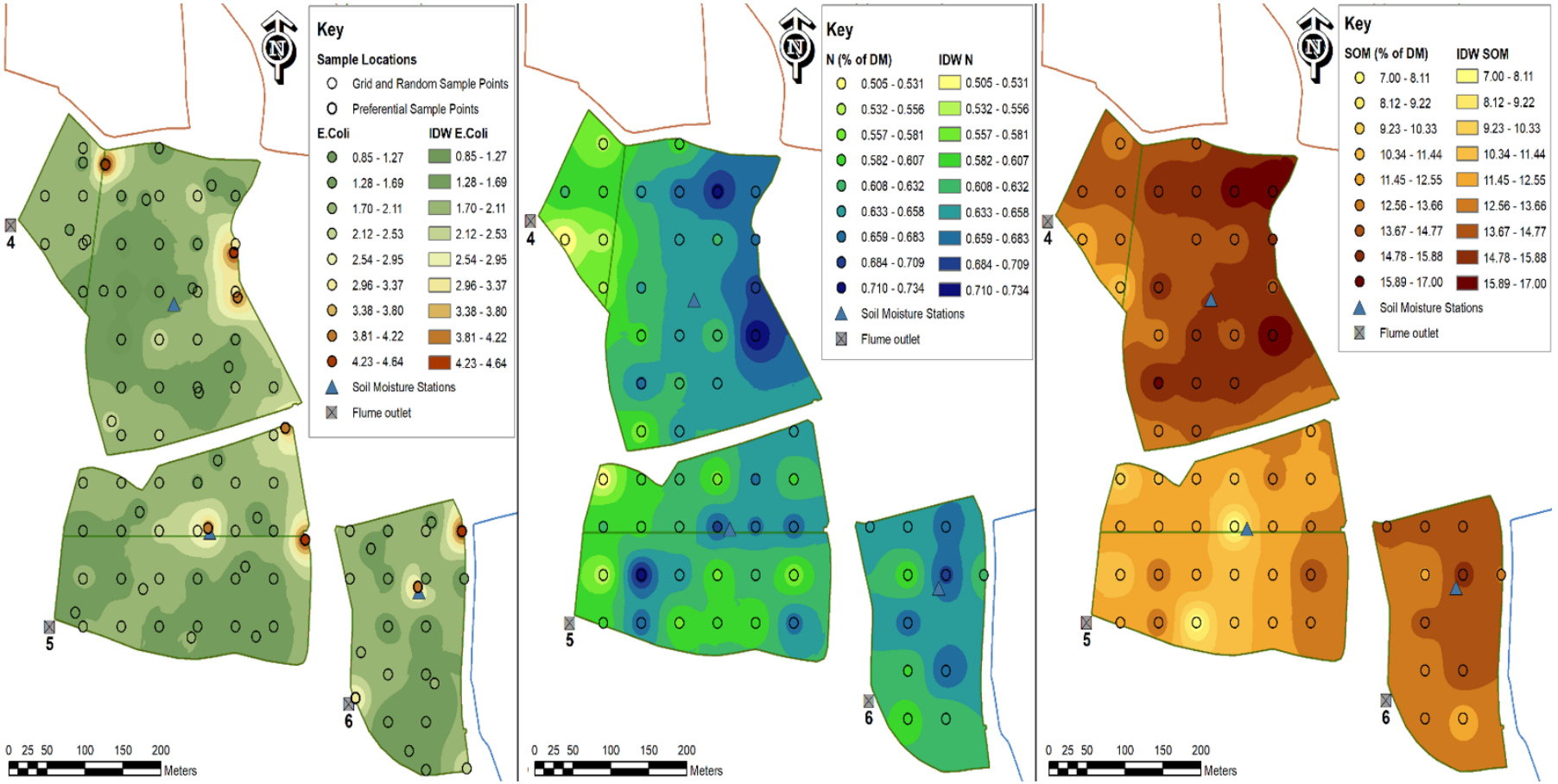

Figure 5. GIS mapping and inverse distance weighted (IDW) extrapolation of *E. coli* (log_{10} $CFUg^{-1}$), Nitrogen (mg N g^{-1}) and soil organic matter (% DM).

Individual spatial mapping of soil field survey parameters by IDW across singular survey area for visual reference.

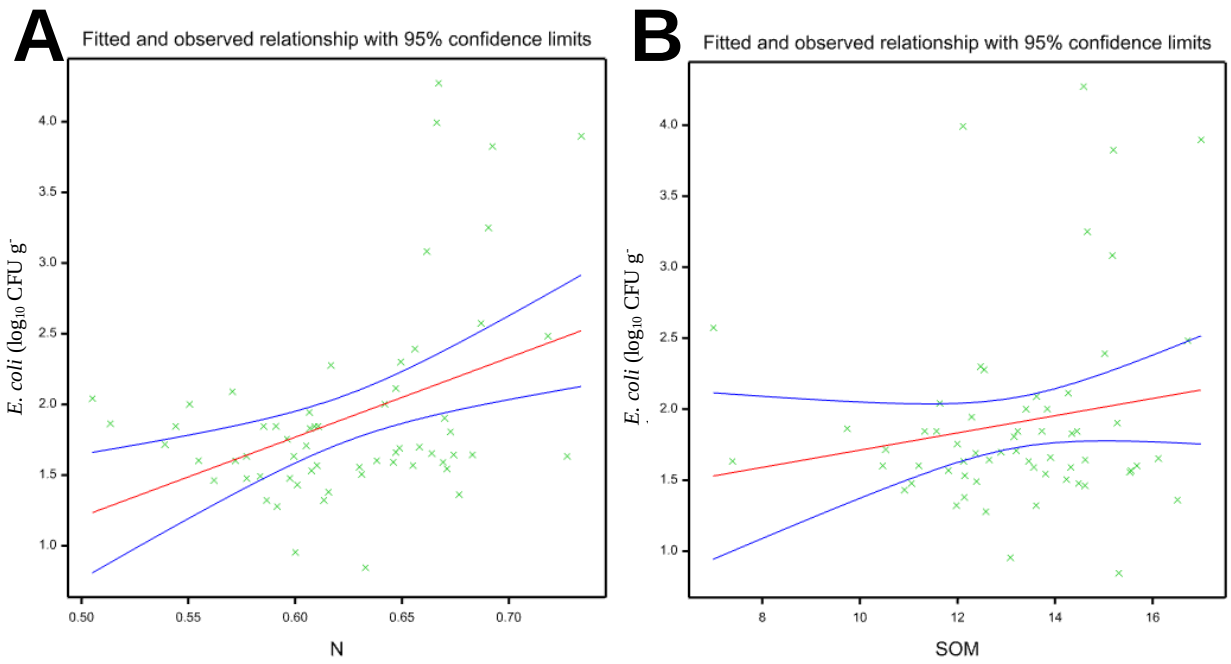

Figure 6. Soil field survey faecal indicator organism concentration (log_{10} CFU g^{-1}) correlation to total Nitrogen (mg N g^{-1}) and soil organic matter (% DM).
A) E. coli log_{10} CFU ml^{-1} correlation to total Nitrogen mg N g^{-1}. B) E. coli log_{10} CFU ml^{-1} correlation to soil organic matter % DM.

Table 5. Soil field survey faecal indicator organism concentration (log_{10} CFU g^{-1}) correlation to total Nitrogen (mg N g^{-1}) and soil organic matter (% DM).

Correlation with manual	*E. coli* r^2	*E. coli* P value
Total Nitrogen	26.2 s.e. 0.59	0.043
Soil Organic Matter	0.90 s.e. 0.690	0.451

Linear mixed model of soil field survey FIO concentration correlation to total Nitrogen and soil organic matter. Coefficient of determination indicated by r^2, significance accepted if $P \leq 0.05$.

3.3.2. Hotspot sampling

3.3.2.1. Faecal indicator organisms

The spatial variation in concentrations of *E. coli* and intestinal enterococci was analysed across all strata. The CFU for intestinal enterococci and *E. coli* were not found to be significantly different from grazed or ungrazed field use samples at any strata. Summary statistics for FIO concentration by grazed and ungrazed field use are presented in Table 6. The CFU for intestinal enterococci and *E. coli* from 'Trough' and 'Station' samples was increased relative to 'non-hotspot' and 'Flume' class samples. The CFU for intestinal enterococci from <10 m samples was increased relative to all distances proximal to defined hotspots. Sample point 'Distance' was also increased for *E. coli* CFU from <10 m samples relative to all distances proximal to defined hotspots. The <20 m distance was also increased for *E. coli* relative 'non-hotspot' samples. Summary statistics for FIO concentrations are presented in Table 7.

Table 6. Variations in faecal indicator organism concentration (log_{10} $CFUg^{-1}$) of soil in-field by sample field use.

Strata	Mean *E. coli* Grazed	Mean *E. coli* Ungrazed	P value	Mean intestinal enterococci Grazed	Mean intestinal enterococci Ungrazed	P value
Field use	1.66	1.61	0.446	1.81	1.87	0.166
sd.	0.727	0.794		0.297	0.224	
Class						
Non-HSP	1.11	1.07	0.551	1.66	1.73	0.403
sd.	0.300	0.357		0.264	0.251	
Flume	1.16	1.19	0.883	1.66	1.70	0.642
sd.	0.109	0.189		0.289	0.270	
Station	2.07	1.82	0.202	2.00	1.93	0.197
sd.	0.837	0.745		0.320	0.296	
Trough	2.31	2.24	0.245	2.11	2.00	0.385
sd.	0.796	0.838		0.353	0.378	
Distance						
Non-HSP	1.11	1.07	0.551	1.66	1.73	0.403
sd.	0.300	0.357		0.264	0.251	
<10m	2.63	2.49	0.196	2.25	2.20	0.302
sd.	1.874	1.902		0.272	0.305	
<20m	1.66	1.58	0.341	1.73	1.68	0.335
sd.	0.461	0.427		0.309	0.276	
<30m	1.14	1.20	0.728	1.74	1.80	0.430
sd.	0.255	0.296		0.165	0.144	

Mean values of soil in-field E. coli *and intestinal enterococci concentration of grazed and ungrazed field use. Blocked ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters within column.*

Table 7. Variations in faecal indicator organism concentration (log_{10} $CFUg^{-1}$) of soil in-field by sample class and distance.

Strata	Mean *E. coli*	sd. *E. coli*	Mean intestinal enterococci	sd. intestinal enterococci
Catchment	1.63	0.849	1.85	0.374
Class				
Non-HSP	1.08[a]	0.382	1.69[a]	0.279
Flume	1.17[a]	0.278	1.67[a]	0.303
Station	1.91[b]	0.901	1.97[b]	0.376
Trough	2.26[b]	0.919	2.02[b]	0.392
P value	0.001		0.001	
Distance				
Non-HSP	1.08[a]	0.382	1.69[a]	0.279
<10m	2.55[c]	1.001	2.21[b]	0.384
<20m	1.61[b]	0.507	1.70[a]	0.339
<30m	1.18[ab]	0.316	1.76[a]	0.186
P value	0.001		0.001	

Mean values of soil in-field E. coli *and intestinal enterococci concentration. Blocked ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters within column.*

3.3.2.2. Antimicrobial resistance

No significant differences in percentage AMR isolates from grazed or ungrazed field use samples were found at any strata. Summary statistics for percentage resistant isolates by grazed and ungrazed field use are presented in Table 8. Differences in percentage AMR isolates across sample 'class' were found. The trough samples presented the highest percentage of resistant isolates for amoxicillin/clavulanate relative to non-hotspot sample 'classes', and for cephalexin and erythromycin relative to non-hotspot and flume sample 'classes'. The highest percentage of resistant isolates for cephalexin and neomycin by 'class' were also found in station samples relative to non-hotspot and flume sample 'classes'. The <10 m 'distance' proximal to hotspots presented the highest percentage of resistant isolates for amoxicillin/clavulanate and tetracycline relative to all other 'distances', and for cephalexine, erythromycin and neomycin relative to the

non-hotspot and <30 m proximal to hotspot ‘distances’. The highest percentage of resistant isolates for cephalexine and erythromycin by ‘distance’ were also found in samples <20 m proximal to hotspots relative to the non-hotspot and <30 m sample ‘distances’. The highest percentage of resistant isolates for neomycin was also found in samples <20 m proximal to hotspots relative to <30 m sample ‘distances’. Zero isolates were identified as exhibiting multiple drug resistance (defined as resistance to 4 or more of the drug panel). Summary statistics for percentage resistant isolates are presented in Table 9.

Table 8. Variations in percentage antimicrobial resistant isolates of soil in-field by sample field use.

	% Resistant Isolates																
Drug type / Strata	AMC Grazed	AMC Ungrazed	P value	CPD Grazed	CPD Ungrazed	P value	CFX Grazed	CFX Ungrazed	P value	ERY Grazed	ERY Ungrazed	P value	NEO Grazed	NEO Ungrazed	P value	TET Grazed	TET Ungrazed	P value
Field use	17.5	19.1	0.667	3.90	4.07	0.891	19.7	17.9	0.754	64.8	68.6	0.756	15.3	12.42	0.774	15.9	12.7	0.651
sd.	19.56	20.66		7.82	6.46		20.07	19.14		22.76	25.11		17.53	18.46		17.82	14.7	
Class																		
Non-HSP	8.10	11.7	0.745	2.02	3.4	0.877	10.6	5.28	0.665	54.1	61.2	0.871	9.03	3.16	0.807	6.43	8.70	0.871
sd.	10.171	12.36		6.441	6.98		13.43	8.099		22.67	24.51		10.741	8.620		9.257	10.903	
Flume	11.6	13.2	0.456	4.12	5.62	0.870	11.2	6.13	0.779	51.2	63.6	0.560	7.92	4.58	0.674	9.77	7.02	0.846
sd.	14.72	12.33		8.799	10.10		12.80	10.771		19.44	20.07		9.883	7.632		11.338	8.409	
Station	25.5	23.7	0.402	4.99	4.67	0.970	26.4	29.6	0.450	75.4	75.0	0.991	21.4	25.7	0.732	24.3	17.1	0.632
sd.	20.40	19.73		10.46	8.655		19.86	22.49		18.63	15.48		22.86	24.39		20.61	8.96	
Trough	26.1	25.3	0.578	4.25	3.14	0.345	34.5	27.6	0.401	78.7	75.9	0.834	22.3	17.6	0.688	23.4	18.2	0.822
sd.	24.87	22.64		9.621	8.192		24.00	18.34		21.36	17.77		18.64	12.07		21.84	19.20	
Distance																		
Non-HSP	8.10	11.7	0.745	2.02	3.4	0.877	10.6	5.28	0.665	54.1	61.2	0.871	9.03	3.16	0.807	6.43	8.70	0.871
sd.	10.171	12.36		6.441	6.98		13.43	8.099		22.67	24.51		10.741	8.620		9.257	10.903	
<10m	37.6	29.4	0.107	6.37	8.18	0.748	34.7	25.2	0.327	79.4	75.3	0.760	23.3	26.5	0.598	31.7	27.2	0.740
sd.	24.16	14.52		11.340	13.491		24.81	17.52		22.06	14.75		19.24	23.06		23.92	16.04	
<20m	16.8	19.9	0.391	5.22	4.37	0.816	25.9	27.8	0.439	73.7	78.5	0.643	21.6	16.1	0.603	12.2	6.46	0.435
sd.	17.42	18.60		10.691	9.252		20.03	22.79		13.51	18.24		19.14	12.32		15.83	9.77	
<30m	8.55	14.8	0.112	1.44	1.01	0.932	8.64	12.9	0.812	54.8	57.9	0.681	6.16	3.65	0.769	12.2	9.70	0.701
sd.	14.12	15.06		5.023	4.978		11.045	14.23		19.73	19.84		10.744	6.710		14.91	7.64	

Mean values of soil in-field percentage antimicrobial resistant isolates of grazed and ungrazed field use. Blocked ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters within column. Only antimicrobials which indicated resistant isolates are presented: DOR (doripenem), EFX (enrofloxacin), FFC (florfenicol) and SXZ (sulphisoxazole) were effective against all isolates tested and no resistance was found. Drug type abbreviations: AMC (amoxicillin/clavulanate), CPD (cefpodoxime), CFX (cephalexin), ERY (erythromycin), NEO (neomycin), TET (tetracycline).

Table 9. Variations in percentage antimicrobial resistant isolates of soil in-field by sample class and distance.

	% Resistant Isolates					
Drug type	AMC	CPD	CFX	ERY	NEO	TET
Strata						
Catchment	18.2	4.01	18.8	67.0	13.9	14.5
sd.	21.56	10.900	22.91	26.77	21.44	21.02
Class						
Non-HSP	9.99[a]	2.70	7.89[a]	57.4[a]	6.05[a]	7.55
sd.	15.511	8.896	14.122	31.78	13.199	14.12
Flume	12.3[ab]	4.86	8.48[a]	57.8[a]	6.21[a]	8.59
sd.	16.40	12.076	14.890	21.86	13.190	14.89
Station	24.6[ab]	4.86	28.2[b]	75.1[ab]	23.5[b]	20.9
sd.	23.74	12.076	23.94	23.74	27.45	22.92
Trough	25.8[b]	3.62	30.7[b]	77.5[b]	19.7[ab]	20.9
sd.	25.04	10.688	26.03	22.65	23.13	26.39
P value	0.007	0.863	0.001	0.003	0.002	0.160
Distance						
Non-HSP	9.99[a]	2.70	7.89[a]	57.4[a]	6.05[ab]	7.55[a]
sd.	15.511	8.896	14.122	31.78	13.199	14.12
<10m	33.3[b]	7.32	29.4[b]	77.5[b]	24.7[c]	29.5[b]
sd.	26.15	14.122	25.04	24.46	27.10	26.69
<20m	18.4[a]	4.86	27.0[b]	76.3[b]	19.7[bc]	9.83[a]
sd.	19.25	12.076	24.52	20.29	23.13	18.06
<30m	11.0[a]	1.15	10.9[a]	56.5[a]	4.98[a]	11.0[a]
sd.	16.01	6.415	18.49	22.29	12.07	16.01
P value	0.001	0.174	0.001	0.001	0.001	0.001

Mean values of soil in-field percentage antimicrobial resistant isolates by sample class and distance. Blocked ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters within column. Only antimicrobials which indicated resistant isolates are presented: DOR (doripenem), EFX (enrofloxacin), FFC (florfenicol) and SXZ (sulphisoxazole) were effective against all isolates tested and no resistance was found. Drug type abbreviations: AMC (amoxicillin/clavulanate), CPD (cefpodoxime), CFX (cephalexin), ERY (erythromycin), NEO (neomycin), TET (tetracycline).

3.3.2.3. DNA analysis

3.3.2.3.1. Sequencing based analysis of microbe populations in soil samples

Final sample concentrations in ng/µl are presented in Table 10. This determined subsequent volumes for pooling of validated libraries. Analysis was conducted across all sequences with quality checked. Producing sequences for diversity analysis (Table 11), totalling 4234709. Randomised subsampling at 41166 cut-off for each experimental grouping was performed.

Table 10. Recorded sample concentration (ng/µl) and quality checked sequences of individual soil samples final validated libraries.

Sample name	Concentration (ng/µl)	Chimera filtered clean sequences
S1	150	93323
S2	586	139051
S3	270	78736
S4	422	74823
S5	390	251324
S6	402	147276
S7	432	226646
S8	530	129660
S9	390	168091
S10	506	122053
S11	498	67779
S12	1060	107982
S13	556	120472
S14	398	158913
S15	696	82652
S16	392	82612
S17	350	267857
S18	954	168830
S19	682	245328
S20	754	163068
S21	806	190800
S22	748	145926
H1	520	85438
H2	686	140897
H3	634	104164
H4	400	148221
H5	348	62762
H6	336	69123
H7	344	241523
H8	146	149379
H9	145	218341

Sample DNA library concentration. Groups are indicated by letters corresponding to S – Random field soil sample and H – Hotspot associated soil sample.

3.3.2.3.2. Analysis of microbial richness and diversity

The quality checked and subsampled sequences were clustered, representing operational taxonomic units (OTUs) of 3% nucleotide dissimilarity. Rarefaction curves were completed on randomised subsample of 41166 sequences to calculate OTU numbers in each sample (Figure 7). The results indicate no significant difference in microbial richness between random and hotspot associated soil sample groups. Rarefaction curves appear to approach saturation plateau, indicating sequencing was sufficiently in-depth to accurately estimate microbe population composition.

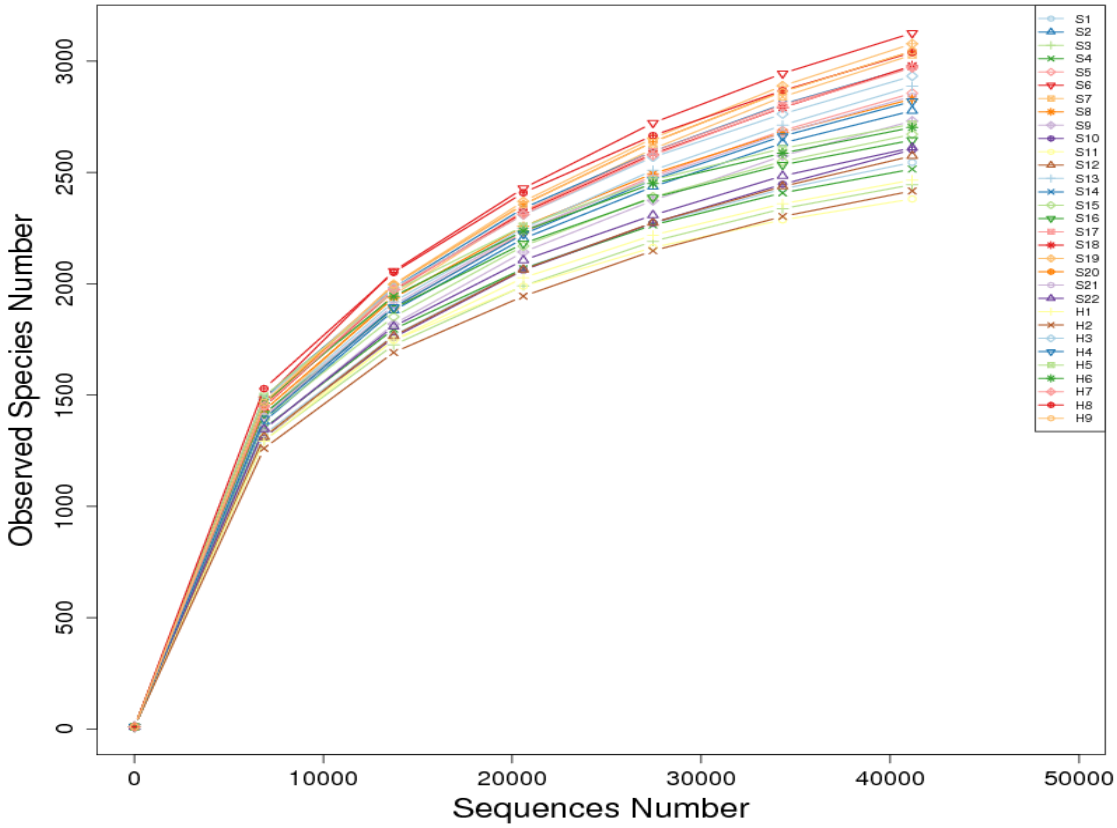

Figure 7. Rarefaction curves based on individual soil samples subsampling of 41166 sequences clustered as operational taxonomic units.
Sample subsampled 41166 *sequences clustered as operational taxonomic units (OTUs). OTUs were defined at 0.03 distances. Groups are indicated by letters corresponding to S – Random field soil sample and H – Hotspot associated soil sample.*

Soil sample groups microbial diversity as was analysed by Shannon diversity index, combining richness and evenness, indicated differences between groups (Figure 8). Significant increase in species diversity was found in random soil sample group relative to hotspot associated sample group (MRRP < 0.01).

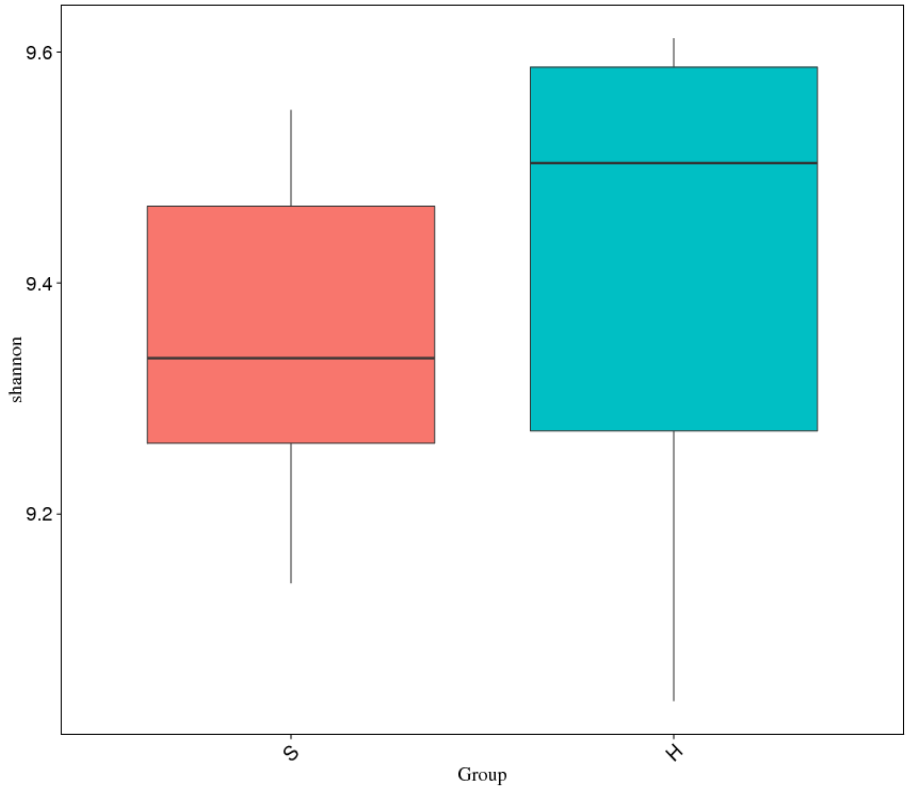

Figure 8. Microbial diversity as determined by Shannon diversity index across soil sample group.
Significant differences between random and hotspot soil sample group was found in Shannon diversity index analyses (MRRP P < 0.01). Groups are indicated by letters corresponding to S – Random field soil sample and H – Hotspot associated soil sample. Box plots represent the mean and standard deviation for Shannon Diversity Index.

3.3.2.3.3. Visualisation of microbial sample similarities

Non-Multidimensional Scaling (NMDS) plot indicated closer clustering of microbial populations in random soil samples relative to the hotspot associated sample group. Variability

between groups also indicated differences random samples and majority of hotspot associated samples. The significance of clustering was computed using ADONIS (PERMANOVA) ($p < 0.01$). Paired analysis indicated statistical differences between microbial populations of groups (Figure 9).

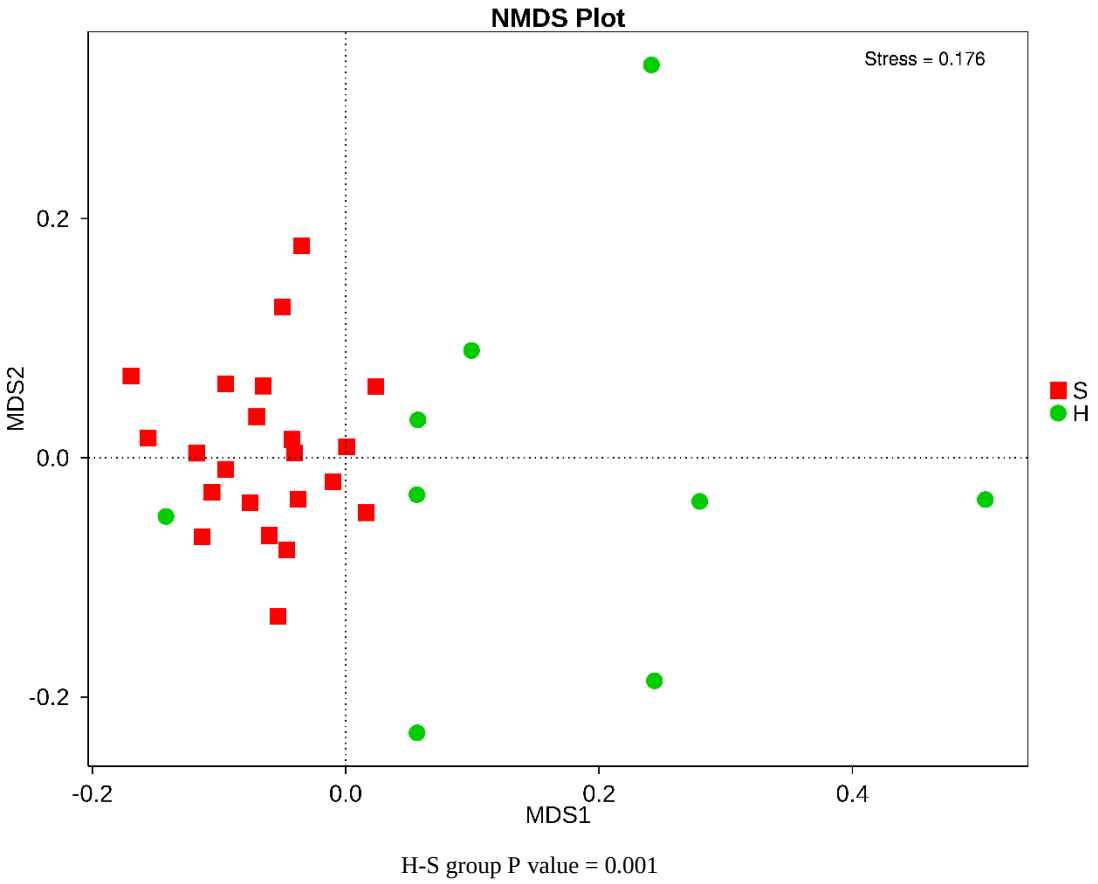

Figure 9. Visualisation of microbial population similarity of soil sample groups by Non-Multidimensional Scaling plot.
Significant differences ($p < 0.01$) between microbial populations of random and hotspot associated soil sample groups was found in paired analysis by ADONIS. Groups are indicated by letters corresponding to S – Random field soil sample and H – Hotspot associated soil sample.

3.3.2.3.4. Analysis of groups microbial composition

The microbe population composition of each soil group was analysed to genus taxonomic level. Percentage relative abundance of the top 10 phyla indicate *Proteobacteria*, *Acidobacteria* and *Actinobacteria* predominate across both sample groups (Figure 10). *Proteobacteria* was the

most abundant (percentage average across samples) both groups. The differences in soil sample microbial composition by the top 35 genera (>0.01 % relative abundance) indicate particular dominance within three most abundant phyla by *Defluviicoccus*, *RB41* and *Pseudonocardia* respectively (Figure 11).

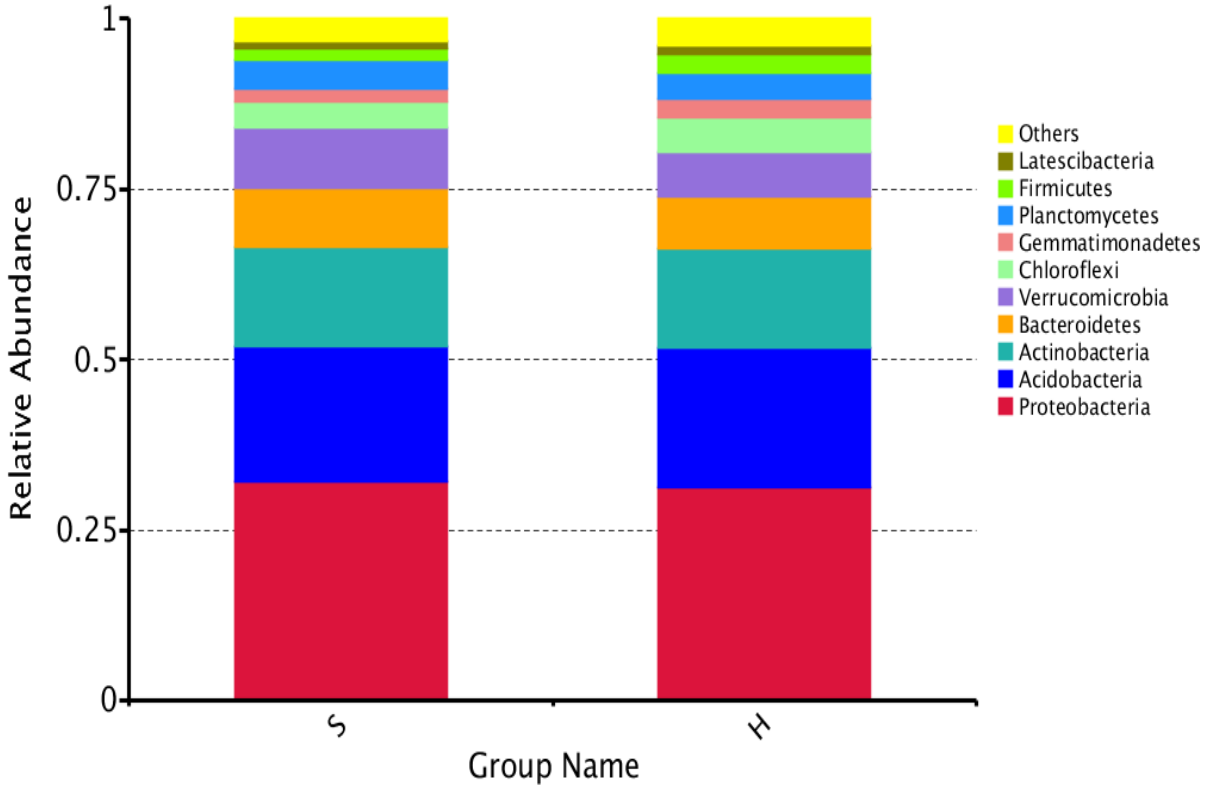

Figure 10. Microbial population composition, phyla percentage relative abundance, of soil group by stacked bar graph.
Bar graph of normalised sequence data for percentage relative abundance by phyla. Groups are indicated by letters corresponding to S – Random field soil sample and H – Hotspot associated soil sample

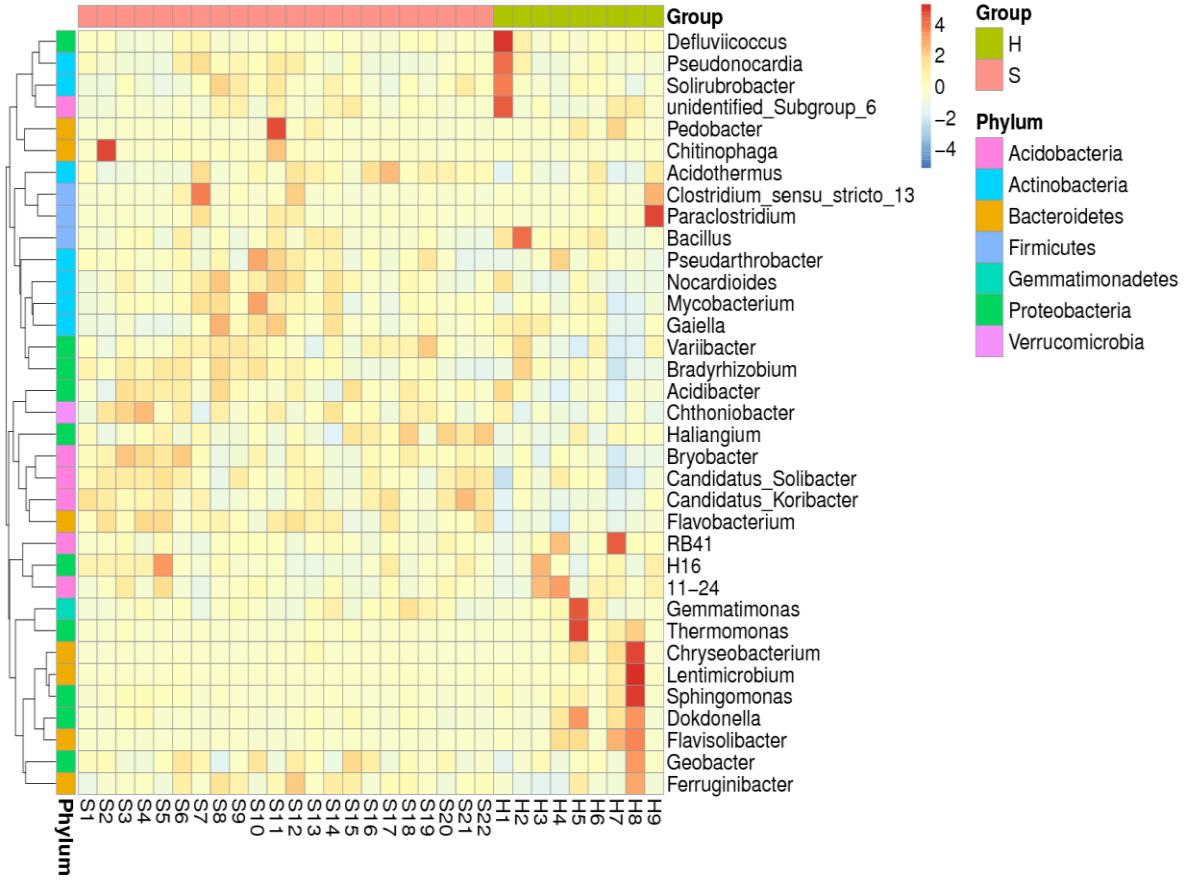

Figure 11. Microbial population composition, genera percentage relative abundance, of individual soil samples by heatmap.
Heatmap presenting the percentage relative abundances of bacterial genera across individual samples. Groups are indicated by letters corresponding to S – Random field soil sample and H – Hotspot associated soil sample.

3.3.2.3.5. Differences in groups microbial composition

The microbe population composition of random and hotspot soil sampling groups was analysed across taxons from phylum to genus level. Significant differences in sample variation are presented alongside the unweighted pair group method with arithmetic mean (UPGMA) cluster tree based on weighted unifrac distance of samples. The tree presents overall sample microbe population relatedness (Figure 12). Statistically significant differences in microbe proportion ($p < 0.01$) between sample groups are indicated in Table 11 alongside percentage relative abundance.

Significant differences between soil group microbe composition were found in phylum and class taxon levels (Table 11).

Sample groups indicated clustering based on weighted unifrac distance for phylum relative abundance apart from hotspot samples 3, 6 and 9. These samples represent the flume hotspot soil samples and the UPGMA clustering with the random soil samples is consistent with the FIO culture based analysis. The clustering of trough and station hotspot soil samples is also consistent with the NMDS results.

Following multiple Wilcoxon signed rank tests with false discovery rate correction comparing median microbe abundances across groups was completed several differences in microbial population were indicated (Table 11). The phylum *Verrucomicrobia* and *Elusimicrobia* were found to be increased in random relative to the hotspot soil sample group. The class *Thermomicrobia* was found to be increased in hotspot relative to the random soil sample group. The phylum *Armatimonadetes* and *Synergistetes* were also found to be significantly increased in the hotspot relative to the random soil sample group. The phylum *Synergistetes* was not found to be present in any of the random soil samples.

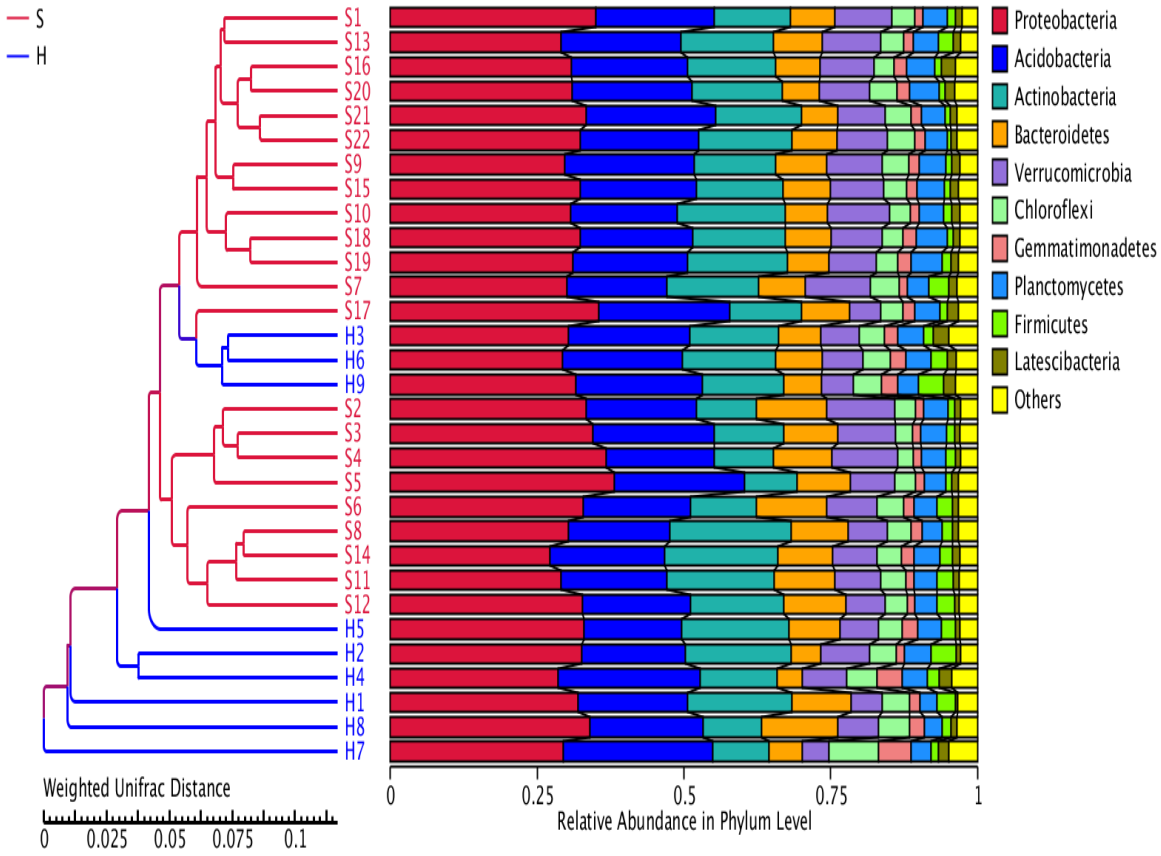

Figure 12. UPGMA cluster tree by weighted unifrac distance for phylum relative abundance. *Soil samples phylum relative abundance and individual sample relatedness by unweighted pair group method with arithmetic mean (UPGMA) hierarchical clustering. Groups are indicated by letters corresponding to S – Random field soil sample and H – Hotspot associated soil sample.*

Table 11. Significant differences between median microbe composition that were not present in comparisons between more than two individual groups.

Microbe name	Taxonomic rank	Random sample group % abundance	Hotspot sample group % abundance	P value
Verrucomicrobia	Phylum	0.0876	0.0682	0.001
Elusimicrobia	Phylum	0.0064	0.0037	0.001
Thermomicrobia	Class	0.0016	0.0033	0.006
Armatimonadetes	Phylum	0.0014	0.0028	0.008
Synergistetes	Phylum	0.00	0.0001	0.001

Significant differences in median microbe percentage abundance of soil group indicated between random and hotspot samples. Taxonomic level and corresponding name is presented alongside group of increased and decreased proportion.

3.4. Discussion

The study assessed potential microbial hazard at a field scale, with results informing targeted sampling of specific spatial hotspots common across management fields and NWFP catchments. These results highlighted the increased microbial risk in soil from potentially pathogenic and percentage AMR isolates proximal to troughs and in-field objects (weather stations). The disproportionate impact of these features at a field scale could be managed by adjustments to simple management techniques, such achieving a consistent grazing pattern by application of true rotational grazing to smaller multiple paddocks and minimizing the concentration of livestock time spent in proximity to specific in-field features by altering water sources.

3.4.1. Field scale variation in soil microbial risk

The preliminary study in Chapter 2 indicated the potential of the soil matrix to capture and maintain significant portions of microbial populations applied to soil through livestock faeces (Calloway et al, 2010, White et al, 2001, Lawlor et al, 2000). This capacity to bind or capture microbes within the soil environment has impacts beyond the impact of individual animal behavior to field and catchment scale implications (Sayah et al, 2005, Hooda et al, 2000). Investigating the persistence of microbes (through sampling on annually alternatively grazed or harvested fields) and the spatial variation resulting from the accumulation at a field scale progresses the initial findings in Chapter 2. This study also contextualizes the subsequent work presented in Chapter 5 investigating the variation in microbial risk at a water catchment scale (Sayah et al, 2005, Hooda et al, 2000). This study aimed to present a comprehensive field scale soil survey that would identify regions of greatest livestock origin microbial accumulation. Presenting a direct link between animal behavior (Betteridge et al, 2010, Oudshoorn et al, 2008, White et al, 2001) by way of faecal

deposition rate to increases in potentially pathogenic and AMR isolates in soils can inform more effective grazing strategies and improve health.

The variation of faecal deposition by livestock, particularly within extensively managed grassland based production systems is of critical economic importance due to its principal role in nutrient cycling, pasture growth and ultimately system productivity (Takahashi et al, 2018, Orr et al, 2016). The direct return of nutrients to pasture is a prerequisite for a functioning management system (Bilotta et al, 2007, Clark et al, 1998). Previous studies have highlighted the importance of faeces and urination in returning Nitrogen to the soil and crop (Mitchell, 2018). The role of soil organic matter as a component of Carbon cycling has been found to be influenced by grazing livestock behavior (Bouman et al, 2018). Soil organic matter content has also been previously linked to animal growth rate and so is itself of economic importance (Takahashi et al, 2018). The influence of animal behavior upon these factors has been suggested and their variability at a field scale investigated. However, correlating differences in Nitrogen and SOM at a field scale as resulting primarily from the influence of grazing livestock is challenging.

This sampling was to inform subsequent high resolution soil studies of the extent of these zones influence within fields. As this relied upon analysing variations in FIO concentrations resulting from livestock defecation there existed an opportunity to investigate any direct correlations between livestock activity and specific soil chemistry parameters. Indicating a strong correlation could enable predictions for soil Nitrogen or organic matter content derived from *E. coli* CFU and vice versa. This could inform stakeholder management strategies for improving pasture productivity with resulting mutual benefits to animal and public health (Craft, 2015, Woolhouse et al, 2015).

The field scale soil survey implemented was designed to be comparable with the previous grid soil sampling conducted on the NWFP (Franklin & Mills, 2003). This aimed to produce results with temporal as well as spatial relevance. The concurrent sampling of FIO concentration by *E. coli* $\log_{10}$ CFU g^{-1}, total Nitrogen mg N g^{-1} and SOM % DM was also completed to enable analysis of correlation between parameters. The correlation between microbe concentration and soil chemistry parameters indicated no association between SOM. A weak correlation between FIO concentration and total Nitrogen was suggested and this should be confirmed by larger scale subsequent studies. No FIO concentration and total Nitrogen correlation was observed in field scale predictions. While the previously described relationships between animal behavior, Nitrogen and SOM undoubtedly exist (Bouman et al, 2018, Mitchell, 2018) their influence at a field scale is less conclusive. In regions of particularly high livestock activity and faecal deposition a corresponding effect upon increased Nitrogen (Mitchell, 2018) or decreased SOM (Bouman et al, 2018) may be observable. Field scale sampling suggests that topography and soil type have greater influence on the studied soil chemistry parameters in areas of lower intensity livestock activity (Van Der Heijden et al, 2007, Crowther et al, 2003).

The field scale survey did support the hypothesis that microbial diversity and FIO are increased in specific hotspots proximal to areas of high intensity livestock activity. This is supported by previous findings both in related extensive grazing and more intensive production systems (Betteridge et al, 2010, Oudshoorn et al, 2008, White et al, 2001). The cumulative temporal impact of these hotspots is demonstrated across fields which at the time of soil sampling had not been grazed that production season. This supports the findings in Chapter 2, and demonstrates the significant role of these zones in retaining microbe populations across grazing seasons. While the hotspots identified were principally the result of increased livestock defaecation rates in those areas

the impact of hydrological transfer of microbes through soil remains a crucial factor (Sayah et al, 2005, Hooda et al, 2000).

This field scale study suggests that similarly to potential microbial hazard, Nitrogen and SOM content in soil are disproportionately influenced at specific hotspots whose impacts could be managed by relatively simple stakeholder interventions. Achieving a consistent grazing pattern by application of true rotational grazing and minimizing the concentration of livestock time spent in proximity to specific in-field features by altering water sources would minimize this.

3.4.2. Increased microbial risk of soil hotspots

3.4.2.1. Faecal indicator organisms

The deposition of livestock faeces on pasture during grazing is a system necessity, particularly in extensively managed production (Bilotta et al, 2007, Clark et al, 1998). The potential impact on animal and human health is an unavoidable result of this process. However, the negative impact of specific hotspots in fields upon disproportionately increased microbial risk by accumulating potentially pathogenic and AMR isolates at high concentrations can be managed. Developing effective mitigation strategies for these soil hotspots requires an understanding of their spatial influence relative to the rest of the grazed land. Utilising rotational and strip grazing to optimize livestock time spent will reduce the development of hotspots occurring through minimising non-grazing time spent. Similarly the application of varying water sources minimizes the development of hotspots by reducing the burden on single sites within grazing fields.

The higher resolution study of defined soil microbial hotspots found evidence for accumulation at these points across grazing seasons with no differences found in FIO concentration between

samples from grazed or ungrazed field use. This is crucial in demonstrating the critical importance of hotspots as sources of microbial hazard whose influence on animal health at a field scale does not readily dissipate. This reinforces the need to address the accumulation at these points through active grazing management strategies as merely removing direct contact with livestock in alternative production seasons is not sufficient. Similarities in the variation of *E. coli* and intestinal enterococci concentration across sample 'class'. Both species concentrations were increased in trough and station relative to flume and non-hotspot soil samples. This correlation between both FIO species clearly demonstrates that these increases result from faecal application and not factors such as preferential growth conditions. *E. coli* and intestinal enterococci CFU were also found to be increased at <10 m proximal to defined hotspots relative to all other hotspot and non-hotspot distances. Increased *E. coli* concentration was also found at <20 m proximal to hotspots relative to non-hotspots.

These results support previous findings which indicate water troughs as subject to increased faecal deposition rates. The result that in-field objects, in this study weather stations, generate sufficient interest to cause similar animal activity as water troughs is not as well characterised (Betteridge et al, 2010, Oudshoorn et al, 2008, White et al, 2001). Previous studies indicated areas within fields as preferred rumination or 'encampment' locations but not suggesting similar faecal deposition patterns (Orr et al, 2012). Interestingly the flume hotspot class, which was included given the hypothesized impact both of surface and subsurface hydrological transfer on the soil FIO concentration, was found to be comparable to non-hotspot samples (Sayah et al, 2005, Hooda et al, 2000). This finding indicates that while microbe risk and FIO concentration may periodically increase in this location associated with water transport (Sayah et al, 2005, Hooda et al, 2000) a process of accumulation within the soil may not occur (Oliver et al, 2005, Hodgson et al, 2009).

This may suggest that soil microbial hotspots driven by livestock defecation are not only accumulators of potentially pathogenic and AMR bacteria (White et al, 2001) but under hydrological action can act as disproportionate sources (Sayah et al, 2005).

The limitations in the extent of the spatial influence of hotspots suggest that management interventions could be effective given the relatively small area of influence. The distribution of faeces has been well characterised in other management systems and similar strategies particularly relating to multiple and variable water source provision could be employed to benefit reductions in microbe risk burden at single points. Reducing the burden of these concentrated hotspots could have both on-farm and wider external benefits to health. The influence of these defined soil hotspots upon grass growing at pasture and subsequent preserved forage crops will be examined fully in Chapter 4. The external transmission risk of increased in-field FIO concentration will be investigated in flume water outflow in Chapter 5.

3.4.2.2. Antimicrobial resistance

The pattern of percentage antimicrobial resistant isolates did not vary between samples from grazed or ungrazed field use. This finding is particularly important in demonstrating the long term influence of livestock derived resistance patterns at point and field scale, similar to sustained concentration of FIO. The removal of direct contact with livestock and their faecal depositions from field in alternative production seasons does not result in a reduced prevalence of AMR bacteria. Actively reducing livestock time spent at these zones while grazing must be prioritized though true rotational or strip grazing strategies and reducing the burden on single water troughs through supply of multiple and variable sources. Variation in percentage antimicrobial resistant isolates was found across sample ‘class’ and ‘distance’ for various drugs. Cephalexin,

erythromycin and neomycin percentage resistant isolates all presented increases in trough or station samples relative to non-hotspot and flume 'classes'. Amoxicillin/clavulanate resistant isolates were increased in trough relative to non-hotspot samples. The <10 m or <20 m 'distance' proximal to hotspots presented the highest percentage of resistant isolates for cephalexin, erythromycin and neomycin relative to the non-hotspot and <30 m proximal to hotspot 'distances'. Amoxicillin/clavulanate and tetracycline percentage resistant isolates indicated increases in <10 m 'distance' relative to all other sample distances. This suggests that the higher rate in livestock faecal deposition proximal to defined hotspots supplies increased concentration of AMR isolates and in turn this intensive accumulation within soil results in greater proliferation of resistance throughout the regional microbe population (Betteridge et al, 2010, Oudshoorn et al, 2008, White et al, 2001). The influence of these defined soil hotspots upon AMR presence on grass growing at pasture and subsequent preserved forage crops will be examined fully in Chapter 4. The increased prevalence of resistance to several antimicrobial drug types associated with hotspot type and distance correlates with the pattern found in FIO concentrations. It is therefore supposed that the increase in livestock defecation in these specified regions and their maintenance within the soil facilitates increased transfer of AMR at microbial population scale (Garbisu et al, 2018). No similar relationship was observed as the hypothesized hydrologically driven flume soil hotspot and the effect of livestock faeces on increased AMR is either transient or does not persist in soil to the same intensity following surface and subsurface water transport (Hodgson et al, 2009, Sayah et al, 2005). The external transmission risk of increased in AMR isolates will be investigated in flume water outflow in Chapter 5.

The identified spatial association between increased FIO (livestock defeacation intensity) and increased prevalence of AMR isolates would generate mutual benefits from intervention strategies.

Reducing the intensive accumulation of potentially pathogenic and AMR bacteria proximal to identified hotspots by improving the grazing management of fields would generate improved economic output and health status. This is particularly true given the likely oral transmission route of livestock grazing at pasture (Duggan et al, 2000), studied in Chapter 4.

3.4.2.3. DNA sequencing

The additional analysis of next generation sequencing to the primarily culture based high resolution soil hotspot testing followed similar application in Chapter 2. This approach enabled identification of differences in microbial composition of soil from hotspot or non-hotspot associated samples. The microbial composition of all sample types was consistent with previous field and laboratory scale studies. The most abundant phyla were present across hotspot and non-hotspot sample groups with *Proteobacteria*, *Acidobacteria* and *Actinobacteria* predominating (Figure 10). The most abundant genera corresponding to these phyla were found to be *Defluviicoccus*, *RB41* and *Pseudonocardia* respectively (Figure 11).

Analysis of microbial alpha diversity of all samples in each soil grouping by Shannon diversity index using MRPP ($P < 0.01$) indicated increases in hotspot associated samples (Figure 8). This supports previous knowledge indicating the increased microbial variation introduced to soil following application of faeces (Calloway et al, 2010, Lawlor et al, 2000) and shows comparable results to those found in Chapter 2 preliminary study. Initial differences between groups soil microbial composition indicated by alpha diversity analysis were visualised by NMDS plots (Figure 9). These indicated high intra group variation of hotspot associated soil samples. Increased degree of group clustering was observed in the non-hotspot random soil samples as assessed by ADONIS (PERMANOVA) ($P < 0.01$). Several hotspot associated samples appeared to cluster

more closely with this group. This may indicate greater stability in the microbial population structure of the wider field soils (Schmid et al, 2018) compared to the significant differences generated by intensive application of faeces at hotspots (Calloway et al, 2010, Lawlor, 2000). Individual sample variation was further investigated by unweighted pair group method with arithmetic mean (UPGMA) and presented as a cluster tree based on weighted unifrac distance of samples (Figure 12). These results highlight flume hotspot samples closer relation to non-hotspot soil microbe composition. The relatedness of trough and station associated hotspot soil samples microbe populations are also presented.

The assessment by multiple Wilcoxon signed rank tests with false discovery rate correction comparing median microbe abundances across groups found several differences in microbial population compositions between groups (Table 11). The phylum *Verrucomicrobia* and *Elusimicrobia* were found to be increased in random relative to the hotspot soil sample group. Interestingly both of these microbe species are faecal associated (Lourenco et al, 2018, Waite et al, 2018) and may indicate the long-term survival of dominant livestock origin microbes within the soil matrix. The increased prevalence in non-hotspots may conceivably result from managed application of faecal material as fertiliser to fields and through deposition while grazing. Due to positioning within fields and practicalities of application, hotspot locations are likely to receive reduced concentration of faecal material via managed application. *Verrucomicrobia* has previously been noted as a dominant constituent of soil microbial communities and it's prevelance in more stable soil locations beyond hotspots influence supports this (Semenov et al, 2018). The class *Thermomicrobia* was found to be increased in hotspot relative to the random soil sample group. This environmental species has been noted for its associated increased growth potential in the presence of specific antimicrobials (Houghton et al, 2015). The increased variation in microbial

composition and the increased prevalence of AMR isolates proximal to hotspots may support the proliferation of this species. The phylum *Armatimonadetes* and *Synergistetes* were also found to be significantly increased in the hotspot relative to the random soil sample group. *Armatimonadetes* species, while relatively poorly characterised are known to possess low nutrient growth potential (Li et al, 2018). The greater variation in soil microbe population proximal to hotspots may enable increased prevalence of species with environmental niches such as *Armatimonadetes*. The phylum *Synergistetes* was only found to be present in hotspot associated soil samples. This species has been previously characterised as a gastrointestinal associated anerobe (Ouyang et al, 2018) and its increased prevalence in hotspot soil samples is considered a function of increased defaecation rates. *Synergistetes* have been suggested as opportunistic pathogens due to their identification at wound sites (Raghupathi et al, 2017). This demonstrates specific pathogenic risks posed by this studies identified soil hotspot locations however this conclusion is limited by identification only to phylum level.

3.5. Conclusions

As previously mentioned grassland grazing livestock systems are a significant source of human food and are of crucial economic importance. The land use required by grazing livestock production results in large portions of utilisable agricultural area being under this management system. The importance of optimising human and animal health through interventions targeting soil should not be underestimated. This study suggests that specific intervention strategies can be applied to minimise the generation on high intensity feacal deposition locations within fields. Experiment 1 demonstrated the disproportionate increase in soil FIO concentrations proximal to specific in-field features, such as water troughs and weather stations, relative to surrounding in-field soil. This informed the design of Experiment 2, where higher resolution testing of defined

hotspots indicated their spatial influence in soil extended to between 10 and 20 m. These hotspots were found to possess increased microbial risk, with higher prevalence of potentially pathogenic and AMR *E. coli* relative to surrounding soils. The persistence of these hotspots was demonstrated across management fields irrespective of their current seasonal use for grazing or forage production demonstrating the long term development and maintenance of these regions.

This study suggests that inadequate grazing management strategies result in the generation and maintenance of these higher microbial risk hotspots within soil. The regions proximal to water troughs and in-field features (weather stations) have the potential to act as sources of potential pathogenic and AMR microbes to grazing livestock and the wider environment. Operating a rotational or strip grazing strategy alongside greater variation in water sources could reduce this high concentration of faecal deposits at hotspots. Similar AMR patterns were demonstrated in grazed and ungrazed field use across production seasons, demonstrating the long term influence of these points. The impact of these identified hotspots on livestock grazing/preserved forage production and wider water course will be examined in Chapter 4 and 5 respectively.

Chapter 4

The role of forage in the farm microbe cycle: Implications for grazed pasture and silage quality, microbial disease risk and antimicrobial resistance

Summary

Temperate grassland based livestock production is dependent on grazed and preserved forage from pastures as the principle source of animal feed. The microbial impacts on grass and forage of livestock grazing on pasture are not as well characterised as those on soil and waters. This whole farm study was undertaken to investigate impacts of grazing livestock on microbial-related disease potential (as indicated by faecal indicator organism (FIO) concentration), antimicrobial resistance (AMR) patterns and forage quality. This study determined the variation in these factors at a field scale, before investigating similar parameters within preserved forage silage bales, including the concentration of zoonotic pathogen *Listeria monocytogenes*. All results were interpreted for their combined health and production impacts. At a field scale, increased FIO concentration and AMR were associated with specific points in fields. Within silage bales a decrease in forage nutritional quality was associated with increased FIO concentration and AMR at the external most aerobic region. Aerobic exposure, and the process of aerobic spoilage, of silage increased concentrations of *Listeria monocytogenes* and decreased forage quality. This work highlights potential stakeholder intervention strategies with mutual benefits to health and production. Management of high concentration FIO and AMR points within fields during grazing could mitigate potential disease and reduce resistance development risks. Similarly, targeted harvesting of grass for ensiling to avoid incorporation of high FIO and AMR regions could reduce resulting disease and resistance risk in preserved forage, while improving feed quality. The variation of parameters within silage bales highlights the importance of ensuring anaerobic conditions in the production process to reduce potential disease, AMR risk, and maintain forage quality. The specific impact of aerobic spoilage on *Listeria monocytogenes* concentration and feed quality indicates the requirements for maintaining anaerobic ensiling conditions and reviewing feeding methods.

4.1. Introduction

In Chapter 3 it was shown how the management of pasture and the interaction of grazing livestock within the environment can generate high intensity focal points in soil of faecal indicator organisms (FIO) such as *Escherichia coli* and intestinal enterococci. The capacity for these microbes to survive within the soil matrix and eventually pass outside individual farm system boundaries by hydrographical transfer will be examined in Chapter 5. However, there exists another major route in the cycling of FIO and associated AMR from identified hotspots and pasture more generally, by grazing and preserved forage.

Grassland based livestock production in the UK represent over 66 % of the utilised agricultural area (UAA), with the majority grazed (Qi et al, 2018, DEFRA, 2016, Smit et al, 2008). Within the UK, silage produced in clamp predominates with 22,152,000 tonnes forage mass (FM) per year relative to 11,928,000 tonnes FM per year baled silage (Wilkinson & Toivonen, 2003). However, the contribution of the different silage types should also be considered on a dry matter (DM) basis, where average grass % DM in bales is 32 compared to 25 in clamp (Wilkinson, 2015, Wilkinson & Fenlon, 2013, Field et al, 1999). Despite the considerable contribution of big bale silage to livestock diets and the potential effects on animal and human health caused by intra-bale microbial dynamics, there is surprisingly limited data available on the intra-bale spatial variation of silage quality, FIO concentrations and AMR. This has direct impact on-farm practice in relation to adequate provision of feed and preservation of animal health. The continual provision of high quality forage is a production necessity within ruminant farming systems. An improved understanding of the extent of avoidable losses through silage spoilage is vital to improve best practice production techniques.

A truly holistic disease control strategy must maximise the impact which relatively simple management decisions can achieve in controlling the transfer of potentially pathogenic and AMR bacteria. The control of livestock disease frequently focusses on the application of veterinary interventions to animals. Mitigating increases in AMR bacteria also primarily focusses on the role of veterinary interventions to the animals by encouraging responsible use. However, this prevailing strategy can only affect a reduction in disease potential or selection for AMR after the event. Its success relies on the effectiveness of this initial control and cannot manage subsequent stages in microbial and AMR cycling on-farms (Sayah et al, 2005, Hooda et al, 2000). The inclusion of effective biosecurity and hygiene as a prerequisite of herd health and farm planning has increased the downstream management of these risks. Improved biosecurity and hygiene can reduce the introduction of external disease and AMR risks into farming systems as well as minimise the avoidable transfer between distinct production stages. There remain significant opportunities to actively disrupt, and not simply manage the potential transfer, of these risks on-farm. One such strategy is the targeted management of grass and preserved forage, beyond simply a feed source, but as a tool for reducing microbial related disease and AMR potential.

4.1.1. Grazing

Microbial disease potential, AMR and forage quality should be considered at two distinct scales: the field and the resulting preserved forage scale. The field scale has obvious relevance in the variation of FIO concentration and AMR potential for grazed grass but also for the harvesting of forage for preservation. Variations at the field scale are driven by the interaction of livestock with the environment, principally in less intensive grassland systems, by the animal's allocation of time spent and excretion distribution. Farms commonly manage the interaction of livestock at

the field scale from a production perspective as discussed in chapter 1. A variety of different grazing strategies can be employed to achieve maximal returns both at grazing and in forage preservation. This results in a tradeoff between yield, land use and necessary stakeholder intervention. Understanding the impact of specific grazing strategies on the interaction between livestock and pasture is critical in determining subsequent field scale impacts in microbial disease potential, AMR expression and forage quality.

The spatial concentration of livestock faeces on pasture directly reflects animal time spent and thus the effectiveness of the grazing strategy. Having identified soil hotspots of livestock origin microbes there is a need to investigate whether similar variations exist on grass at pasture and subsequent preserved forage. More than any other aspect of the on-farm microbial cycle, the consumption of grazed and preserved forage has the greatest potential to return potentially pathogenic and AMR bacteria back to livestock.

The consumption of grass at pasture is crucial both in livestock nutrition and in the cycling of microbes on-farm. As discussed in chapter 1, the grazing strategy can significantly drive both forage quality, availability and exposure to livestock associated microbes through the dispersion of faecal deposits on pasture. It is important to note the differences in behaviors of grazing livestock as these directly influence microbial cycling from pasture (Scimone et al, 2007, Hodgson et al, 1991).

Grazing cattle will use their tongue to wrap around grass, tearing off mouthfuls often including root and soil matter. Cattle can avoid grazing near manure deposits although this is only true when other pasture is plentiful (Hutchings and Harris, 1997, Cazcarra et al, 1995). Through effective grazing management cattle can graze pasture uniformly and to a low level.

Sheep use teeth and lips to slice grass while grazing, consuming material proximal to crop bases. Sheep do preferentially graze areas or crops but proximity to flock is a stronger driver, which can result in overgrazing if forage strategy is inefficient (Dumont & Boissy, 2000). However increased prevalence of fresh faeces does reduce grazing in these areas (Hutchings et al, 2001, Cooper et al, 2000, Hutchings et al, 1998) when uncontaminated pasture is available elsewhere.

The tearing action of cows and basal grazing of sheep can result in both species exposure and ingestion of varying amounts of soil (Smith, 2009 et al, Judson and McFarlane, 1998, Kreulen, 1985). This is important when considering the impact of higher disease risk areas of pasture, particularly those where higher rates of faecal deposition are expected, such as the hotspots initially identified in Chapter 3.

4.1.2. Ensiled feed

At a field scale the harvesting of forage is a critical step in potential microbial cycling and subsequent forage quality. The harvesting and preservation of grass is required in most pasture based production systems as the primary over-wintering feed source (Wilkins, 2000, Wilkinson, 2000). In many systems, particularly high yielding dairy production, year-round housing necessitates continual harvesting and preservation of grass (Meul et al, 2012). The prevalence of such production systems and the corresponding importance of harvested forage and silage as feed sources is increasing globally (Van den Pol-van Dasselaar et al, 2008). The climate over the winter period in the UK can result in effective cessation of grass growth and pasture conditions not appropriate for livestock. Cattle are typically housed and fed silage, and supplemented with grain or manufactured feed to varying degrees (Beukes et al, 2011, Gordon, 1984). Sheep may be housed

or remain on pasture and will also commonly be fed silage/hay occasionally with some supplementation (Kendall et al, 2001, Robinson et al, 1973).

At a field scale harvesting of grass at pasture is principally driven by grass heading date. Heading is when grass crops produce a seed head and harvesting grass for silage should be applied to control this process. A timing strategy for harvesting should optimise the lower yields of digestible younger plant material against the higher yields of less digestible older crops (Kuoppala et al, 2010, Marten & Hovin, 1979). The height of cut can also affect resulting silage quality, particularly through soil and thus unwanted microbial inclusion. This focus on soil and microbe contamination is primarily on too low-cut height or uneven ground (Rafferty et al, 1994). This would be of greatest risk from grass in areas of deemed higher faecal deposition rates. Soil origin microbes significant negative impacts upon silage fermentation and resulting nutritional quality, livestock origin microbes in addition also negatively impact silage hygienic quality posing risk of disease and AMR. The inclusion of soil and unwanted microbes in silage results in inefficient fermentation of forage and poses significant disease risks. Following cutting crops are wilted in-field, rapid wilting reduces nutritional losses by permitting earlier ensiling and cessation of respiration.

The role of preserved feed within on-farm microbial cycling has consistently been underestimated. This stage in microbial cycling effects feed quality, animal health and can elicit selective pressure on microbial population diversity (Dunière et al, 2013, Driehuis & Oude Elferink, 2000, Muck, 1988). Greater understanding of the effects of inadequate silage production will highlight improved processing opportunities for farmers and the demonstrable extent of avoidable losses.

Following wilting crops are consolidated to create anaerobic conditions and sealed either wrapped in individual bales or collected and sealed in a large clamp (potentially containing most of the harvested crop). Subsequent to bale wrapping or clamp sealing, grasses will ferment under anaerobic conditions, corresponding to a drop in pH associated with lactic acid producing bacteria. The fermentation process can occur for several weeks until the silage is considered in a stable state. If acidic and anaerobic conditions are successfully achieved and maintained through storage, good forage quality can be expected.

Within the farm microbial cycle silage has the potential as a unit for improving system production through increased forage quality and reducing potential disease risk by impacting the proliferation and return to livestock of undesirable microbes. Utilising silage bales as the experimental unit to assess this is logical as interventions are relatively inexpensive and sampling can be conducted at a higher resolution than is possible within clamp silage.

Studies have previously assessed inter bale differences, focusing on resulting forage quality in relation to the impacts of storage conditions, fermentation, bale density and moisture content (Müller et al, 2007, Huhnke et al, 1997, Collins et al, 1987). This previous work highlights the impacts on forage quality and corresponding financial implications which suboptimal silage production can result in (Dunière et al, 2013, Driehuis et al, 1999). As a consequence, significant attention has been given to the effects which targeted silage additives can elicit in preserving silage quality and reducing concentration of microbial contaminants (Bolsen et al, 1996, Östling et al, 1993, Jacobs & McAllan, 1991). The success of these interventions is clear, however a significant portion of losses in silage quality and increased microbial contaminants remain avoidable through relatively simple adjustments to farm management practices.

Far less research has investigated the consequences of production factors upon intra bale variation in silage quality and microbial contaminants (Naoki & Yuji, 2008, McEniry et al, 2006). This assessment of spatial variation is important because it has the greatest potential to indicate the direct effects of imperfect production of individual bales at different zones (depths and positions from top, middle and base as the bale was stored). Identifying these effected horizons is critical in improving feed provision, reducing feed loses and maintaining livestock health..

Opportunities also exist to optimise forage quality and reduce potential disease risk at point of feeding. As described the process of achieving and maintaining anaerobic conditions is crucial to improving silage quality and eliminated microbial contaminants. However at feeding silage is exposed to oxygen and the process of aerobic spoilage occurs. Aerobic spoilage is driven by yeasts, in the presence of oxygen, which can thrive on high nutritional quality silage. Yeasts convert sugars and lactic acid to carbon dioxide and water, resulting in production of heat and high DM crop losses (Wilkinson, & Davies, 2012). This change in conditions enable undesirable microbe concentrations to increase, further accelerating spoilage. Some silage associated potentially pathogenic organisms such as *L. monocytogenes* and *E. coli* are considered the primary result of soil inclusion at the field scale. However, the disease risk is more complex and managing aerobic exposure/spoilage at point of feeding could present another strategy for disease control.

4.1.3. Research aims

The principle aim of this study is to investigate and quantify the extent which consumption of grazed and preserved forage enables transmission of potentially pathogenic and AMR bacteria from faecal material to livestock. Within all aspects of the study microbial parameters were assessed against forage quality, allowing the development of more robust and persuasive strategies for farmers that could improve livestock health and production. In this chapter the North Wyke Farm Platform permanent pasture is utilised to test the overall hypothesis that: Consumption of grazed and preserved forage is a significant transmission pathway of potentially pathogenic and AMR bacteria from faecal material to livestock.

Hypothesis 1 - There are hotspots of increased faecal indicator organisms on grass at pasture, related to livestock activity and field scale hydrology.

Hypothesis 2 - The concentration of faecal indicator organisms is increased in poor quality silage.

Hypothesis 3 - Increases in *L. monocytogenes* and *E. coli* concentrations in silage can occur due to aerobic spoilage in the absence of significant soil contamination.

4.2. Materials and methods

4.2.1. Experiment 1: Grass sampling and design- to test Hypothesis 1.

Sampling of grass at pasture, prior to harvesting for silage making, was completed across catchments 4, 5, 6 of the North Wyke Farm Platform (Orr et al., 2016) as shown in Figure 1 (constituting fields: Bottom Burrows and Burrows, Orchard Dean North and Orchard Dean South, Golden Rove). These catchments are predominantly perennial ryegrass (*Lolium perenne L*) dominated permanent pasture which has not been resown for 20 years+. Conventional NPK fertiliser application is applied to all (following Nutrient Management Guide (RB209) supported by annual soil testing). These catchments represent the principal basis of the North Wyke Farm Platform permanent pasture functional 'farmlet' and have the widest external stakeholder relevance as the most common grassland system within the UK (DEFRA, 2016). The 'semi-rotational continuous (variable)' grazing strategy utilised in this study results in multifunctionality of fields within catchments. As described chapter 3, at the time of sampling catchment 6 and 5 (Orchard Dean South only) were not grazed and were soon to be harvested for silage production. Catchment 4 and 5 (Orchard Dean North only) were under grazing rotation of the beef herd and sheep flock, respectively. Sampling was conducted across grazed and ungrazed field use to enable analysis of any differences in FIO concentration and % AMR resulting from temporal differences between periods since livestock contact. Ungrazed field use had not had livestock contact for over 6 months, since the previous production season. Grazed field use had livestock present for over 3 months prior and during sampling but had no contact in the previous production season.

Grass sampling was conducted at a field scale across a 'W'-transect for considered 'non-hotspot' areas. Sampling of each individual point consisted of replicate-sampling in triplicate. This resulted in each catchment non-hotspot area being represented by n=13 sample points in triplicate,.

This sampling plan was mapped to optimise relevance with previously conducted soil sampling, the overlap of sample points is highlighted in Figure 1. Individual replicates, to grass heights equivalent of practiced silage harvesting (10 cm), were placed in separate sealed bags. Sterility was maintained between replicates by washing shears with ethanol. Each replicate was apportioned 5 g fresh weight (FW) for faecal indicator organisms (FIO) and subsequent antimicrobial resistance (AMR) analysis. Individual sample point replicates were mixed apportioning 10 g FW for pH and 100 g FW for forage quality analysis.

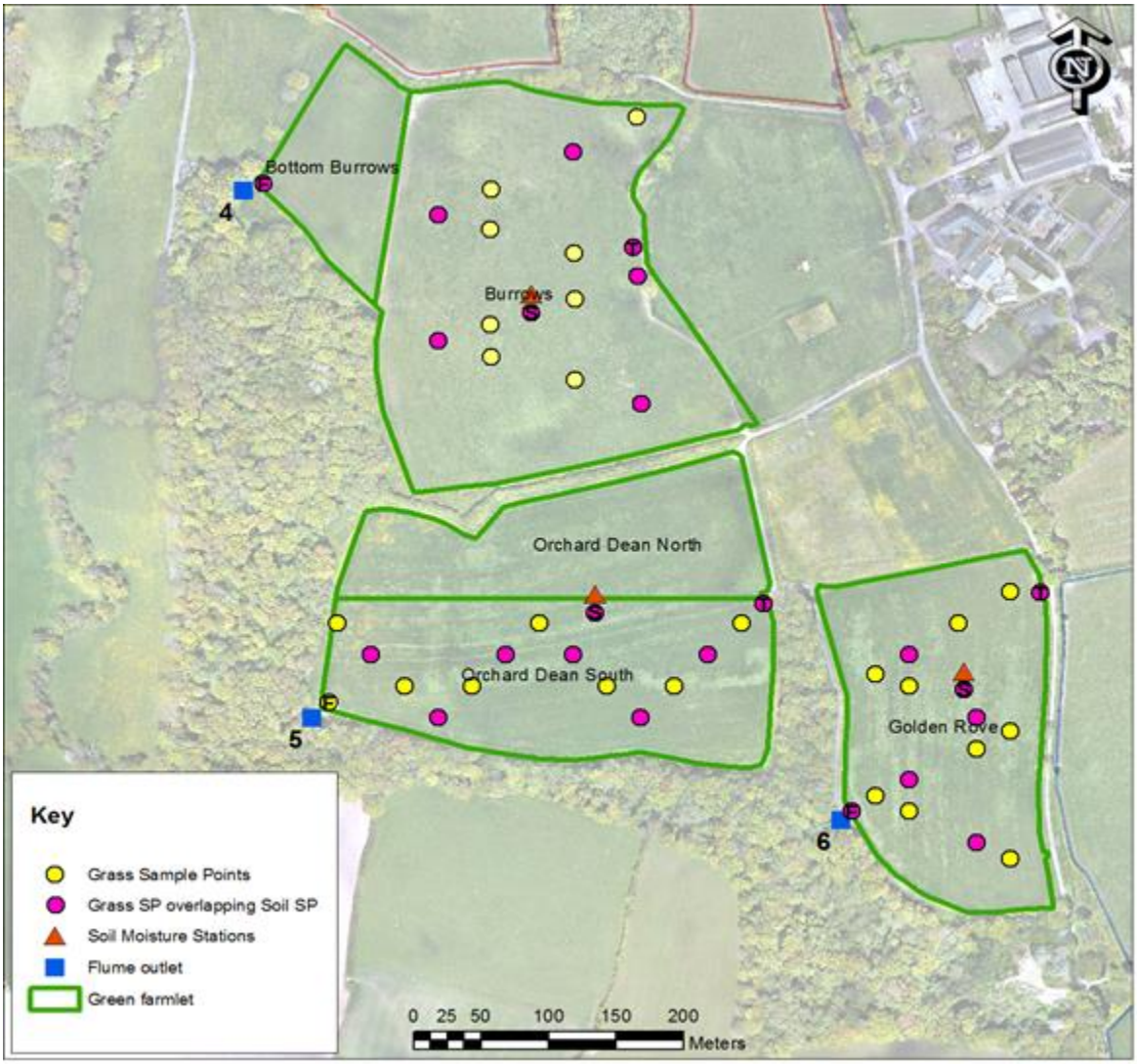

Figure 1. GIS mapping of individual sampling points of grass at pasture on Rothamsted Research North Wyke Farm Platform.
Sample points are yellow, points overlapping with soil sampling are highlighted in pink. Letters 'T, S, F' indicate hotspot sampling sites: Trough, Station, Flume and correspond to the described spatial sampling at <10, <20 and <30 m associated to the individual point.

Grass sampling at considered 'hotspots' was completed similarly to the protocol described in Chapter 3 for soil, and is presented in Figure 2. Hotspots were designated in classification as Trough (water trough in each field), Station (soil moisture/weather station on each catchment) and Flume (boundary of catchment adjacent to flume outlet). The hypothesized hotspots were

identified as deriving from direct animal interaction (Trough and Station) or driven by field scale hydrology (Flume). Sample points within the 10, 20 and 30 m regions were randomly allocated by GIS mapping. Within in each region n=3 sample points were allocated and grass sampling in triplicate as described for non-hotspot areas. In this manner individual hotspots were sampled n=9 times in triplicate, with n=3 hotspots considered on each catchment and n=3 of each hotspot classification across all catchments.

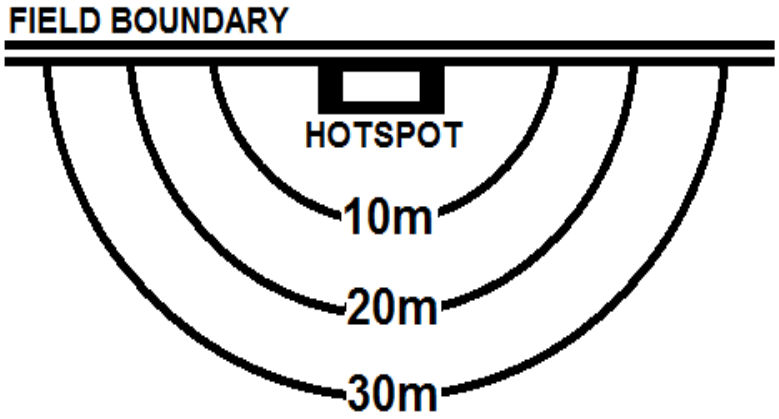

Figure 2. Hotspot spatial sampling.
Radiating sampling regions of <10, <20 and <30 m, Trough and Flume hotspots were situated at field boundaries as presented but Station hotspots were situated within fields. Three sample points were taken within each region.

4.2.2. Experiment 2: Silage bales and design- to test Hypothesis 2 and 3.

Silage was produced using standard farm procedure with grass chopped from catchment 5 (Orchard Dean North and South) and catchment 6 (Golden Rove). Grass was cut to approximately 7 cm lengths and baled before being wrapped in 4 layers of plastic (750 Black 125 μm, Visqueen, Rushden, Northamptonshire, UK). Baling occurred less than 24 hour after wilting in-field and maintained in a stack for 8 weeks (Keles et al, 2009).

Silage sampling was conducted alongside standard farm feeding practice. Bales were selected by farm staff for consecutive days' feeding (n=5). The bale position in stack, source field label and

qualitative metrics for assessing silage bale quality were noted. Quantitative assessment of bale wrap integrity was measured with a custom-built pressure gauge, providing comparative assessment of anaerobicity by indicating bale seal (Jacobsson et al, 2002). Coring was completed through the bale wrap, with the number of wraps counted to confirm farm practice, in the sampling pattern indicated in Figure 3. Cores were taken to the centre of the bale (60.0 cm ± 5.00 cm). Variation was defined by bale, section (side or centre), position (top, mid or base) and depth (inner or outer).

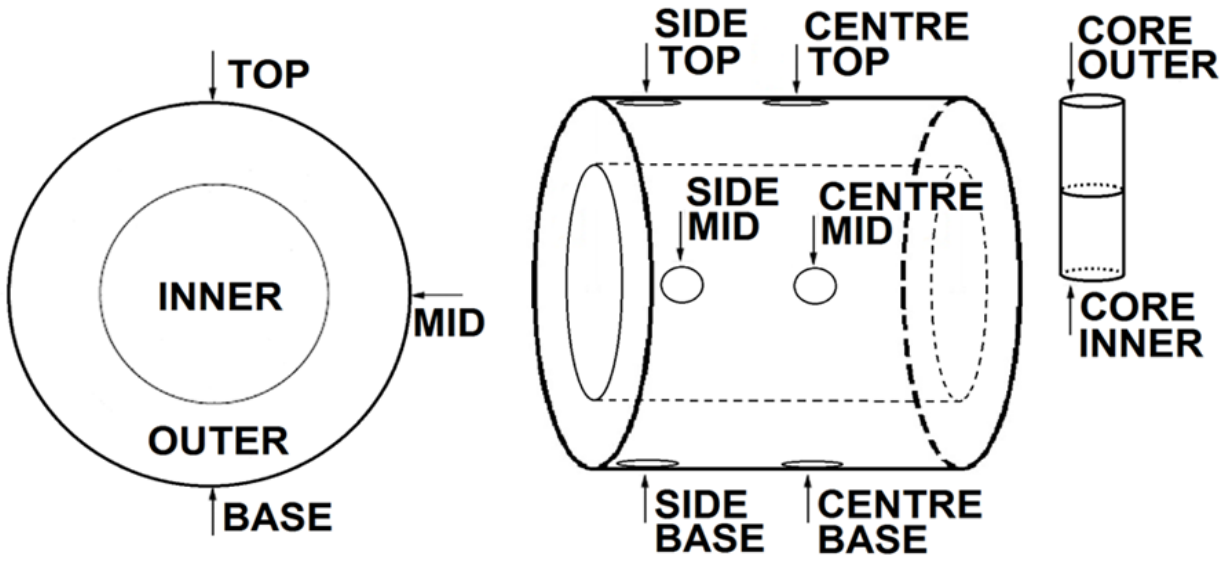

Figure 3. Individual silage big bale core sampling plan.
Spatial horizons defined as 'section': side or centre, 'position': top, mid or base, 'depth': inner or outer. All cores were taken to a depth of 60 cm ± 5 cm and split into inner and outer constituents.

Individual cores to constituent depths were placed in separate sealed bags. Sterility was maintained between sampling points by washing corer with ethanol. Core samples were mixed and sub-sampled apportioned: 5 g FW for faecal indicator organisms (FIO), 10 g FW for pH, 100 g FW for DM and ash component testing with the remainder for forage quality analysis by NIRS (Park, 1998). Subsamples for NIRS analysis were vacuum packed (CD969 vacuum pack machine, Buffalo appliances, Bristol, UK) and conventionally frozen at -20℃ for whole batch analysis.

4.2.3. Experiment 3: Silage aerobic stability and design- Hypothesis 3.

Separate silage bales from Experiment 2 were selected from North Wyke farm supply (n = 6). The bale position in stack, visible condition of bales and integrity of wrap was noted. Quantitative assessment of bale wrap integrity was measured as described above. These assessments enabled sampling of bales that were deemed representative.

The systematic sampling of the 6 bales was completed by mechanical silage coring of 14 cores per bale to a total of 7 kg of silage from each bale. Sterility was maintained between different bale sampling. The 14 core samples from the same bale were immediately recombined and mixed. From these, representative samples of each bale, 750 g FW were apportioned into 6 sterilised aerobic storage vessels (ASVs) and an additional 750 g FW sub-sample was retained for analysis of time point 0. This totaled 6 ASVs for each of the 6 bales, 36 ASVs for the entire study. All ASVs included an individual temperature data logger (SL52T / SL52T-A, Signatrol, Gloucestershire, UK), whose results were recorded on study completion. All ASVs were maintained in controlled environment conditions throughout the study at 20.3 (±0.25 °C) The temperature of the controlled environment cabinet was recorded by a data logger (SL52T / SL52T-A, Signatrol, UK).

Destructive sampling was undertaken at timepoints: 0, 1, 2, 4, 8, 16, 32 days after the experiment commenced. At each time point one sample for each of the 6 bales was analysed. Processing of samples was undertaken immediately at sample points, except for NIRS analysis, for which 300 g FW was vacuum packed (CD969 vacuum pack machine, Buffalo appliances, UK) and conventionally frozen at -20 (±0.5 °C) to enable whole batch analysis. Analysis was completed on 25 g FW for *Listeria* culturing, 10 g FW for pH, 50 g FW for DM and ash component testing

and 300 g FW for forage quality analysis by NIRS (Park et al, 1998). Remaining sample was utilised in concurrent research.

4.2.4. Forage quality analysis

Forage quality analysis was completed by a combination of methodologies, utilising a variety of techniques across samples from grass at pasture and from silage. All sample analysis was completed for pH from 10 g FW sample in 90 ml Milli-Q water by pH meter (Jenway 3320, Cole-Parmer, St. Neots, Cambridgeshire, UK).

4.2.4.1. Experiment 1: Grass at pasture specific forage quality analysis

Samples from grass at pasture were first prepared for forage quality analysis by oven drying to a constant weight at 80℃. Samples were then finely ground (<2 mm). DM content was calculated by weight loss.

Sample OM, and conversely ash content, were measured by weight loss at 505℃ for 12 hours (Thermolyne F6000, Thermo Scientific, Waltham, Massachusetts, USA), of 1 g FW samples in pre-furnaced and desiccated crucibles. The remaining sample was determined as ash and weight lost as OM.

Fibre content was determined by FOSS wet (FT 121 Fibertec) and cold (Fibertec 8000) extraction protocol as recognized by EN ISO 16472. Analysis of samples neutral detergent fibre (NDF) content was completed first. Samples were weighed and 0.5 g DM added to FOSS unit glass filter, pore 2 μm, crucibles with 0.5 g, anti-protein clumping, sodium sulphite. Crucibles were inserted into the cold extraction unit and acetone was applied along with agitation to clean samples and remove excess non-polar lipids if present. After a period of 5 min the acetone was removed by air vacuum and the process repeated twice more. Crucibles were then inserted to the hot

extraction unit and samples digested in 20 ml of neutral detergent solution (NDS) (FND20C, Ankom Technology, New York, USA) and 2-4 drops of the foam preventing n-octanol (Ankom Technology, USA). The mixture was again agitated by air and heated with another 20 ml of NDS, 2 min subsequent to the mixture achieving boiling a further 10 ml of NDS was added. Boiling continued, and 2 min after the final 10ml of NDS had been added, 2 ml of α-amylase (Ankom Technology, USA) was combined to the mixture to aid in digestion. Boiling and evaporation continued for 55 min before crucibles and remaining sample were removed from the unit and drained by vacuum. A further addition of 2 ml α-amylase and 15 ml deionized water solution was then agitated and drained. Crucibles were rinsed and drained twice more with deionized water before being reinserted into the cold extraction unit and soaked without agitation with 25 ml of acetone for 5 min. The acetone was drained by vacuum and this process repeated twice more. The crucible and sample were dried to a constant weight at 105 ℃ with the remaining sample determined as the NDF content of the original sample.

Analysis of sample acid detergent fibre content (ADF) was completed similarly to the described NDF protocol. During the hot extraction for ADF 45 ml of acid detergent solution (ADS) (Ankom FAD20C) and 2-4 drops of n-octanol was added. The mixture was again agitated by air and heated with another 45 ml of ADS, 2 min subsequent to the mixture achieving boiling a further 10 ml of ADS was added. Boiling and evaporation continued for 55 min before crucibles and remaining sample were removed from the unit and drained by vacuum. Pre- boiled deionized water solution (30 ml) was then added, agitated and drained. Crucibles were rinsed and drained twice more with deionized water before being reinserted into the cold extraction unit and underwent the same process as the NDF protocol to determine ADF content as the portion of remaining sample.

Following ADF analysis, acid detergent lignin (ADL) content was measured on the same sample by immersion of the crucible in 72% sulphuric acid (Ankom Technology, USA) for 3 hrs with periodic agitation. Acid was drained by vacuum from the crucibles and washed 5 times with warmed deionized water. Crucibles were dried at 105℃, weighed, and furnace at 505℃, with the ADL content of the original sample calculated by weight loss.

The content of modified acid detergent fibre (MADF) was analysed using the same experimental procedure as that described for ADF, except that the ADS used was prepared to half the original concentration used in the ADF procedure. The predicted metabolisable energy (ME) content is determined by calculation using Clancy and Wilson (1966) conversion factor ME (MJ/Kg DM) = 16.20 - 0.0185 (MADF).

Crude protein was measured by Kjeldahl determination (Bradstreet, 1940) and Kjeldahl %N by calculation with a conversion factor. Following established procedure for plant material, 0.5 g DM, 2 Kjeltabs CK tablets and 15 ml of 98% sulphuric acid (Ankom Technology, USA) were added to a digestion tube. Fumes were exhausted using prepared Gerhardt TURBOSOG centrifugal scrubber run on plant material appropriate programme 3. On completion of programme acid digested sample tubes underwent distillation by prepared Gerhardt Vapodest 40. Distilled samples were then auto-titrated by prepared Metrohm 716 DMS Titrino for Kjeldahl %N. The %N was calculated as: (titrant volume [ml] x acid normality x 1.40067)/initial sample weight [g]. Crude protein was then calculated by applying a conversion factor of 6.25 (Sanz, 2005, Hayes, 2004).

The determination of major and trace elements (P, K, Ca, Mg, Na, S, Al, Fe, Ti, Zn, Cu, Ni, Cd, Cr, Pb, Mo, Co, Mn) were analysed by inductively coupled plasma - optical emission spectrometer (ICP-OES), Optima-Perkin Elmer. An optimised sample set targeting hotspot and non-hotspot differences was initially included for this analysis, hotspot n=9 and non-hotspot n=9.

In preparation for ICP-OES analysis forage samples were freeze dried, finely ground (<2 mm) and 1 g DM of forage samples were digested using a mixture of nitric acid and perchloric acid (85:15 V/V) in open tube digestion blocks. The acids were removed by volatilisation and the residue dissolved in nitric acid (5% V/V). Primar (or equivalent) grade acids and 18 MΩ H_2O was used throughout. Within the ICP-OES unit the induction system aerosols the sample and mixes it with the plasma gas, causing the excitation of elements and the emitting of light. The wavelength of light is element specific and the concentration of light corresponds to the elements concentration. The selected wavelengths of light are transferred through an echelle diffraction grating, detected by a solid state segmented array charged coupled detector, converted to photoelectrons whose charge is ultimately converted to digital information on elemental concentrations. All results are reported as elements PPM, 0.250 g in 25 ml.

4.2.4.2. Experiment 2 and 3: Silage specific forage quality analysis

Samples from silage were first assessed by weight loss for DM and ash content from 100 g sample by weight loss at 80 °C for 24 hours and 505 °C for 12 hours (Thermolyne F6000, Thermo Scientific, Waltham, Massachusetts, USA), respectively. Silage quality parameters were assessed by NIRS, the whole batch of study samples were defrosted uniformly and removed from vacuum bags before scanning was undertaken and results recorded. The NIRS used was a commercially available mobile NIR4Farm (Aunir, ABVista Group, Towcester, Northamptonshire, UK). The instrument scans over a wavelength ranging between 920-1650 nm and based on the equations held in Aunir database calculates predicted analyses for various parameters (pH, DM, ash, ME, protein, VFA, lactic acid) (Baker, 1994).

4.2.5. Faecal indicator organism culturing: Experiment 1 and 2.

The determination of FIOs of each 5 g sub sample, from grass at pasture or silage, first required liquidising in 45 ml Ringers buffer solution (Thermo Scientific) in a sterilized blender capsule (35BL59, Waring, Stamford, Connecticut, USA). A serial dilution was then completed to enable standard membrane filtration as described by Hodgson et al (2016). Samples were washed through the filtration manifold unit with 20 ml of sterile Ringer's solution. Membrane filters of 0.45µm (Sartorius Stedim Biotech, Goettingen, Lower Saxony, Germany) were placed onto species selective sterile agars for FIO culturing. *E. coli* growth was targeted on Membrane Lactose Glucuronide Agar (Oxoid, Basingstoke, Hampshire, UK) and incubated for 18-24 h at 44.5 °C (±0.20 °C; Hodgson et al, 2016). Intestinal enterococci growth was targeted on Slanetz and Bartley agar (Oxoid) and incubated for 44-48 h at 37.5 °C (±0.20 °C; Hodgson, 2016). Confirmation of intestinal enterococci concentrations were cultured on Kanamycin aesculin azide agar (Oxoid) plates pre-warmed to room temperature, and incubated at 37.5 °C (±0.20 °C) for 4 h (±5 minutes). All samples were processed in triplicate throughout culturing. The initial FIO counts were transformed to Log_{10} colony forming units (CFU) g^{-1} DM.

4.2.6. Antimicrobial resistance analysis: Experiment 1 and 2.

Assessment of AMR prevalence, from samples of grass at pasture and silage, was tested by Kirby-Bauer disc diffusion test (Bauer et al, 1959). *E. coli* colonies deemed representative were isolated in triplicate from each experimental unit on non-selective, Mueller-Hinton agar (Oxoid), incubated for 24 h at 35.0 °C (±0.20 °C; Tenover et al, 2001). Cells were then suspended in phosphate-buffered saline (PBS) to a concentration of 1.5×10^8 CFU ml^{-1}, visually assessed against a 0.5 McFarland standard (Wiegand et al, 2008). Mueller-Hinton agar plates were immediately

inoculated by spread plating prior to the application of 6 antimicrobial susceptibility testing disks (Oxoid) and incubated for 18 h at 35.0 °C (±0.20 °C; Jorgensen and Turnidge, 2007). Antimicrobials used and their amounts were: amoxicillin/clavulanate (30 µg), cefpodoxime (10 µg), cephalexin (30 µg), doripenem (10 µg), enrofloxacin (5 µg), erythromycin (15 µg), florfenicol (30 µg), neomycin (30 µg), sulphisoxazole (300 µg) tetracycline (30 µg). These were selected to best capture veterinary and human antimicrobials, and resistance patterns of critical or high importance (WHO AGISAR, 2017). The panel was correlated against farm specific history. Each antimicrobial's zone of inhibition was measured to the nearest millimetre. Inhibition zones were assessed against EUCAST (http://www.eucast.org), BSAC (http://www.bsac.org.uk; Andrews et al, 2001) and CLSI (https://clsi.org) agreed clinical breakpoints, indicated in Table 1.

Table 1. Antimicrobial breakpoint assessments for Kirby-Bauer disc diffusion test used in experiments 1 and 2.

Antimicrobial	Resistance breakpoint (mm)	Source
amoxicillin /clavulanate	19.0	EUCAST[a]
cefpodoxime	19.0	BSAC[b]
cephalexin	14.0	EUCAST
doripenem	18.0	BSAC
enrofloxacin	16.0	CLSI[c]
erythromycin	13.0	CLSI
florfenicol	32.0	EUCAST
neomycin	13.0	CLSI
sulphisoxazole	12.0	CLSI
tetracycline	14.0	CLSI

Resistance was defined as visible isolate growth measured to be equal to or less than the specified breakpoint radiating from antimicrobial inoculated disk. a- European Committee on Antimicrobial Susceptibility Testing (EUCAST), b- British Society for Antimicrobial Chemotherapy (BSAC), c- Clinical and Laboratory Standards Institute (CLSI).

4.2.7. Listeria *culturing: Experiment 3.*

The concentration of *Listeria* species, from samples of the silage tested for aerobic stability, was determined as absolute presence or absence using the Listeria Precis method (Oxoid, UK). Each 25 g sub sample, representing an individual sampling unit, was added to 225 ml of ONE Broth-Listeria (Oxoid, UK). The sample and broth enrichment were mixed for 1 minute at ambient temperature by undergoing stomaching (Stomacher 400 circulator, Seward, UK) (Sharpe and Jackson, 1972). The mixed sample and enrichment broth was then incubated for 24 h at 30.0°C (±0.20°C). Following initial incubation Brilliance Listeria (Oxoid, UK) selective plates were inoculated with 10 µl of enriched sample liquid and further incubated for 24 h targeting *Listeria* spp. growth at 37.0°C (±0.20°C) (Kathariou et al, 1994, Young, & Foegeding, 1993). Presumptive *Listeria* spp. (blue/green colony) and *L. monocytogenes* (blue/green colony with opaque halo) isolates were confirmed by combination of gram test and selective API biochemical testing (Setiani et al, 2015). Results were reported as presence or absence of *Listeria* spp. and were used primarily to confirm absolute positive or negative samples.

The enumeration of both total *Listeria* spp and *L. monocytogenes* isolates was undertaken by the Rapid enumeration method (Oxoid, UK). Each 25 g sub sample, representing an individual sampling unit, was added to 225 ml of sterile Ringer's solution. The mixed sample and ringers solution was then incubated for 1 h at 20.0°C (±0.20°C). A serial dilution was then completed, permitting calculation of CFU, before inoculation of Brilliance Listeria (Oxoid, UK) selective plates. Inoculated plates were incubated for 48 h targeting Listeria spp. growth at 37.0°C (±0.20°C) (Kathariou et al, 1994, Young, & Foegeding, 1993). Presumptive *Listeria* spp. (blue/green colony) and *L. monocytogenes* (blue/green colony with opaque halo) isolates were confirmed by combination of gram test and selective API biochemical testing (Setiani et al, 2015). The initial

Listeria spp. and *L. monocytogenes* CFU counts from all log dilutions were normalized by transforming to Log_{10} colony forming units (CFU) g−1.

4.2.8. Statistical Analysis

All statistical analysis was completed in Genstat (16th Edition, 2013). The variation of FIO CFU g^{-1} was calculated by blocked general ANOVA. Correlation of NIRS forage quality analysis to the measured metrics of pH, DM and ash was by linear mixed modelling. Variation in forage quality parameters was calculated by blocked general ANOVA.

4.2.8.1. Experiment 1: Grass at pasture statistical analysis

The ANOVA model used was:

Y variate: Forage quality value, FIO CFU g^{-1} and AMR

Treatment: Class*Distance

Block: Catchment

Where: Class = non-hotspot, flume, station or trough, Distance = non-hotspot, <10 m, <20 m or <30 m, Catchment = number (4 to 6).

Significance was accepted if $P \leq 0.05$.

4.2.8.2. Experiment 2: Silage bale statistical analysis

The ANOVA model used was:

Y variate: Forage quality value , FIO CFU g^{-1} and AMR

Treatment: Position*Section*Depth

Block: Bale/Core/Subsample

The linear mixed model used was:

Y variate: Measured pH, DM and ash value

Fixed model: NIRS pH, DM and ash value (+Depth)

Block: Bale/Core/Subsample

Where: Position = top, mid or base, Section = side or centre, Depth = inner or outer, Bale = number (1 to 5), Core = individual core effect (side/centre, top/mid/base) Subsamble = combined core and depth effect.

Significance was accepted if $P \leq 0.05$.

4.2.8.3. Experiment 3: Silage aerobic stability statistical analysis

The ANOVA model used was:

Y variate: Forage quality value, temperature and Listeria log CFU g^{-1}

Treatment: Timepoint

Block: Bale

The post hoc testing was Tukey test (significance 0.05).

The linear mixed model used was:

Y variate: Calculated pH, DM and ash value

Fixed model: NIRS pH, DM and ash value

Block: Bale

Where: Timepoint = 0, 1, 2, 4, 8, 16 or 32 days post aerobic exposure, Bale = number (1 to 6), Listeria log CFU g^{-1} = total *Listeria* spp. or *L. monocytogenes* (when stated).

Significance was accepted if $P \leq 0.05$.

4.3. Results

4.3.1. Grass at pasture analysis: Experiment 1.

4.3.1.1. Measured forage quality parameters

No significant differences were indicated for NDF, ADF, ADL or ME at ‘class’ or ‘distance’. Significant variation in forage OM content was found at ‘class’, with lower OM content sampling points associated with ‘Trough’ compared to ‘Flume’. OM content of grass at pasture was also significantly different across sample point ‘distance’, with lower OM content found at <10 m compared to <30 m from defined hotspots. The CP content was also found to be significantly different across sample point ‘distance’, with the highest CP found at <10 m compared to <30 m from defined hotspots.

No significant differences were found in elemental concentrations of micro and macro mineral nutrients for grass at pasture between hotspot (means of all hotspot classes) and non-hotspot. Significant variation at the field scale was indicated. Summary statistics are presented in Table 2 and 3.

Table 2. Variations in measured forage quality parameters of grass at pasture due to sample class and distance.

Strata	OM (g kg^{-1} DM)	NDF (g kg^{-1} DM)	ADF (g kg^{-1} DM)	ADL (g kg^{-1} DM)	ME (MJ kg DM)	CP (g kg^{-1} DM)
Catchment	918	462	249	39.5	11.5	159
sd.	7.2	5.6	13.9	3.29	0.25	3.7
Class						
Non-HSP	919[ab]	463	251	40.1	11.5	159
sd.	6.4	5.9	14.0	3.22	0.25	4.0
Flume	922[b]	463	251	40.7	11.4	158
sd.	2.4	4.9	13.2	2.80	0.23	2.8
Station	917[ab]	461	242	37.3	11.6	159
sd.	9.3	5.4	10.6	2.28	0.18	2.8
Trough	913[a]	461	248	38.1	11.5	160
sd.	9.1	5.6	16.2	3.78	0.28	3.7
P value	0.048	0.751	0.547	0.168	0.368	0.653
Distance						
Non-HSP	919[ab]	463	251	40.1	11.5	159[a]
sd.	6.4	5.9	14.0	3.22	0.25	4.0
<10m	912[a]	459	242	38.9	11.6	160[b]
sd.	9.4	4.9	16.0	3.50	0.28	3.7
<20m	918[ab]	462	250	38.9	11.5	158[ab]
sd.	7.6	5.0	11.9	3.36	0.21	2.6
<30m	921[b]	463	249	39.0	11.5	159[a]
sd.	5.0	4.9	12.8	3.28	0.22	3.0
P value	0.038	0.23	0.657	0.972	0.501	0.012

Mean values of grass at pasture measured forage quality parameters. Blocked ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters within column.

Table 3. Variations in elemental concentration (ppm) of grass at pasture at hotspot and non-hotspot sampling.

Strata	P	K	S	Ca	Mg	Na	Fe	Mn	Cu	Zn	Co	Se	Mo
Reference mean	3700	38000	3470	4200	1730	1820	151	99	7.90	22.0	0.19	0.02	0.64
Catchment	3365	8662	2778	3147	2623	2450	423	214	7.98	23.7	0.55	0.04	0.77
sd.	187.4	1534.0	161.7	398.8	423.8	414.9	65.1	55.0	0.90	4.41	0.227	0.016	0.098
HSP	3320	8180	2826	3215	2582	2485	426	193	8.12	23.1	0.53	0.04	0.76
sd.	180.3	1768	169.1	340.8	380.0	392.5	72.4	63.0	1.010	4.59	0.197	0.018	0.073
Non-HSP	3410	9144	2731	3078	2664	2414	420	235	7.84	24.3	0.57	0.04	0.79
sd.	193.8	1163	148.0	459.8	483.2	457.0	61.2	37.7	0.823	4.42	0.264	0.015	0.121
P value	0.321	0.194	0.202	0.448	0.698	0.708	0.852	0.079	0.548	0.612	0.732	0.454	0.536

Blocked ANOVA test was applied to analyse the data. Reference mean was derived from Germinal, 2015.

4.3.1.2. Faecal indicator organisms

The spatial variation in concentrations of *E. coli* and intestinal enterococci was analysed across all strata. The CFU across the sample points 'class' was again not significant for intestinal enterococci but was significant for *E. coli* with 'Trough' higher than 'Flume' or 'non-hotspot' class samples. The differences in CFU at sample point 'Distance' were significant for intestinal enterococci with, <10 m higher than <30 m proximal to defined hotspots. Sample point 'Distance' was also different for *E. coli* CFU with <10 m proximal to defined hotspots higher than the non-hotspot distance. Summary statistics for FIO concentrations are presented in Table 4.

Table 4. Variations in faecal indicator organism concentration (log_{10} $CFUg^{-1}$) of grass at pasture by sample class and distance.

Strata	Mean *E. coli*	sd. *E. coli*	Mean intestinal enterococci	sd. intestinal enterococci
Catchment	2.29	0.555	1.71	0.448
Class				
Non-HSP	2.10^{ab}	0.469	1.67	0.381
Flume	2.09^{a}	0.340	1.62	0.121
Station	2.60^{ac}	0.504	1.69	0.300
Trough	2.97^{c}	0.490	1.98	1.022
P value	0.007		0.322	
Distance				
Non-HSP	2.10^{a}	0.469	1.67^{abc}	0.381
<10m	2.93^{b}	0.643	2.17^{b}	0.916
<20m	2.38^{ab}	0.540	1.61^{ab}	0.265
<30m	2.35^{ab}	0.328	1.51^{a}	0.195
P value	0.001		0.01	

Mean values of grass at pasture E. coli and intestinal enterococci concentration. Blocked ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters within column.

4.3.1.3. Antimicrobial resistance

Differences in percentage resistance isolates across sample 'class' were also found. The trough samples presented the highest percentage of resistant isolates for amoxicillin /clavulanate and cefpodoxime relative to all other sample 'classes', and for erythromycin and neomycin relative to non-hotspot and flume sample 'classes'. The highest percentage of resistant isolates for tetracycline by 'class' were found in station samples relative to non-hotspot and flume sample 'classes'. The <10 m 'distance' proximal to hotspots presented the highest percentage of resistant isolates for cephalexin and neomycin relative to all other 'distances', and for tetracycline relative to the non-hotspot and <30 m proximal to hotspot 'distances'. The highest percentage of resistant isolates for cefpodoxime by 'distance' were found in samples <20 m proximal to hotspots relative to all other sample 'distances'. The <10 m and <20 m 'distances' proximal to hotspots presented the highest percentage of resistant isolates for erythromycin relative to all other sample 'distances'. Zero isolates were identified as exhibiting multiple drug resistance (defined as resistance to 4 or more of the drug panel). Summary statistics for percentage resistant isolates are presented in Table 5.

Table 5. Variations in percentage antimicrobial resistant isolates of grass at pasture by sample class and distance.

	% Resistant Isolates					
Drug type	AMC	CPD	CFX	ERY	NEO	TET
Strata						
Catchment	9.09	3.03	3.03	12.12	4.54	7.57
sd.	0.513	0.262	0.262	0.498	0.318	0.404
Class						
Non-HSP	1.70[a]	0.00[a]	0.00	1.70[a]	0.00[a]	0.85[a]
sd.	7.449	0.000	0.000	7.449	0.000	5.338
Flume	0.00[a]	0.00[a]	0.00	0.00[a]	0.00[a]	0.00[a]
sd.	0.000	0.000	0.000	0.000	0.000	0.000
Station	0.00[a]	0.00[a]	3.70	7.40[ab]	3.70[ab]	11.1[b]
sd.	0.000	0.000	11.111	14.700	11.111	16.67
Trough	14.8[b]	7.40[b]	3.70	14.8[b]	7.40[b]	3.70[ab]
sd.	24.22	14.700	11.111	17.57	14.700	11.111
P value	0.006	0.004	0.148	0.005	0.019	0.012
Distance						
Non-HSP	1.70	0.00[a]	0.00[a]	1.70[a]	0.00[a]	0.85[a]
sd.	7.449	0.000	0.000	7.449	0.000	5.338
<10m	7.40	0.00[a]	7.40[b]	11.1[b]	11.1[b]	11.1[b]
sd.	22.222	0.000	14.700	16.67	16.67	16.67
<20m	7.40	7.40[b]	0.00[a]	11.1[b]	0.00[a]	3.70[ab]
sd.	14.700	14.70	0.000	16.67	0.000	11.111
<30m	0.00	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.00[a]
sd.	0.000	0.000	0.000	0.000	0.000	0.000
P value	0.269	0.004	0.004	0.014	0.001	0.012

Mean values of grass at pasture percentage antimicrobial resistant isolates by sample class and distance. Blocked ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters within column. Only antimicrobials which indicated resistant isolates are presented: DOR (doripenem), EFX (enrofloxacin), FFC (florfenicol) and SXZ (sulphisoxazole) were effective against all isolates tested and no resistance was found. Drug type abbreviations: AMC (amoxicillin /clavulanate), CPD (cefpodoxime), CFX (cephalexin), ERY (erythromycin), NEO (neomycin), TET (tetracycline).

4.3.2. Silage bale analysis: Experiment 2.

4.3.2.1. Measured forage quality parameters

No significant differences were indicated for pH at 'section', 'position' or 'depth'. For DM, variation was found at 'depth', with higher DM in the outer depth, but not at 'section' or 'position'. The ash indicated no differences at 'section', 'position' or 'depth'. Summary statistics are presented in Table 6.

Table 6. Variations in measured forage quality parameters of silage bales by sample section, position and depth.

Strata	Mean pH/kg DM	sd. pH/kg DM	Mean DM %	sd. DM %	Mean ash %	sd. ash %
Bale	4.81	0.551	59.0	9.61	7.82	0.647
Section						
Centre	4.83	0.672	59.7	9.78	7.90	0.632
Side	4.78	0.405	58.2	9.55	7.74	0.662
P value	0.778		0.596		0.270	
Position						
Top	4.95	0.725	61.4	12.0	8.05	0.679
Mid	4.77	0.345	59.2	6.61	7.78	0.669
Base	4.70	0.514	56.3	9.12	7.62	0.540
P value	0.557		0.316		0.071	
Depth						
Inner	4.75	0.532	56.7[a]	8.48	7.79	0.520
Outer	4.86	0.572	61.3[b]	10.23	7.85	0.761
P value	0.213		0.001		0.647	

Mean values of silage bales measured forage quality parameters (bale, section, position and depth). Blocked ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letter within columns.

4.3.2.2. NIRS forage quality parameters

The accuracy of NIRS forage quality prediction analysis was confirmed by linear mixed modelling to be significantly associated to measured pH, DM and ash. The success of this comparison provided confidence in associated NIRS values. In the absence of measured analyses for ME and CP the subsequent assessment by ANOVA of these key forage quality parameters along with pH utilised NIRS predicted values (Beever and Mould, 2000). Summary statistics of NIRS analysis is presented in Table 7.

Corresponding to measured pH the analysed NIRS pH values also indicated no differences at ‘section’, ‘position’ or ‘depth’. The NIRS ME analysis also indicated no differences at ‘section’, ‘position’ or ‘depth’. ME (MJ/kg DM) indicated a non-significant trend across positions with lowest concentration at the base and higher at the top. CP as predicted by NIRS was increased at the ‘outer’ ‘depth’. CP showed a trend across positions with lowest content at the top and highest at the base but was not significant at ‘section’ or ‘position’. Analysis indicated no differences in volatile fatty acids (VFA) (g/kg DM) at ‘section’, ‘position’ or ‘depth’. Similarly, no differences were associated with lactic acid concentration (g/kg DM) at ‘section’, ‘position’ or ‘depth’. Both VFA and lactic acid indicated a similar trend across positions as CP, with lowest concentration at the base.

Table 7. Variations in predicted NIRS forage quality parameters of silage bales by sample section, position and depth.

Strata	Mean pH/kg DM	sd. pH/kg DM	Mean DM %	sd. DM %	Mean ash %	sd. ash %	Mean ME MJ/kg DM	sd. ME MJ/kg DM	Mean protein %	sd. protein %	Mean VFA g/kg DM	sd. VFA g/kg DM	Mean Lactic g/kg DM	sd. Lactic g/kg DM
Bale	4.64	0.345	59.0	11.73	7.61	0.916	9.58	0.596	14.5	2.69	16.5	6.10	19.3	12.25
Section														
Centre	4.61	0.423	60.0	12.56	7.49	1.041	9.62	0.717	14.1	2.85	16.3	6.33	22.3	14.06
Side	4.68	0.246	58.1	10.97	7.73	0.770	9.53	0.452	15.0	2.49	16.7	5.96	16.4	9.44
P value	0.522		0.546		0.274		0.642		0.193		0.874		0.108	
Position														
Top	4.67	0.330	64.1	11.61	7.45	1.181	9.58	0.789	14.0	3.57	15.6	6.10	16.3	10.69
Mid	4.61	0.238	58.0	10.61	7.86	0.892	9.76	0.464	14.8	2.34	15.2	6.34	17.6	13.98
Base	4.64	0.449	54.9	11.60	7.53	0.565	9.39	0.440	14.8	1.93	18.8	5.47	24.1	10.91
P value	0.902		0.073		0.301		0.333		0.500		0.273		0.178	
Depth														
Inner	4.63	0.330	56.6[a]	11.10	7.60	0.687	9.55	0.659	14.1[a]	2.16	16.8	5.97	20.1	13.94
Outer	4.65	0.365	61.4[b]	11.90	7.62	1.112	9.60	0.535	14.9[b]	3.12	16.2	6.31	18.6	10.48
P value	0.711		0.025		0.932		0.553		0.05		0.593		0.554	

Mean values of silage bales predicted NIRS forage quality parameters (bale, section, position and depth). Blocked ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters within column.

4.3.2.3. Faecal indicator organisms

The spatial variation in concentrations of *E. coli* and intestinal enterococci was analysed individually, as with forage quality parameters, accounting for source of variation resulting from between 'bale' differences. The differences in CFU at 'section' were not different for *E. coli* but were for intestinal enterococci with higher concentrations in the 'side' section. While the observed trend indicated an increase in FIO concentration from top to base 'position' this was not found to be significant in either *E. coli* or intestinal enterococci. The 'depth' was different for both *E. coli* and intestinal enterococci concentrations. The highest CFU of *E. coli* were in the 'outer' and highest CFU of intestinal enterococci in the 'inner' depth. Summary statistics for FIO concentrations are presented in Table 8.

Table 8. Variations in faecal indicator organism concentration (log_{10} CFU g^{-1}) of silage bales by sample section, position and depth.

Strata	Mean *E. coli*	sd. *E. coli*	Mean intestinal enterococci	sd. intestinal enterococci
Bale	2.79	0.616	5.03	0.635
Section				
Centre	2.81	0.593	4.87[a]	0.637
Side	2.78	0.647	5.20[b]	0.598
P value	0.848		0.007	
Position				
Top	2.64	0.697	4.96	0.689
Mid	2.76	0.483	4.96	0.697
Base	2.98	0.627	5.18	0.508
P value	0.208		0.214	
Depth				
Inner	2.48[a]	0.392	5.28[b]	0.535
Outer	3.11[b]	0.645	4.79[a]	0.637
P value	0.001		0.001	

Mean values of silage bales faecal indicator organism concentration (log10 CFU g-1) (bale, section, position and depth). Blocked ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters within column.

4.3.2.4. Antimicrobial resistance

Statistical significance is indicated in percentage resistance isolates for both cephalexin and erythromycin at 'depth'. Higher percentage resistant isolates were indicated in the 'outer' depth. One isolate was identified as a presumptive Extended Spectrum Beta-Lactamases (ESBL) - producer, indicated by amoxicillin/clavulanate and cefpodoxime resistance (Bean et al, 2008). However, with a single isolate caution is exercised in the absence of genetic confirmation (Oliver et al, 2002). One isolate was identified as exhibiting multiple drug resistance (defined as resistance to 4 or more of the drug panel), presenting as resistance to amoxicillin/clavulanate, cephalexin, erythromycin and neomycin. Summary statistics for percentage resistant isolates are presented in Table 9.

Table 9. Variations in percentage antimicrobial resistant isolates of silage bales by sample section, position and depth.

	% Resistant Isolates													
Drug Type	Mean AMC	Sd. AMC	Mean CPD	Sd. CPD	Mean CFX	Sd. CFX	Mean ERY	Sd. ERY	Mean FFC	Sd. FFC	Mean NEO	Sd. NEO	Mean TET	Sd. TET
Srata														
Bale	3.88	10.790	1.11	6.034	5.55	13.950	30.5	26.25	2.22	8.385	3.88	12.410	5.00	12.000
Section														
Centre	4.44	11.520	1.11	6.086	7.77	14.340	27.7	26.38	1.11	6.086	3.33	13.420	5.55	12.630
Side	3.33	10.170	1.11	6.086	3.33	13.420	33.3	26.26	3.33	10.170	4.44	11.520	4.44	11.520
P value	0.666		1.000		0.308		0.456		0.335		0.757		0.726	
Position														
Top	1.66	7.454	0.00	0.000	1.66	7.454	41.6	26.21	1.66	7.454	1.66	7.454	5.00	12.210
Mid	5.00	12.210	1.66	7.454	5.00	12.210	25.0	26.21	3.33	10.260	6.66	13.680	0.00	0.000
Base	5.00	12.210	1.66	7.454	10.0	19.040	25.0	23.88	1.66	7.454	3.33	14.910	10.0	15.67
P value	0.477		0.628		0.294		0.125		0.786		0.515		0.053	
Depth														
Inner	2.22	8.457	1.11	6.086	2.22[a]	8.457	21.1[a]	20.50	1.11	6.086	3.33	10.170	3.33	10.170
Outer	5.55	12.630	1.11	6.086	8.88[b]	17.360	40.0[b]	28.23	3.33	10.170	4.44	14.470	6.66	13.560
P value	0.233		1.000		0.033		0.001		0.327		0.709		0.213	

Mean values of silage bales percentage antimicrobial resistant isolates (bale, section, position and depth). Blocked ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters within column. Only antimicrobials which indicated resistant isolates are presented: DOR (doripenem), EFX (enrofloxacin) and SXZ (sulphisoxazole) were effective against all isolates tested and no resistance was found. Drug type abbreviations: AMC (amoxicillin /clavulanate), CPD (cefpodoxime), CFX (cephalexin), ERY (erythromycin), FFC (florfenicol), NEO (neomycin), TET (tetracycline).

4.3.3. Silage aerobic stability analysis: Experiment 3.

4.3.3.1. Measured forage quality parameters

The accuracy of NIRS forage quality predictions to the calculated values for pH and DM was validated to be significantly correlated as described previously for Experiment 2. NIRS predicted value for ash content was not significantly correlated to calculated values.

Measured pH indicated no significant difference at 0, 1, 2, 4, 8, 16, 32 days post aerobic exposure. Measured DM was significantly increased at the 32 day time point relative to preceding time points. Measured ash % indicated no significant difference across time points. Summary statistics are presented in Table 10.

Table 10. Variations in measured forage quality parameters of silage samples by time points (days post aerobic exposure).

Time point	Mean pH/kg DM	sd. pH/kg DM	Mean DM %	sd. DM %	Mean ash %	sd. ash %
0	4.92	0.244	28.7[a]	3.81	8.90	0.889
1	4.84	0.145	29.5[a]	3.80	8.7	0.375
2	4.76	0.142	29.3[a]	3.45	9.12	1.050
4	4.73	0.144	29.8[a]	3.08	8.77	0.267
8	4.66	0.097	29.0[a]	2.82	9.22	0.407
16	4.66	0.118	29.9[a]	4.78	8.99	0.658
32	4.98	0.730	33.1[b]	4.45	9.13	0.435

Mean values of silage samples measured forage quality parameters between time points, days post aerobic exposure (column). Blocked ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters within column.

4.3.3.2. NIRS forage quality parameters

Significant variation for forage quality indicators resulted from between ‘bale’ differences and this was accounted for in ANOVA design. Corresponding to calculated parameters analysed, NIRS pH and ash values presented no significant differences across time points. The NIRS DM analysis was significantly increased at 32 days relative to 1 day post aerobic exposure. A significant decrease in ME MJ/kg was found by NIRS at 32 days relative to 0, 2 and 8 days post aerobic exposure. The NIRS predicted protein %, VFA g/kg and lactic g/kg were not indicated as significantly different across any time points. Summary statistics are presented in Table 11.

Table 11. Variations in predicted NIRS forage quality parameters and measured temperature of silage samples by time points (days post aerobic exposure).

Time point	Mean pH/kg DM	sd. pH/kg DM	Mean DM %	sd. DM %	Mean ash %	sd. ash %	Mean ME MJ/kg DM	sd. ME MJ/kg DM	Mean protein %	sd. protein %	Mean VFA g/KG DM	sd. VFA g/kg DM	Mean lactic g/kg DM	sd. Lactic g/kg DM	Mean temp. °C	sd. temp. °C
0	4.37	0.030	31.5^{ab}	2.72	8.41	0.106	10.2^{b}	0.15	16.0	0.44	33.7	2.64	49.8	2.50	20.4^{ab}	0.75
1	4.34	0.092	31.1^{a}	2.73	8.38	0.230	10.0^{ab}	0.11	16.1	0.40	34.4	1.92	47.6	5.34	20.5^{ab}	0.22
2	4.38	0.062	31.3^{ab}	2.60	8.37	0.180	10.1^{b}	0.12	16.0	0.26	34.6	2.44	47.2	6.16	20.1^{a}	0.83
4	4.33	0.060	32.2^{ab}	3.16	8.28	0.223	10.0^{ab}	0.23	15.8	0.42	34.2	2.57	47.9	3.49	19.7^{a}	0.37
8	4.31	0.030	31.4^{ab}	2.59	8.19	0.170	10.1^{b}	0.17	15.6	0.30	35.0	2.51	51.4	5.40	20.2^{a}	0.52
16	4.28	0.068	31.5^{ab}	3.60	8.24	0.079	10.0^{ab}	0.06	15.7	0.17	35.1	3.19	52.0	3.90	20.6^{ab}	0.30
32	4.37	0.137	33.2^{b}	3.33	8.17	0.318	9.77^{a}	0.346	15.7	0.75	34.1	2.48	55.0	6.93	21.4^{b}	1.02

Mean values of silage samples predicted NIRS forage quality parameters and measured temperature between time points, days post aerobic exposure (column). Blocked ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters within column.

4.3.3.3. Temperature

The temperature of individual ASV was recorded continuously and values presented are those associated with experimental time points. The temperature was found to be significantly increased at day 32, relative to days 2, 4 and 8 post aerobic exposure. Temperature appears relatively high at 0 and 1 day before decreasing over days 2, 4 and 8. Temperature increases over day 16 before significantly increasing at day 32 post aerobic exposure. The largest cumulative increases in temperatures by bales peaked at either 16 or 32 days post aerobic exposure.

4.3.3.4. Listeria isolates

The differences in CFU of total *Listeria* spp. isolates were found to be statistically significant across the time points (P = 0.002). The CFU of total *Listeria* spp. was significantly higher at day 32 relative to days 0, 2, 4, 8 and 16 post aerobic exposure. Significant variation in CFU of *L. monocytogenes* similarly found across time points. The CFU of *L. monocytogenes* was significantly higher at day 32 relative to days 0, 2, 4, 8 and 16 post aerobic exposure. Summary statistics are presented in Table 12.

Table 12. Variations in total *Listeria* spp. and *L. monocytogenes* concentration (Log CFU g^{-1}) of silage samples by time points (days post aerobic exposure).

	Concentration (Log CFU g^{-1})			
Time point	Mean total *Listeria* spp.	sd. total *Listeria* spp.	Mean *L. monocytogenes*	sd. *L. monocytogenes*
0	0.47^{a}	0.411	0.31^{a}	0.350
1	1.20^{ab}	1.532	0.55^{ab}	0.877
2	0.56^{a}	0.760	0.36^{a}	0.566
4	0.08^{a}	0.210	0.00^{a}	0.000
8	0.81^{a}	1.000	0.30^{a}	0.533
16	0.90^{a}	1.265	0.41^{a}	0.555
32	2.97^{b}	2.301	1.55^{b}	1.210

Mean values of silage samples total Listeria spp. and L. moncytogenes concentration (Log CFU g^{-1}) between time points, days post aerobic exposure (column). Blocked ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters within columns.

4.4. Discussion

The study assessed potential microbial transmission risk at grazing and from preserved forage, highlighting spatial variation at both, which could be managed by adjustments to simple management techniques. Specific disease risks resulting from the aerobic spoilage of silage was further investigated, highlighting the impact on zoonotic *L. monocytogenes*.

4.4.1. Higher risk microbial contamination zones of grass at pasture: Experiment 1.

4.4.1.1 Forage quality

The variation in forage quality across a farming production system is most commonly considered at a field scale. Individual units of fields, or possibly separate paddocks, grazing or harvesting potential are assessed for their total production value not the variation they possess within their boundaries. This study identifies the spatial variation of grass at pasture in terms of forage quality, indicating specific potential production losses resulting from management strategies (Dorrough et al, 2004, Schwinning & Parsons, 2002, Wallis de Vries & Daleboudt, 1994). This study indicates associations between regions of high time spent by livestock (defined hotspots), both in transit and rest, with impacts upon forage quality and microbial contamination. OM (g kg^{-1} DM) was found to be different across the 'class' and 'distance' of sample points analysed. These differences in OM content may result from increased soil incorporation on the grass at pasture (Bilotta et al, 2007, Belnap & Eldridge, 2001, Scholefield & Hall, 1986). The lowest OM content of sample 'class' was found to be associated to trough and distance from defined hotspot found to be <10 m. As the trough 'class' and <10 m distance from hotspots are visibly the most disturbed by livestock locomotion the increased presence of soil is not surprising.

The likely negative impact of increased soil incorporation of these regions both at grazing and harvesting is particularly noteworthy, given the associated disease risks (Santamaría & Toranzos, 2003).

CP (g kg^{-1} DM) was also found to vary across distance from hotspots. The highest CP content indicated at sampling points <10 m proximal to defined hotspots compared to <30 m from defined hotspots and non-hotspot sampling zones. This may be the result of variations in DM content of grasses in the <10 m hotspot distance or due to the relative increased availability of nitrogen at this distance due to the higher livestock defecation and urination rates (Schuman et al, 1999). This further demonstrates the impact which high frequency livestock activity causes in relatively small defined regions within a field scale.

No differences were found in the elemental analysis of grass at pasture comparing hotspot and non-hotspot concentrations of various micro and macro mineral nutrients. The study concentrations found were considered in the satisfactory production range for equivalent pasture type compared to reference values (Germinal, 2015). This indicated that nutrient uptake did not differ significantly between hot spot and non-hotspot regions, although variation at a field scale was notable. Higher resolution elemental sampling may indicate spatial differences associated to defined regions within the field (Gupta et al, 2008, White & Zasoski, 1999, Reid & Horvath, 1980).

4.4.1.2. Faecal indicator organisms

The presence of FIO on pasture grazed by livestock is an unavoidable result of this management system. Indeed, the managed return of nutrients from faecal deposits to the soil is considered a beneficial source to support the growth of pasture, as discussed previously (Bilotta et

al, 2007, Clark et al, 1998). The concentrated faecal deposition in regions of high time spent by livestock does pose a potential disease risks.

A species difference was found in the concentration of *E. coli* and intestinal enterococci across sample 'class'. While intestinal enterococci concentration was not found to differ across sample 'class', *E. coli* CFU was higher in 'Trough' samples than 'Flume' or 'Non-hotspot' sample classes. This may be the result of the environmental resilience of *E. coli*, resulting in an observable difference between sample 'classes' (Van Elsas et al, 2011, Anderson et al, 2005). As the sampling was conducted on grass at pasture there is also potential for increased crop associated *E. coli* to result in a measurable difference between the sample classes (Jeter, & Matthysse, 2005, Islam et al, 2004, Solomon et al, 2002). A process of retainment is also considered likely in the soil matrix, where several factors permit the capture and survival of *E. coli* (Topp et al, 2003, Cools et al, 2001). The differences in species characteristics described is considered the principle cause of the disparity in *E. coli* and intestinal enterococci spatial variation.

The CFU of *E. coli* and intestinal enterococci were both found to be increased at <10 m proximal to defined hotspots, in *E. coli* relative to non-hotspot 'distance' and in intestinal enterococci relative to <30 m proximal to defined hotspots. Both FIO species exhibiting higher concentrations on grass immediately surrounding hotspot regions of increased livestock time spent indicates that this distance is particularly crucial in the transmission pathway of faecal contamination from pasture returning to livestock. The results support the findings of White et al (2001), who found significantly higher defecation rates within a 30 m surrounding of field water sources. Experiment 1 highlights the 'Trough' hotspot primary driver in field scale variation being livestock time spent, corresponding to increased defecation rate and subsequent higher faecal contamination of pasture (Betteridge et al, 2010, Oudshoorn et al, 2008).

The spatial variation in FIO concentrations at a field scale highlights the importance of an individually appraised grazing management strategy. These organisms are markers of livestock defecation rate and the findings in this study indicate a non-uniform distribution across the grazed fields, and consequently a non-uniform grazing and behavior pattern. As discussed previously, achieving consistent pasture utilisation is a key component in achieving optimal usage of grazed land. These results suggest that improved production rates could be attained by the introduction of further paddocks. The assessment of FIO concentration on pasture should supplement estimated yield measurement when making decisions to optimize grazing management.

There is also potential for significant health benefits derived from adjusted management techniques. The greatest risk of ingestion of potentially pathogenic organisms occurs proximal to defined hotspots. Applying additional paddocks, in a rotational grazing strategy, would reduce the continual burden and concentrated disease risk posed by these regions during grazing at a field scale. Additional and varing water sources would also be required. The survival and transmission of specific silage microbial contaminants, such as *E. coli*, through the rumen (Grauke et al, 2002, Donkersgoed et al, 1999) with persistence in the hind gut (Bertin et al, 2010, Callaway et al, 2003) is also well described.

The managed application of faecal material via slurry is also crucial in minimising risk for harvested of grass. In the absence of significant rainfall events Fenlon et al (2012), suggested *E. coli* from slurry application could be incorporated and proliferate to concentrations exceeding 10^6 g^{-1} in silage. Islam et al (2004), indicated the survival of *E. coli* originating from contaminated manure application for more than 5 months in soil regardless of crop type. However, Vinten et al (2006), found that the risk of wider water contamination was greater from land grazed by livestock

(drainage water *E. coli* counts represented 0.4% of estimated input) than that which had managed slurry application (drainage water *E. coli* counts represented 0.03% of estimated input).

4.4.1.3. Antimicrobial resistance

Variation in percentage antimicrobial resistant isolates was found across sample 'class' and 'distance' for various drugs. Amoxicillin /clavulanate, cefpodoxime, erythromycin, neomycin and tetracycline percentage resistant isolates all presented increases in trough or station samples, relative to non-hotspot and flume 'classes'. The <10 m or <20 m 'distance' proximal to hotspots presented the highest percentage of resistant isolates for cefpodoxime, cephalexin, erythromycin, neomycin and tetracycline relative to the non-hotspot and <30 m proximal to hotspot 'distances'. The greater prevalence of antimicrobial resistant isolates in specific sample 'classes' and 'distances' is considered the likely result of an increased transfer potential in regions of higher livestock origin microbe concentrations (Tenover, 2006, Lorenz & Wackernagel, 1994). This hypothesis is supported by the patterns of FIO concentrations on grass at pasture. The implication of specific regions of high livestock defecation rates, such as those proximal to the defined trough and station hotspots and not the passive hydrology driven flume hotspots is noteworthy (Singer et al, 2006). Previous studies have focused primarily on resistance patterns in soil (Joy et al, 2013, Knapp et al, 2010, Sayah et al, 2005). Experiment 1 confirms the AMR pattern found in soil in Chapter 3 translates to a similar distribution of grass at pasture.

This spatial variation in antimicrobial resistance presents similar opportunities for positive management interventions as described for FIO. Improved uniformity of grazing and livestock time spent could reduce the transfer potential of antimicrobial resistance genes, by minimising regions of high concentration faecal deposition. This could reduce the risk of livestock ingesting

grass contaminated with higher prevalence of AMR microbes and minimise potential for transfer orally (Duggan et al, 2000). The ability of *E. coli* to survive and thrive in the gastrointestinal tract of livestock, described in previous section 4.4.1.2., increases potential of AMR transfer and this experiment highlights the risk of transmission from high contamination zones of grass at pasture. The transfer of AMR in the digestive tract has been highlighted in human (Schjørring,& Krogfelt, 2011, Salyers et al, 2004) and animal studies (McCuddin et al, 2006, Russell & Houlihan, 2003, Scott, 2002). Eliminating regions of pasture with identified increased antimicrobial risk from inclusion in silage production could similarly benefit overall farm health. This is a consistently underestimated source of antimicrobial resistant microbes to livestock and one which unless actively targeted is self-perpetuating.

4.4.2. Effects of spatial variation and aerobic exposure on silage: bale quality and microbial contamination risk: Experiment 2 and 3:

4.4.2.1. Forage quality (Experiment 2 and 3)

The demonstration of forage quality variation within bales related to management of anaerobic conditions is of most immediate production importance. Highlighting the lack of uniformity both in the harvested crop and the individual bale ensiling process should be identified as an opportunity to reduce production losses. This study indicates associations between microbial contaminants, considered indicative of poor anaerobic stability, and parameters of forage quality.

The significant correlation across most measured forage quality values and NIRS analysis gave greater confidence in spatial differences observed. The NIRS prediction for key forage quality analyses was used in the absence of specific measured values (Beever and Mould, 2000). While

measured ash content and NIR prediction were not correlated in Experiment 3, both datasets indicated low values as reliable markers for low soil incorporation in all samples analysed (Wallsten, & Martinsson, 2009, Van Soest et al, 1991).

A notable association in increased CP in the 'outer' horizon was noted through application of NIRS predicted values in Experiment 2. This is not deemed of relevance to feeding potential and is hypothesised to be an artifact of higher DM losses (Savoie & Jofriet, 2003), possibly partially the result *E. coli* driven VFA production. In Experiment 2 CP also suggested a trend increasing from 'top' to 'base' position, as did lactic acid (g/kg DM) and VFA (g/kg DM). This is hypothesised to be the result of water flow associated concentrations. Similarly, ME indicated a non-significant upward trend from 'base' to 'top' position which could follow the same hypothesis as being driven by water movement to the bale base increasing relative fibre and thus ME content.

As described in Muck et al (2003), the high DM and resulting increased porosity of this silage resulted in greater susceptibility to aerobic spoilage. This study suggests that poor anaerobic stability negatively effecting forage quality in specific horizons of silage bales associated with microbial contaminants. This was supported by findings in Experiment 3 where silage DM increased and ME decreased at 32 days relative to days 0 and 1 post aerobic exposure, respectively. In neither Experiment 2 or 3 was pH found to have increased over the course of the study period, as may have been expected during aerobic spoilage (Wilkinson & Davies, 2012, Weinberg et al, 1993). The effects on DM and ME content are indicative of this process occurring (Tabacco et al, 2011). Although Honig (1987) indicated correlation between high silage DM and density this did not account for a dynamic process of DM losses as occurs during aerobic exposure.

However, even in undamaged anaerobically stable bales air ingress can occur due to the relatively high oxygen permeability of commonly used polyethylene wraps (Borreani & Tabacco

2010[a]). This can supply facultative anaerobes with oxygen in the external regions of silage bales as demonstrated in both Experiment 2 and 3. The low concentration of soil incorporation in both Experiment 2 and 3 indicates that the principle driver of reduced forage quality in the external horizon of silage bales is the process of aerobic exposure (Woolford, 1990, Muck et al, 1988)

4.4.2.2. Temperature (Experiment 3)

Temperature is the key indicator of aerobic spoilage of silage (Wilkinson, & Davies, 2012, Borreani & Tabacco, 2010[b]). Experiment 3 found non-uniformity in temperature across the bales sampled over the days post aerobic exposure. Results found cumulative increases of 1.70 °C, peaking at 16 or 32 days post aerobic exposure. Thus although aerobic spoilage is found to have occurred, the variation between bales in increased temperatures suggests that certain bales received previous aerobic challenge during storage prior to experimental exposure. This previous aerobic challenge is suggested by the variance in temperatures of certain bales at days 0 and 1 post aerobic exposure and the corresponding microbial profiles support this hypothesis. The temperature results for all silage bales presented a pattern of reduced and stable temperature from days 2 - 8 post aerobic exposure. The measured initial decrease or lag in silage temperature when placed into an experimental aerobic stability vessel is well documented (Jungbluth, 2017, O'Kiely & Muck, 1992). Wang & Nishino 2013, indicated a similar lag period in temperature increase or aerobic spoilage following 90 days ensiling. This is supported by Koc et al (2009) who found significantly higher aerobic spoilage in silage storage at 30-37 °C compared to 20 °C after 45 days ensiling. The variation in temperature profiles across bales and the elevated temperature in some would appear to suggest that specific bales were previously exposed to oxygen during the ensiling process while anaerobicity was maintained in others. This result affirms the occurrence of aerobic spoilage

occurring between 16 – 32 days post aerobic exposure. Andrieu & Demey 2015 indicated that significantly lower temperature in higher density silage storage vessels (238 ± 48 kg DM/m^3) compared to those of lower density (209 ± 47 kg DM/m^3). The variation in temperature profiles found in Experiment 3 is suggested in part to result from this variation in original silage bale density and compaction within the aerobic stability vessels.

4.4.2.3. Faecal indicator organisms and Listeria isolates (Experiment 2 and 3)

It is not feasible or indeed beneficial to attempt removing all non-fermentation beneficial microbial contaminants from silage (Wardrope and Macleod, 1983). While sterile production should not be a management strategy neither should the maintenance of optimised environments for proliferation. Both Experiment 2 and 3 indicated low soil incorporation (Wagner et al., 2005, Jiang et al, 2004, Low and Donachie, 1991) and no increases in pH (Ryser et al, 1997, Clarkson and Faull, 1987; Low and Renton, 1985), commonly associated with proliferation of undesirable microbes in silage. Ash content of over 10 % is generally considered an indication of soil contamination with corresponding impacts upon fermentation (Cherney et al, 2016). The spatial variation of FIO and the increased *Listeria* isolate concentrations following aerobic challenge, found in Experiment 2 and 3 respectively, are thus considered to principally result from favourable growth conditions in the presence of increased oxygen.

Modifications to harvesting strategy are considered to offer mutual health and feed quality benefits. The negative influences these regions of pasture elicit upon resulting silage is not proportional to the perceived yield benefit that their inclusion would justify. Precision harvesting would eliminate the most contaminated regions of grass, both in terms of FIO and soil concentration, from silage production. The beneficial impacts on silage yield are considered

significant due to improved silage quality and reduced wastage at point of feeding. Animal health would also be positively impacted from implementing such a winter feed harvesting strategy.

The significant association of increased *E. coli* concentration to the 'outer' horizon along with the converse relationship by intestinal enterococci found in Experiment 2 supports the assessment of particularly poor bale wrap integrity. As this external horizon is vulnerable to air and oxygen ingress even in well maintained bales, as found by Borreani & Tabacco (2010[a]), increased concentrations of microbial contaminants may always be considered (Wilkinson and Davies, 2012). A combination of diffusion and pressure differences will result in increased aerobic conditions in this horizon (McEniry et al, 2007, Huhnke et al, 1997, Parsons, 1991). Increased oxygen content results in a less efficient fermentation process.

Increased aerobic conditions such as those studied spatially in Experiment 2 or over time post exposure as in Experiment 3 result in a more enterobacterial driven fermentation process with corresponding increased acetic and lower lactic acid concentrations. Inefficient fermentation would also impact the described trends of increased DM and ME of bales through increased DM losses. This in itself would impact the observed localization of undesirable microbial species (Naoki and Yuji, 2008).

The increased lactic acid concentrations resulting from more efficient fermentation is considered a more favorable environment for intestinal enterococci growth. As lactic acid producers, this environment may partially explain the increased concentration found in Experiment 2 of intestinal enterococci at the 'inner' depth (Li et al, 2015) and the corresponding localisation of the relatively less lactic acid tolerant mixed acid fermenter *E. coli* to the 'outer' (Förster and Gescher, 2014, Chen et al, 2005). The results of Experiment 2 align with previous findings by Naoki & Yuji (2008), who indicated a trend of increased lactic acid bacteria concentration in the

internal compared to external horizon of baled silage. The findings of Experiment 2 also further those from the spatial assessment of baled silage conducted by McEniry et al (2006), who found significantly increased *Enterobacteria* in the external (1.44 $\log_{10}$ CFU g^{-1}) compared to internal (1.07 $\log_{10}$ CFU g^{-1}) horizon of baled silage. A similar association of increased concentration of *Listeria* isolates was found in Experiment 3 as aerobic spoilage occurs and fermentation becomes less lactic acid driven. This is supported by the mixed fermentation characteristics of *Listeria* (Orsi & Wiedmann, 2016, Romick et al, 1996, Daneshvar et al, 1989) and its preference of acetic (Gonzalez-Fandos & Herrera, 2014, Buchanan et al, 1993) over lactic acid conditions (Scatassa et al, 2017, Hwang et al, 2012). This also aligns with the findings of Pauly & Tham 2003, who demonstrated *L. monocytogenes* survival following 30 days ensiling only in silages of high DM (430 and 540 g DM kg^{-1}) compared to those of low DM (200 g DM kg^{-1}).

The significance in aerobic spatial localisation of *E. coli* found in Experiment 2 as well as in *L. monocytogenes* concentration correlating with temperature and thus aerobic spoilage in Experiment 3 are both considered to be driven by preferred growth conditions and superior competitive fitness (Bachmann et al, 2017, Fisher & Phillips, 2009, Byrne et al, 2002). These findings while acknowledging that minimising the incorporation of soil must be a priority in silage production (Vivant et al, 2013, Fenlon et al, 2012, Nicholson et al, 2004), highlight the critical role of achieving and maintaining anaerobicity. This study suggests that in the absence of excessive soil inclusion and at non-elevated pH, *L. monocytogene*s and *E. coli* can occur and proliferate in silage due to aerobic exposure. This finding has immediate relevance within industry and reinforces the necessity to practice and review aerobic stability of silage production to storage. This work also highlights the importance of a managed strategy for silage at point of feeding, as a pre-requisite in addition to optimising production techniques. Extended feeding out periods,

without the removal of older material, will expose livestock to silage with an increased risk of elevated *L. monocytogenes* concentrations as demonstrated in Experiment 3. Consumption of silage previously aerobically challenged, possibly during storage, risks increased exposure to microbial contaminants and potential pathogens such as *E. coli* as found in Experiment 2.

4.4.2.4. Antimicrobial resistance (Experiment 2)

The association in higher percentage cephalexin and erythromycin resistant isolates, in addition to the presumptive ESBL- producing and multiple drug resistant isolates with the 'outer' depth has implications for effective protection of animal health. As described in section 4.4.1.3. the potential for AMR transfer in the gastrointestinal tract of human and animals is significant. The exchange of AMR elements has been indicated in model rumen environments by Toomey et al (2009), and *in vitro* by Carvalho et al (2011). Both these studies focus on lactic acid bacteria, whose interaction with faecal indicator organism during ensiling is significant (Driehuis et al, 2002, Cai et al, 1999) and its subsequent survival in the rumen (Weinberg & Ashbell, 2003) could increase risk of AMR transfer. However, lactic acid bacteris are considered extremely beneficial in silage fermentation and ultimately its aerobic stability (Pang et al, 2011, Cai et al, 1999, Weinberg et al, 1993), often being applied as additives to improve production (Reich & Kung, 2010, Filya, 2003, Driehuis et al, 2002). The impact of lactic acid bacteria on reducing the likely survival of microbial contaminants during ensiling is therefore beneficial for silage nutritional and hygienic quality.

The role of a forage based diet has long been identified by Brownlie & Grau 1967, more recently by Callaway et al (2009), in reducing the survival and shedding of *E. coli* in livestock through altered rumen digestion conditions. However, Jacob et al (2009), does identify that many

such studies are often not replicable, attributing this to the complex and individual animal specific interactions of forage based diets on lower digestive tract ecosystems. As described in section 4.4.1.2. the capacity of *E. coli* to survive and thrive in the gastrointestinal tract of livestock poses a risk for greater AMR transfer and this study highlights the role of silage in fueling that process.

The increased percentage of cephalexin and erythromycin resistant isolates in the 'outer' depth corresponds to the increased *E. coli* concentration of this horizon. As indicated in section 4.4.1.3. there is significant variance in resistance patterns across environments and individual systems drug usage. While the results must acknowledge limitations in isolate numbers tested, the resistance pattern is consistent with that found on grass at pasture in of Experiment 1 and in previous studies (Sayah et al, 2005). An increased likelihood of AMR conferring genes occurring at a population level in these higher microbial concentrations, and thus greater potential of transfer is considered the principal reason for spatial variation (Tenover, 2006, Lorenz & Wackernagel, 1994). McMahon et al (2007), indicated evidence of increased rate of specific AMR plasmids transfer in *E. coli* in the presence of environmental stresses such as low pH 4.3 and low 5 °C to moderate 37 °C temperatures. These stresses correspond to pH and temperature in Experiment 2 silage bales and to ensiled conditions more generally (Pakarinen et al, 2008, Williams et al, 2008), further indicating the important role silage plays in AMR development.

It is of note that both the presumptive ESBL- producing (Wellington et al, 2013, Hartmann et al, 2012, Reinthaler et al, 2010) and multiple drug resistant isolates (D'Costa et al, 2007, Reinthaler et al, 2003) were cultured from the 'outer' depth 'base' position. This may further support the hypothesis of an increasing trend in *E. coli* concentration from 'top' to 'base' position and as discussed, increase potential of AMR development. To definitively establish spatial

resistance patterns in silage would require subsequent studies to test increased number of isolates and silage from additional sources potentially applying selective isolation techniques.

4.5. Conclusions

Grazing and preserved forage supply the majority of nutritional requirements for UK ruminant livestock diets. This study suggests specific management interventions exist at the field and preserved forage scale, to optimise productivity and health within grassland livestock production.

Increased faecal origin microbe concentrations, antimicrobial resistance prevalence and reduced forage quality of grass growing at pasture within 10 m proximal to defined field hotspot regions (water trough and weather stations) was found. The findings of grass at pasture correlate with Chapter 3, highlighting the interconnectivity between crop and soil at hotspots. These findings clearly demonstrate the impact of sup-optimal grazing management on specific regions in fields. In addition to adjusting grazing management precision forage harvesting which avoids these regions could provide production and health benefits. The impact of field microbe risk hotspots on resulting water outflow is examined in Chapter 5

The designated ‘outer’ bale depth has critical economic importance to farmers, not merely by volume but often as the initial portion consumed by livestock (Stockdale, 2010, Weinberg & Ashbell, 2003). The correlation between microbial contaminant and nutritional differences of bales, depths, and temperatures, highlight the opportunity to achieve mutual benefits to health and production through improved farmer management at wrapping, stacking and feeding out. Ensuring initial anaerobicity is maintained throughout storage and minimizing the period of aerobic exposure during feeding should be reviewed as a production priority. Microbial conditions on pasture can influence silage production. Aerobic exposure of silage is critical in generating conditions supportive of microbial contaminant survival and proliferation, decreased nutritional quality, increased disease and AMR potential. The role of silage as a transmission route of potentially pathogenic and AMR bacteria to livestock is currently underestimated.

Chapter 5

Water- Assessing the influence of field scale hydrology on transmission of livestock origin microbes, antimicrobial resistance and key environmental markers.

Summary

The hydrological transmission of potentially pathogenic and AMR bacteria from agricultural pasture to natural water courses is well recognised as a crucial route for the wider dissemination of microbial hazard. This catchment scale study investigated the accuracy of automated water sampling in microbial monitoring, compared the microbial risk of differing climatic and associated outflow conditions, and developed an alternative model for predicting potentially pathogenic microbial concentrations in storm outflow. Utilising the unique hydrological infrastructure of the North Wyke Farm Platform, sampling was conducted across multiple catchments, simultaneously recording flow conditions and microbial parameters. The accuracy of two alternative automated microbial water sampling protocols was trialed against manual sampling. Applying pre-sampling sterilisation to all sample contactable components of the automated sampled was found to produce microbial concentration results comparable to manual sampling. This protocol could be employed within monitoring programmes to better target mitigation schemes. The assessment of potential microbial hazard was measured across designated seasonal baseflows and winter stormflow events. Highlighting the resulting outflow following storm events as possessing increased risk of potentially pathogenic microbe concentrations and percentage AMR isolates. This has potential in informing future research and stakeholder management strategies during specific high risk periods such as the predicted increase in winter storm events. The development of an accurate model of *E. coli* concentration based on flow rate and hydrograph slop suggests a possible alternative method to provide rapid and economical predictions on microbial risk.

5.1. Introduction

In Chapters 3 and 4 the cumulative impacts of grazed livestock management upon pasture were investigated. These Chapters highlighted the potential of soil and growing pasture to capture microbes of faecal origin and facilitate their reintroduction to livestock on-farm. However, the preliminary lysimeter study in Chapter 2 indicated the significant transmission possible through soil and into a modelled watercourse. The role of hydrological transfer from agricultural land into natural watercourses and the wider environment is well documented (Woolhouse et al, 2015, Worral et al, 2009, Hooda et al, 2000). This route is considered the principle mechanism by which microbes and antimicrobial resistance of agricultural origin is disseminated off farm (Craft, 2015, Woolhouse et al, 2015). Hydrological transfer is also the most significant factor in environmental pollution resulting from chemicals of agricultural origin (Worral et al, 2009, Jordan & Smith, 2005).

Previous research acknowledges the duality of surface and subsurface water flows in contributing to the total discharge of water and associated solutes at landscape scale (Oliver et al., 2018). The unique engineering and soil classification of the North Wyke Farm Platform ensures that at a field catchment scale a more complete capture of all water flows is possible (Takahashi et al, 2018, Orr et al, 2016). As described in Chapter 1, the soil type and infrastructure of the North Wyke Farm Platform (NWFP) enables studies to assess characteristics of water discharge from fields by overland and inter flow.

This Chapter will examine the route of external transfer and attempt to indicate specific temporal and spatial indicators for increased microbial risk. The negative externality for public health (Craft, 2015, Woolhouse et al, 2015) and the environment (Eccard et al, 2000, Milchunas & Lauenroth, 1993) driven by hydrological transfer originates from farm processes indicated in

Chapters 3 and 4. In this research the role of soil and grazed or fed pasture on-farm is principally one of maintaining and reintroducing microbes of livestock origin. The discharge of water associated microbes from agricultural land is the quantifiable result of insufficient management of these sources in soil and pasture, as described in Chapters 3 and 4. The success of any on-farm management of microbial cycling will be widely perceived by the effects upon the risk of water discharge to the wider environment (Woolhouse et al, 2015). This is particularly true in mitigating the dissemination and proliferation of environmental AMR (Sayah et al, 2005, Hooda et al, 2000). Hydrological transfer is the major exposure pathway for AMR genes accumulated on-farm, principally on pasture, to be transferred beyond system boundaries and impact the viable treatment of clinically relevant microbes. This Chapter will combine microbial assessments along with detailed flow metrics and chemical characteristic to provide a holistic assessment of the external risk from fields water discharge.

5.1.1. Field scale outflow sampling

There is a need to describe the role of hydrological action in the dissemination of agricultural origin microbes at field and catchment scale (Oliver et al, 2018, Hodgson et al, 2009). Evaluating the impact of these units upon the wider external environment is of vital importance to the farmer in assessing the success of upstream management strategies employed to minimise risk perpetuated on-farm (Calloway et al, 2010, Lawlor et al, 2000). This is crucial in contextualising microbial dissemination risk at a field or farm and not the notional landscape scale for stakeholders.

The NWFP catchments were developed to emphasise natural watersheds and are discrete to one or two management fields. This produces results with greatest production system relevance, and when combined to the findings of Chapters 3 and 4 indicates the external microbial hazard from the well characterised on-farm microbial cycle.

The constructed flumes channelling each catchment's out flow enable the simultaneous measurement of a variety of metrics (Takahashi et al, 2018, Orr et al, 2016). This Chapter investigates the correlation between specific microbe concentrations and these supplemental flow measures across multiple catchments flumes. Combining measures of out flow characteristics such as flow rate, dissolved oxygen, pH, turbidity, with analysis of Nitrogen and Phosphorus concentrations to microbial concentrations provides a comprehensive assessment of conditions. Alongside the primary information concerning agricultural origin microbe dissemination to the wider environment, the additional factors describe the water conditions which facilitate organism survival (Anderson et al., 2005). The additional metrics also provide meaningful information for stakeholders regarding budgeting of Nitrogen and Phosphorus losses alongside microbe concentrations (Vinten et al, 2017, Worral et al, 2009, Jordan & Smith, 2005). Correlations between the various flow characteristic metrics and microbe concentrations could allow a more dynamic management strategy and provide a predictive marker of microbe dissemination risk.

5.1.2. Sampling method: automated and manual

Due to the farm scale sampling proposed and simultaneous analysis of multiple metrics, the protocols were optimised for time and materials. Automated sampling of water is well established across industry and research (Harmel et al, 2018), particularly for concurrent sampling, however not within regulatory microbial sampling. Manual sampling of water for microbial analysis, involving the collection of samples by individual containers isolated from each other, remains the most established methodology (Harmel et al, 2018). Manual sampling eliminates the risk of cross contamination between samples and ensures results are a true representation of water conditions at point of sampling. Automated sampling principally reduces reliance on personnel,

particularly when sampling concurrently across several sites. Automated sampling also improves the accuracy of sampling points at specified criteria such as temporally and at given flow rates or temperature. Automated sampling and analysis of flow characteristics such as rate, dissolved oxygen, pH and turbidity can be programmed to trigger in given conditions or temporally (Orr et al, 2016). This allows flexibility in sampling protocol and when coupled to sampling of Nitrogen, Phosphorus and microbes can trigger sampling in specified flow conditions.

However automated sampling of water associated microbes introduces complications in ensuring accuracy and validity of sequential sample results. Issues such as carry over between sample points and the effect of biofilms (Richards et al, 2018) have been cited as key factors in reduced reliability compared to individual manual sampling (Harmel et al, 2018, Searcy et al, 2018). Several studies have indicated that given sufficient prerequisites are applied to automated methodology result accuracy compared to manual sampling is not compromised (Searcy et al, 2018, Farnleitner et al, 2017). A lack of consensus remains as to the validity of automated compared to established manual microbe water sampling and to the most accurate methodology for automated sampling. This necessitates most studies, particularly larger scale sampling protocols, provide an experimental comparison of their designated automated sampling accuracy with manual sampling.

As the application of automated microbe water sampling becomes more prevalent in industry, government and research there is an increasing need to demonstrate methods which provide comparable accuracy to manual techniques (Harmel et al, 2018, McGonigle et al, 2012, Sudol & Ambrose, 2002). The widespread adoption of economic and accurate automated water sampling could enable real-time landscape scale monitoring of water flows microbe risk. This

could better inform targeted interventions to manage specific spatial and temporal risks arising from dissemination of agricultural origin microbes.

5.1.3. Research aims

The role of hydrological transfer, with water acting as the principal vehicle in external transmission for the on-farm microbial cycle, is acknowledged as critically important in the preservation of human and animal health. In this chapter the North Wyke Farm Platform permanent pasture is utilised to test the overall hypothesis that: the risk posed by the hydrological transmission of potentially pathogenic and AMR bacteria from pasture to the wider watercourse varies significantly with rainfall, land management and water flow conditions.

Hypothesis 1 – Automated water sampling protocols accurately correlate with manual sampling results for concentration of faecal indicator organisms.

Hypothesis 2 - The concentration of faecal indicator organisms and antimicrobial resistant bacteria in natural water courses correlates with specific water flow conditions.

5.2. Materials and methods

Water sampling for all metrics was conducted utilising the engineered flume outflows for NWFP catchments 4, 5 and 6 of the North Wyke Farm Platform (Orr et al, 2016) as shown in Figure 1 (constituting fields: Bottom Burrows and Burrows, Orchard Dean North and Orchard Dean South, Golden Rove). These catchments and their constituent management fields represent the principal production units of the Permanent Pasture (PP) system. The 'semi-rotational continuous (variable)' grazing strategy utilised in this study results in multifunctionality of fields within catchments. At time of water sampling catchments 4 (Bottom Burrows only) and 5 (Orchard Dean North only) were not grazed and under silage production. Catchment 4 (Burrows only), 5 (Orchard Dean South only) and 6 were under grazing rotation of the beef herd (catchment 4) and sheep flock (catchment 5 and 6). Sampling was conducted across grazed and mixed grazed/ungrazed catchment field use to enable analysis of any differences in FIO concentration and % AMR resulting from temporal differences between periods since livestock contact. The catchments soil moisture station/rain gauge are calibrated to allow comparison and informed sampling protocol and sampling event designation at described in Sections 5.2.1. and 5.2.2.

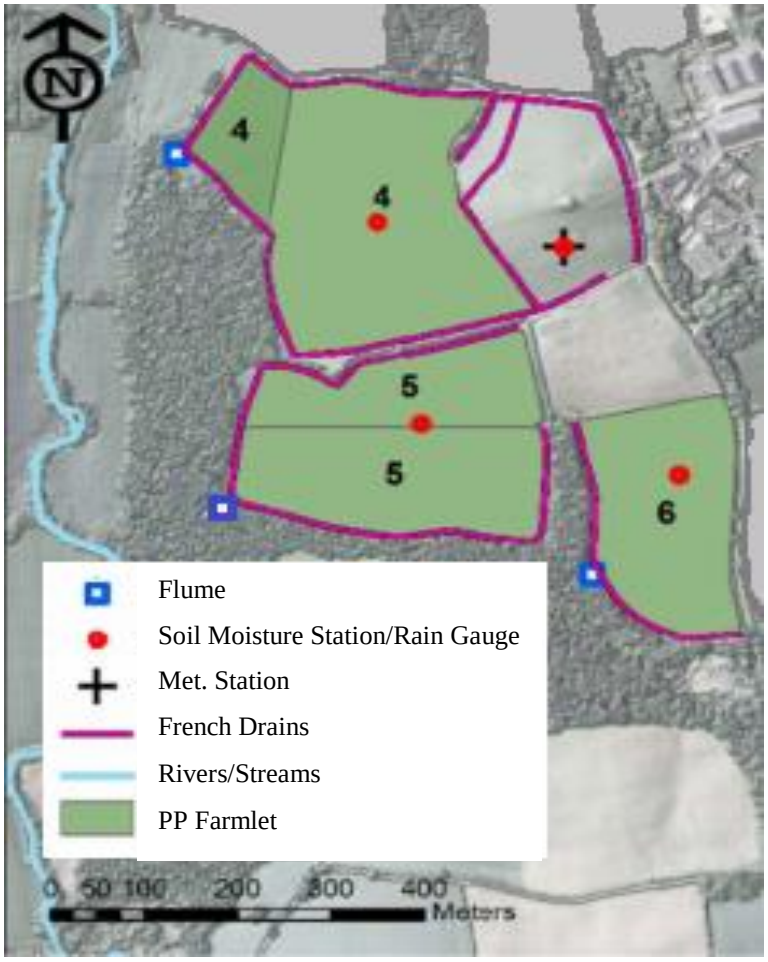

Figure 1. Study catchments 4, 5 and 6 of the NWFP Permanent Pasture (PP) farmlet. *Constituent management fields of catchments indicated by corresponding number. Terrain derived french drain boundaries and catchment flume out flow points are indicated. Locations of catchment and PP farmlet associated soil moisture station/rain gauge and Meteorological station, respectively, are presented.*

The engineered flumes and water sampling sites, shown in Figure 2, permit the singular sampling of the associated catchments representative outflow, as described in Chapter 1. All microbial, water flow characteristics, Nitrogen and Phosphorus sampling was conducted at the same location within the water sampling site across catchments 4, 5 and 6 and all sample events.

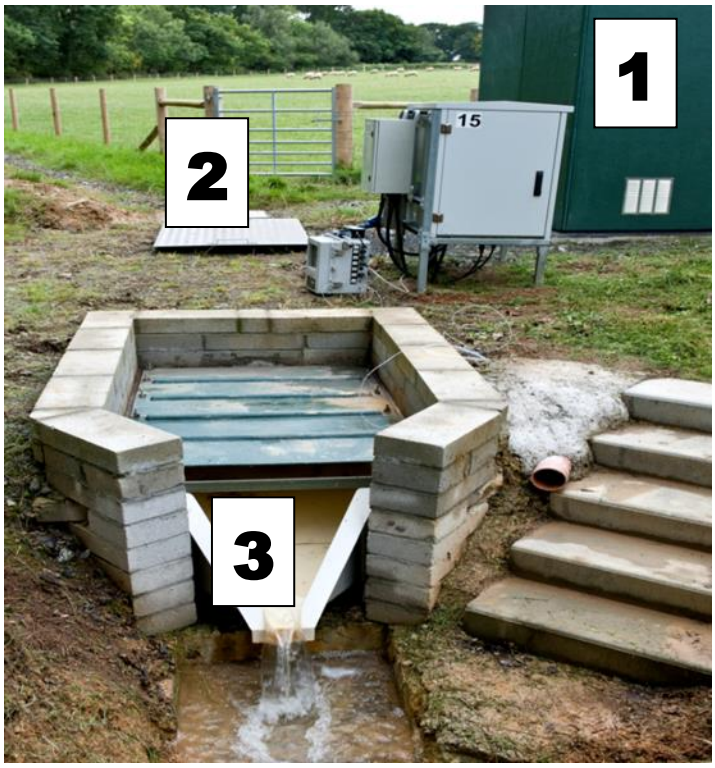

Figure 2. NWFP representative catchment outflow and study sampling point.
1) Catchment associated water flow analysis station. 2) Water outflow sampling site. 3) Flume out flow to wider natural water course

5.2.1. Categorisation of water flow sampling

The process of water sampling remained uniform throughout all work as described in Sections 5.2.3.-5.2.6. Sampling events were categorised as summer baseflow, summer stormflow, winter baseflow and winter stormflow. These definitions and their associated parameters enabled separation of study periods and targeting of resources to periods of greatest interest. Combining NWFP site records (Flow rate, soil moisture and rain gauge measures) and archive meteorological data (Meteorological Office, 2018, Orr et al, 2016) the parameters of sampling period categories were defined as presented in Table 1.

Table 1. Definitions of water flow sampling category parameters
Categories of water flow sampling as characterised by sampling period and flow rate. Parameters were defined through compiling NWFP site records and meteorological data.

Category	Sampling period	Flow rate (l/s)
summer baseflow (SB)	1 May – 30 November	<0.5
summer stormflow (SS)	1 May – 30 November	>0.5
winter baseflow (WB)	1 December – 30 April	<0.5
winter stormflow (WS)	1 December – 30 April	>0.5

These defined categories aimed to maximise the recording of specific seasonal variations such as infiltration excess flow, primarily in periods of dry soils (soil moisture deficit), and saturation excess flow, in periods of high moisture content soils. This distinction in sample events contextualised results and informed subsequent targeted sampling of categories indicated as posing the greatest risk in microbial dissemination. Categorisation of sampling events as either base or storm flow was designated based on flow rate through catchment outflow. Sampling conducted in conditions not exceeding 0.5 l/s were designated as summer or winter baseflow according to the corresponding sampling period. This base flow rate is in accordance with previously recorded values at NWFP. Baseflow samples were taken every 15 minutes. Storm sampling targeted the outflow following rain events, as described in Section 5.2.2. This required flow rate exceeding the base flow of 0.5 l/s for two or more consecutive sample points. Sample points were triggered based on flow thresholds, presented in Table 2. When flow rate crossed from one boundary to another, either increasing or decreasing, a sample was triggered. Storm events included sample points immediately prior and subsequent to flow rate exceeding the base flow threshold of 0.5 l/s, to ensure comprehensive measurement of parameters on hydrograph rising and falling limbs.

Table 2. Water flow rate sampling thresholds
Water sampling flow rate thresholds. Individual sample points triggered upon flow rate change from one boundary to another.

Threshold	Flow rate (l/s)
A	<0.3
B	0.5-1.0
C	1.1-1.5
D	1.6-2.0
E	2.1-2.5
F	2.6-3.0
G	3.1-4.0
H	4.1-5.0
I	5.1-7.0
J	7.1-9.0
K	9.1-12.0
L	12.1-15.0
M	15.1-18.0
N	18.1-21.0
O	21.1-25.0
P	25.1-30.0
Q	30.1-40.0
R	40.1-50.0
S	50.1-70.0
T	70.1-100.0
U	100.1-160.0

5.2.2. Meteorological sampling parameters

Sampling periods were planned using meteorological estimates, these were combined with previous NWFP data on outflow lag period related to total precipitation and intensity. This allowed predictions of when to initiate stormflow sampling enabling capture the rising limb of the hydrograph. The example outflow rate and precipitation, shown in figure 3, indicate that high volume and intensity rainfall event required outflow sampling to be initiated simultaneously with precipitation. Less intensive rainfall required a delay in initiating outflow sampling, to minimise the capture of unwanted pre-stormflow baseflow samples. Given discrepancies in meteorological forecasts, caution was exercised and sampling was initiated to ensure transition from baseflow to stormflow was fully captured. Baseflow sampling proved simpler in planning with only

requirement to avoid periods of expected precipitation. The weather conditions over the sampling period resulted in summer stormflow events occurring with extreme infrequency. The volume and intensity of precipitation events required to generate summer stormflow conditions did not occur on sufficient occasions to produce required replication for inclusion in the study. Analysis and results comparing the accuracy of automated and manual sampling methods are therefore limited to SB, WB and WS conditions.

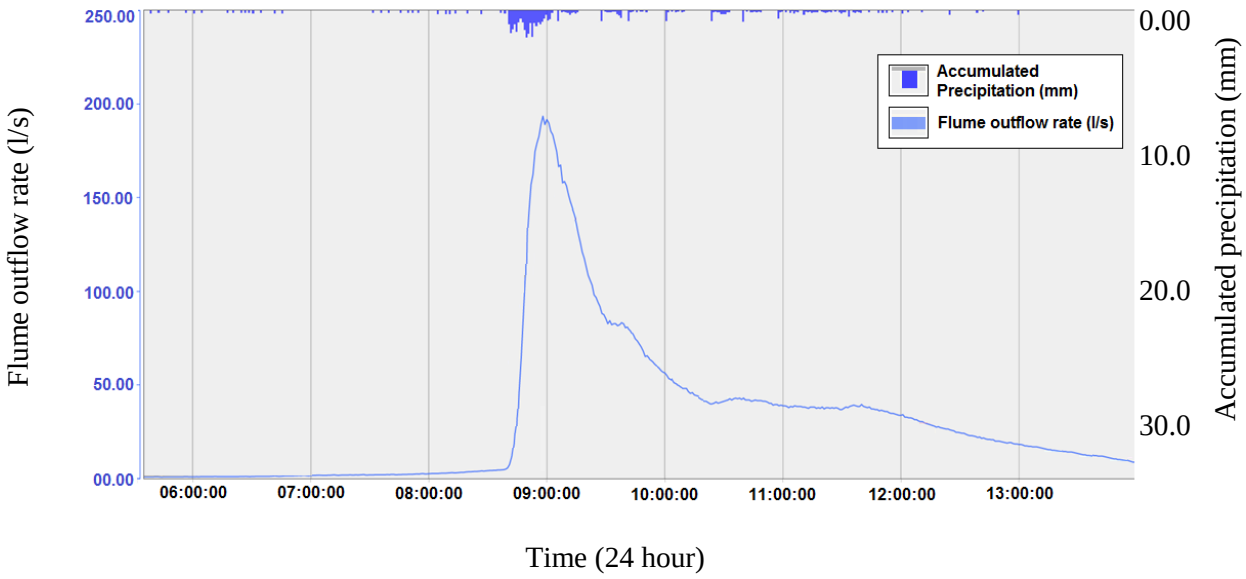

Figure 3. Example of NWFP catchment 4 representative stormflow with corresponding precipitation.
Catchment 4 associated outflow rate (l/s) against time and associated accumulated precipitation (mm/minute).

5.2.3. Automated and manual water sampling, experimental protocol

Automated and manual *Escherichia coli* and intestinal enterococci water sampling was conducted during the designated event categories (SB, WB, WS) to compare the accuracy of the two automated sampling protocols relative to manual sampling. This comparison enabled future sampling to have confidence in automated sampling protocol accuracy. The results from the event

sampling also directed future sampling to target specific event category (WS). The subsequent comprehensive automated sampling of WS events included concurrent automated sampling for Nitrogen and Phosphorus concentration analysis alongside automated microbe sampling and measurements of outflow water conditions.

Automated sampling utilised the ISCO 3700 full-size sampler (Teledyne ISCO, Lincoln, Nebraska, USA). The samplers were stored in the catchment associated analysis station and sample pipe attached within the water outflow sampling site. Samplers were programmed based on the sample event, temporally sampling every 15 minutes during baseflow and sampling based on outflow rate during stormflow conditions (as described in section 5.2.1). The 'existing' automated protocol required no amendments were made to the sampler set up and samplers were left *in situ* between events as was normal practice. The 'treated' automated protocol required all parts of sampler in contact with water samples were sterilised by autoclave 121.0 °C (±0.25 °C) for 20 min. This included sample piping and peristaltic piping. Both existing and treated sample collection bottles were sterilised by autoclave between samplings. Automated samplers were limited to 24 sample points and this was the maximum taken during individual sampling events. Manual sampling, undertaken alongside automated sampling, was conducted using individual sterile sample bottles submerged at the same position within the water outflow sampling site. Thus, manual sampling was collected from the same position and same time as associated automated sampling.

All water samples collected were 400.0 ml (±5.00 ml) volume and stored either within the automated sampler insulated collection compartment or within an insulated storage box until sampling completion. Water samples were returned immediately to the laboratory for analysis of microbe, Nitrogen or Phosphorus concentration.

The evaluation of existing and treated automated sampling accuracy compared to manual sampling was a methodological requirement in this study. This concurrent *E. coli* and intestinal enterococci sampling by triplicate was completed over 5 instances of SB, WB and WS conditions over all three catchments. Comprehensive sampling of *E. coli*, intestinal enterococci, *L. monocytogenes*, Nitrogen and Phosphorus concentrations alongside measurement of flow rate, dissolved Oxygen content, flow temperature and flow turbidity outflow water conditions, were subsequently undertaken across all three catchments. This sampling was conducted over WS events using the sterilised treated automated sampling protocol.

5.2.4. Microbial analysis

The analysis of *E. coli* and intestinal enterococci concentration was completed on all samples: evaluating automated and manual sampling protocol accuracy; comparing microbial hazard of SB, WB, WS event categories; and comprehensive sampling of WS. The analysis of percentage antimicrobial resistant isolates was completed using the manual samples and used to compare the risk of SB, WB, WS event categories only. Analysis of *L. monocyctogenes* concentration was completed during the comprehensive sampling of WS only.

5.2.4.1. Faecal indicator organism culturing

A serial dilution of water samples was completed to enable standard membrane filtration as described by Hodgson et al (2016). Samples were washed through the filtration manifold unit with 20 ml of sterile Ringer's solution. Membrane filters of 0.45µm (Sartorius Stedim Biotech, Goettingen, Lower Saxony, Germany) were placed onto species selective sterile agars for FIO culturing. *E. coli* growth was targeted on Membrane Lactose Glucuronide Agar (Oxoid,

Basingstoke, Hampshire, UK) and incubated for 18-24 h at 44.5 °C (±0.20 °C) (Hodgson et al., 2016). Intestinal enterococci growth was targeted on Slanetz and Bartley agar (Oxoid) and incubated for 44-48 h at 37.5 °C (±0.20 °C) (Hodgson et al, 2016). Confirmation of intestinal enterococci concentrations were cultured on Kanamycin aesculin azide agar (Oxoid) plates pre-warmed to room temperature, and incubated at 37.5 °C (±0.20 °C) for 4 h (±5 minutes). All samples were processed in triplicate throughout culturing. The initial FIO counts were transformed to Log_{10} colony forming units (CFU) ml^{-1}.

5.2.4.2. Listeria *culturing*

The enumeration of both total *Listeria* spp and *L. monocytogenes* isolates was undertaken by the rapid enumeration method (Oxoid, UK). Each 25 ml sub sample, representing an individual sampling unit, was added to 225 ml of sterile Ringer's solution. The mixed sample and ringers solution was then incubated for 1 h at 20.0°C (±0.20°C). A serial dilution was then completed, permitting calculation of CFU, before inoculation of brilliance *Listeria* (Oxoid, UK) selective plates. Inoculated plates were incubated for 48 h targeting *Listeria* spp. growth at 37.0°C (±0.20°C) (Kathariou et al, 1994, Young & Foegeding, 1993). Presumptive *Listeria* spp. (blue/green colony) and *L. monocytogenes* (blue/green colony with opaque halo) isolates were confirmed by combination of gram test and selective API biochemical testing (Setiani et al, 2015). The *L. monocytogenes* CFU counts from all log dilutions were normalized by transforming to Log_{10} colony forming units (CFU) ml^{-1}.

5.2.4.3. Antimicrobial resistance analysis

Assessment of AMR prevalence, from automated water samples, was tested by Kirby-Bauer disc diffusion test (Bauer et al, 1959). Representative *E. coli* colonies were isolated in triplicate from each experimental unit on non-selective, Mueller-Hinton agar (Oxoid), incubated for 24 h at 35.0 °C (±0.20 °C) (Tenover et al, 2001). Cells were then suspended in phosphate-buffered saline (PBS) to a concentration of 1.5 x 10^8 CFU ml^{-1}, visually assessed against a 0.5 McFarland standard (Wiegand et al, 2008). Mueller-Hinton agar plates were immediately inoculated by spread plating prior to the application of 6 antimicrobial susceptibility testing disks (Oxoid) and incubated for 18 h at 35.0 °C (±0.20 °C) (Jorgensen & Turnidge, 2007). Antimicrobials used and their amounts were: amoxicillin/clavulanate (30 µg), cefpodoxime (10 µg), cephalexin (30 µg), doripenem (10 µg), enrofloxacin (5 µg), erythromycin (15 µg), florfenicol (30 µg), neomycin (30 µg), sulphisoxazole (300 µg) tetracycline (30 µg). These were selected to best capture veterinary and human antimicrobials, and resistance patterns of critical or high importance (WHO AGISAR, 2017). The panel was correlated against farm specific history. Each antimicrobial's zone of inhibition was measured to the nearest millimetre. Inhibition zones were assessed against EUCAST (http://www.eucast.org), BSAC (http://www.bsac.org.uk) (Andrews et al, 2001) and CLSI (https://clsi.org) agreed clinical breakpoints, indicated in Table 3.

Table 3. Antimicrobial breakpoint assessments for Kirby-Bauer disc diffusion testing

Antimicrobial	Resistance breakpoint (mm)	Source
amoxicillin /clavulanate	19.0	EUCAST[a]
cefpodoxime	19.0	BSAC[b]
cephalexin	14.0	EUCAST
doripenem	18.0	BSAC
enrofloxacin	16.0	CLSI[c]
erythromycin	13.0	CLSI
florfenicol	32.0	EUCAST
neomycin	13.0	CLSI
sulphisoxazole	12.0	CLSI
tetracycline	14.0	CLSI

Resistance was defined as visible isolate growth measured to be equal to or less than the specified breakpoint radiating from antimicrobial inoculated disk. a- European Committee on Antimicrobial Susceptibility Testing (EUCAST), b- British Society for Antimicrobial Chemotherapy (BSAC), c- Clinical and Laboratory Standards Institute (CLSI).

5.2.5. Nitrogen and Phosphorus analysis

Following identification of WS as event category of interest, automated water samples for Nitrogen and Phosphorus concentration were taken concurrently alongside microbe samples and measurements of outflow water conditions. This chemical sampling used separate automated samplers during the designated WS events from flumes 4, 5 and 6. It was ensured that samples for N and P analysis were taken simultaneously to those for microbial and outflow conditions determination.

Prior to the completion of individual event sampling, 4.75 g sodium hydroxide and 22.5 g potassium persulfate were dissolved in ultrapure water made up to total 500 ml volume digestion reagent. Into an autoclavable digestion tube 6.0 ml of water sample and 3.0 ml of digestion reagent was added. This process was completed with blank and standard samples to enable sample calibration. Digestion tubes are autoclaved at 121.0 °C (±0.25 °C) for 1 h (Fu et al., 2018). Samples

are then analysed for either total Nitrogen concentration by applying Nitrate-Nitrite Nitrogen colourimetry method (Saha et al, 2018) or for total Phosphorus concentration by malachite green Phosphorus colourimetry method (Khoi et al, 2018).

5.2.6. Outflow water conditions

During the comprehensive water sampling of the WS event category, outflow water characteristics were recorded simultaneously to automated water samples for microbe, Nitrogen and Phosphorus concentrations. This sampling used separate automated samplers during the designated WS events from flumes 4, 5 and 6. Outflow water characteristics sampling occurred continuously at 15 min intervals throughout sampling periods.

A bi-directional peristaltic pump is permanently fitted within the outflow sampling site. This is connected to a purpose designed flow cell, within the catchment associated analysis station, fitted with optical and thermistor sensors enabling measurement of dissolved Oxygen, turbidity and temperature, as seen in Table 4. The water outflow rate is measured by OTT PLS pressure transducers (OTT Hydromet, Loveland, Colorado, USA). The sensor measures the depth of water by means of an integrated controller and ceramic pressure-measuring cell. The output data are converted to flow (l/s) externally by formula. This sampling occurs every 15 minutes and gives continuous data on outflow water conditions.

Table 4. Outflow water conditions measured at each NWFP catchment flume

Outflow water characteristics	Method	Units	Range	Resolution	Accuracy
Dissolved Oxygen	Optical sensor (YSI EXO 2)	%	0-500	0.1	0–200 %: ±1 % of reading, 200–500%: ± 5 % of reading.
Turbidity	Optical sensor (YSI EXO 2)	FNU	0-4000	0.1	0–999 FNU: ±2 % of reading, 1000-4000 FNU: ±5 % or reading.
Temperature	Thermistor (YSI EXO 2)	°C	-5-50	0.001	-5-35 °C: ±0.01 °C, 35-50 °C: ±0.05 °C.

Outflow water characteristics recorded during comprehensive study of WS conditions. Detailing each parameters analysis method.

5.2.7. Statistical analysis

All statistical analysis was completed in Genstat (16th Edition, 2013). Correlation of automated water sample (existing or treated method) FIO CFU ml^{-1} to manual water sample was performed by linear mixed modelling. The variation of automated water sample FIO CFU g^{-1} and percentage antimicrobial resistant isolates was calculated by blocked general ANOVA.

5.2.7.2. Automated and manual microbe water sampling trial

The linear mixed model used was:

Y variate: Automated sampling (existing or treated) FIO CFU ml^{-1}

Fixed model: Manual sampling FIO CFU ml^{-1}

Block: Event type/Sampling

Where: Event Type = SB, WB, or WS, Sampling = Sampling event number.

Significance was accepted if $P \leq 0.05$.

5.2.7.2. Automated microbe water sampling: event type variation

The ANOVA model used was:

Y variate: FIO CFU ml^{-1} and AMR

Treatment: Event Type

Block: Sampling

Where: Event Type = SB, WB, or WS, Sampling = Sampling event number.

The post hoc testing was Tukey test (significance $P \leq 0.05$).

5.2.7.3. Catchment WS conditions outflow; predictive modelling of potential microbial risk

The model development process used was:

Hydrograph slope was derived through 'difference in flow rate/sample time difference'. Non-microbe parameters were Log transformed to enable correlations with *E. coli*, Intestinal enterococci and *L. monocytogenes* concentrations (log_{10} CFU ml^{-1}). Potential grouping factors such as sample event number and catchment numbers were combined as 'Sampling_Catchment'.

Initial linear regression model used was:

Y variate: CFU ml^{-1}

X variates: DO, Flow_rate, Hydrograph_slope, N_concentration, P_concentration, Temp, Turbidity.

Where: DO = Dissolved Oxygen, Flow_rate = Outflow rate (l s^{-1}), Hydrograph_slope = Product of (difference in flow rate/sample time difference), N_concentration = µg N l^{-1}, P_concentration = µg P l^{-1}, Temp = Outflow water temperature (ºC), Turbidity = Outflow turbidity (Formazin Nephelometric Units).

Significance was accepted if $P \leq 0.05$.

Initial grouped linear regression model used was:

Y variate: CFU ml^{-1}

X variates: DO, Flow_rate, Hydrograph_slope, N_concentration, P_concentration, Temp, Turbidity.

Grouping factor: Sampling_Catchment

Significance was accepted if $P \leq 0.05$.

All-subsets linear regression model used was (Forward stepwise, search options: In-4, Out-2):

Y variate: CFU ml^{-1}

Listed explanatory data: DO, Flow_rate, Hydrograph_slope, N_concentration, P_concentration, Temp, Turbidity, Sampling_Catchment.

Multiple linear regression model used was:

Y variate: CFU ml^{-1}

X variates: ‘Corresponding final model structure’

Significance was accepted if $P \leq 0.05$.

5.3. Results

5.3.1. Automated and manual microbe water sampling trial

Multiple event sampling was successfully completed during SB, WB and WS conditions. The accuracy of both automated sampling protocols (see section 5.2.3) FIO CFU measurement was assessed by linear mixed modelling (see section 5.2.7.2) to be significantly correlated to the manually sampled measures. Both the existing and treated automated sampling results were found to correlate with manual sampling results at SB and WB. There was significant variation between the existing automated sampling results and manual during the WS events recorded. The success of the correlation between the treated automated sampling and the manual sampling, in all conditions, provided confidence in subsequent application of the methodology.

5.3.1.1. Summer baseflow

The accuracy of the existing and treated automated sampling protocols compared to manual sampling during SB conditions was assessed by linear mixed modelling, see Figure 3 and Table 5. The treated automated sampling for *E. coli* ($P = 0.001$, $r^2 = 95.1$) and intestinal enterococci ($P = 0.001$, $r^2 = 95.6$) concentrations were both found to be significantly correlated to the associated manual sampling results. The existing automated sampling for *E. coli* ($P = 0.001$, $r^2 = 64.1$) showed significant correlation to manual sampling results, however results for intestinal enterococci ($P = 0.01$, $r^2 = 38.5$) correlated poorly.

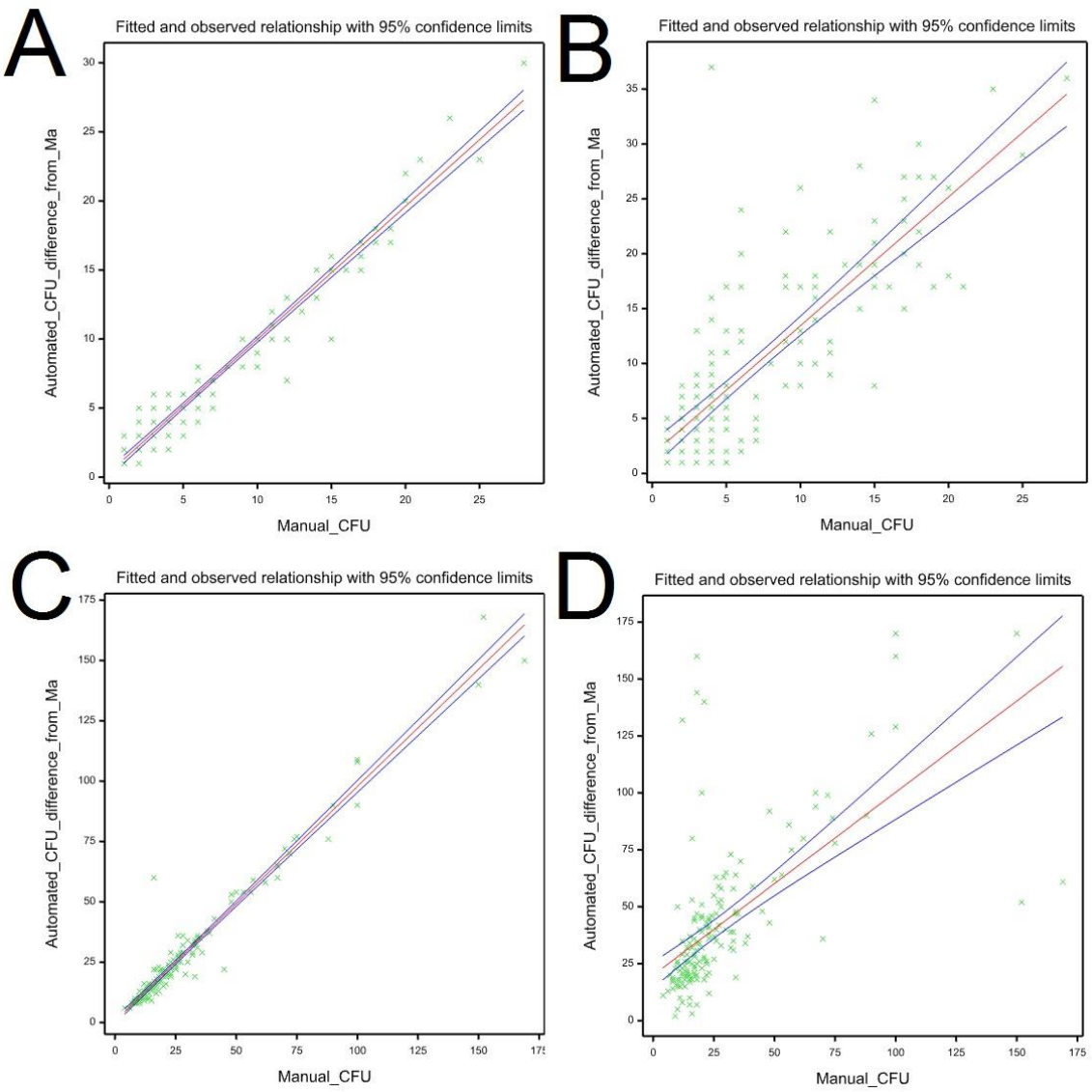

Figure 3. Summer baseflow automated water sampling correlation to manual sampling faecal indicator organism concentration (CFU ml^{-1})

A) Treated automated sampling E. coli CFU ml^{-1}. B) Existing automated sampling E. coli CFU ml^{-1}. C) Treated automated sampling intestinal enterococci CFU ml^{-1}. D) Existing automated sampling intestinal enterococci CFU ml^{-1}.

Table 5. Summer baseflow automated water sampling correlation to manual sampling faecal indicator organism concentration (CFU ml^{-1})

Correlation with manual	*E. coli* r^2	*E. coli* P value	Intestinal enterococci r^2	Intestinal enterococci P value
treated automated	95.1 s.e. 1.24	0.001	95.6 s.e. 5.36	0.001
existing automated	64.1 s.e. 4.99	0.001	38.5 s.e. 25.9	0.01

Linear mixed model of summer baseflow treated and existing automated sampling correlation to manual sampling FIO CFU. Coefficient of determination indicated by r^2, significance accepted if $P \leq 0.05$.

5.3.1.2. Winter baseflow

The accuracy of the existing and treated automated sampling protocols compared to manual sampling during WB conditions was assessed by linear mixed modelling, see Figure 4 and Table 6. Both treated automated sampling for *E. coli* (P = 0.001, r^2 = 96.0) and intestinal enterococci (P = 0.001, r^2 = 97.6) concentrations were found to be significantly correlated to manual sampling results. The existing automated sampling for *E. coli* (P = 0.001, r^2 = 62.2) and intestinal enterococci (P = 0.001, r^2 = 68.5) showed significant correlation to manual sampling results.

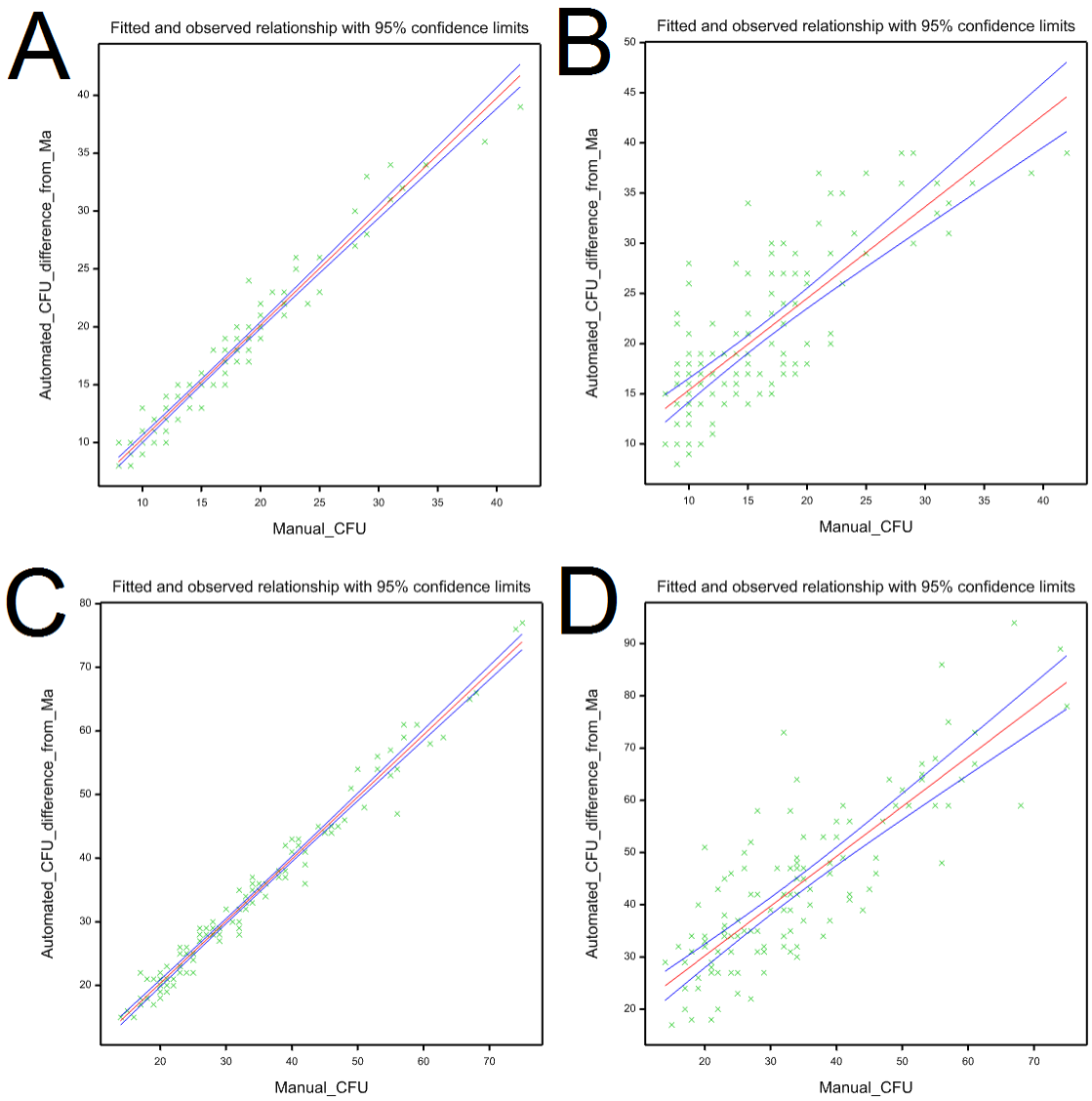

Figure 4. Winter baseflow automated water sampling correlation to manual sampling faecal indicator organism concentration (CFU ml^{-1})
A) Treated automated sampling E. coli CFU ml^{-1}. B) Existing automated sampling E. coli CFU ml^{-1}. C) Treated automated sampling intestinal enterococci CFU ml^{-1}. D) Existing automated sampling intestinal enterococci CFU ml^{-1}.

Table 6. Winter baseflow automated water sampling correlation to manual sampling faecal indicator organism concentration (CFU)

Correlation with manual	*E. coli* r^2	*E. coli* P value	Intestinal enterococci r^2	Intestinal enterococci P value
treated automated	96.0 s.e. 1.37	0.001	97.6 s.e. 2.13	0.001
existing automated	62.2 s.e. 4.83	0.001	68.5 s.e. 8.79	0.001

Linear mixed model of winter baseflow treated and existing automated sampling correlation to manual sampling FIO CFU. Coefficient of determination indicated by r^2, significance accepted if $P \leq 0.05$.

5.3.1.3. Winter stormflow

The accuracy of the existing and treated automated sampling protocols compared to manual sampling during WS conditions was assessed by linear mixed modelling, see Figure 5 and Table 7. Both treated automated sampling for *E. coli* (P = 0.001, r^2 = 72.0) and intestinal enterococci (P = 0.001, r^2 = 78.4) concentrations were found to be significantly correlated to manual sampling results. The existing automated sampling for *E. coli* (P = 0.01, r^2 = 47.6) and intestinal enterococci (P = 0.01, r^2 = 40.5) showed poor correlation to manual sampling results.

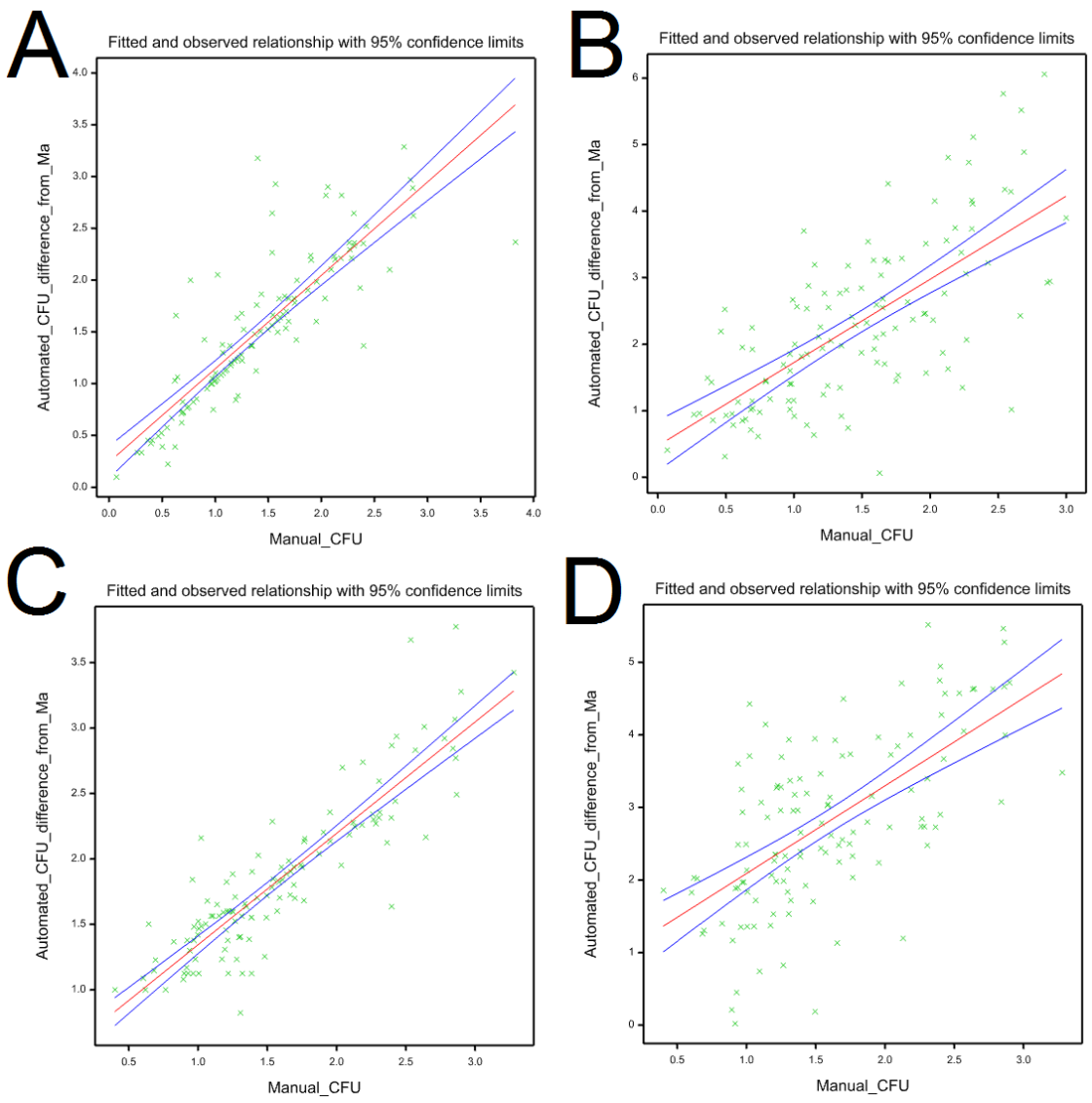

Figure 5. Winter stormflow automated water sampling correlation to manual sampling faecal indicator organism concentration (log_{10} CFU ml^{-1})
A) Treated automated sampling E. coli log_{10} CFU ml^{-1}. B) Existing automated sampling E. coli log_{10} CFU ml^{-1}. C) Treated automated sampling intestinal enterococci log_{10} CFU ml^{-1}. D) Existing automated sampling intestinal enterococci log_{10} CFU ml^{-1}.

Table 7. Winter stormflow automated water sampling correlation to manual sampling faecal indicator organism concentration (log_{10} $CFUml^{-1}$)

Correlation with manual	*E. coli* r^2	*E. coli* P value	Intestinal enterococci r^2	Intestinal enterococci P value
treated automated	72.0 s.e. 0.38	0.001	78.4 s.e. 0.27	0.001
existing automated	47.6 s.e. 0.89	0.01	40.5 s.e. 0.90	0.01

Linear mixed model of winter stormflow treated and existing automated sampling correlation to manual sampling FIO log_{10} $CFUml^{-1}$. Coefficient of determination indicated by r^2, significance accepted if $P \leq 0.05$.

5.3.2. Event type variation

Variation in potential microbial risk of different event types outflow, as predicted by faecal indicator organism concentration (CFU) and percentage antimicrobial resistant isolates was assessed. Increased concentrations of both faecal indicator organisms were present in water samples collected during winter stormflow relative to summer and winter baseflow. Summary statistics for FIO concentrations are presented in Table 8.

Significant differences between event type samples for percentage resistance isolates was indicated for both Erythromycin (P = 0.008) and Tetracycline (P = 0.001). Increased percentage resistant isolates were found in winter stormflow relative to summer and winter baseflow. Zero isolates were identified as exhibiting multiple drug resistance (defined as resistance to 4 or more of the drug panel). Summary statistics for percentage resistant isolates are presented in Table 9.

Table 8. Variations in faecal indicator organism concentration (log_{10} CFU ml^{-1}) of automated water sampling by event type.

Strata	*E. coli*	Intestinal enterococci
Sampling	1.34	1.68
sd.	0.475	0.424
Event Type		
SB	1.08[a]	1.48[a]
sd.	0.132	0.209
WB	1.12[a]	1.49[a]
sd.	0.136	0.183
WS	1.82[b]	2.07[b]
sd.	0.550	0.487
P value	0.008	0.003

Mean values of automated water sampling E. coli *and intestinal enterococci concentration by event type. Blocked ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters within columns.*

Table 9. Variations in percentage antimicrobial resistant isolates of automated water sampling by event type.

	% Resistant Isolates					
Drug type	AMC	CPD	CFX	ERY	NEO	TET
Strata						
Sampling	4.80	8.14	1.85	19.6	6.29	8.51
sd.	11.780	16.810	7.678	22.29	15.720	16.240
Event Type						
SB	3.33	6.66	2.22	12.2[a]	3.33	5.55[a]
sd.	10.170	13.560	8.457	16.34	13.420	12.630
WB	4.44	4.44	1.11	15.5[a]	4.44	2.22[a]
sd.	11.520	14.470	6.086	16.91	11.520	8.457
WS	6.66	13.3	2.22	31.1[b]	11.1	17.7[b]
sd.	13.560	20.720	8.457	27.59	20.22	20.96
P value	0.252	0.143	0.850	0.008	0.160	0.001

Mean values of automated water sampling percentage antimicrobial resistant isolates by event type. Blocked ANOVA test was applied to analyse the data and a post hoc Tukey test with significance <0.05 indicated by superscript letters within columns. Only antimicrobials which indicated resistant isolates are presented: DOR (doripenem), EFX (enrofloxacin), FFC (florfenicol) and SXZ (sulphisoxazole) were effective against all isolates tested and no resistance was found. Drug type abbreviations: AMC (amoxicillin /clavulanate), CPD (cefpodoxime), CFX (cephalexin), ERY (erythromycin), NEO (neomycin), TET (tetracycline).

5.3.3. Predictive modelling of catchment outflows potential microbial risk

Following the completion of all-subsets linear regression model the outflow parameters dissolved oxygen, Nitrogen concentration, Phosphorus concentration, temperature, turbidity, sampling event number and catchment number were all discounted as potential explanatory variables for *E. coli*, intestinal enterococci and *L. monocytogene* concentration. Final predictive models are shown in Table 10.

The application of the final predictive model (Flow rate + Hydrograph slope) for *E. coli* concentration ($P = 0.001$, $r^2 = 88.3$) was found to be significantly correlated. The corresponding predictive model (Hydrograph slope + Flow rate) for intestinal enterococci concentration (P =

0.001, r^2 = 66.8) was also significantly correlated. The final predictive model (Flow rate) for *L. monocytogenes* concentration (P = 0.001, r^2 = 57.3) was found to be significantly correlated but relative to *E. coli* and intestinal enterococci indicated a weaker association.

Table 10. Optimised catchment outflow predictive models of *E. coli*, Intestinal enterococci and *L. monocytogenes* concentration (log_{10} CFU ml^{-1})

Model structure	r^2	P value
***E. coli*:** 'Flow rate + Hydrograph Slope'	88.3 s.e. 0.25	0.001
Intestinal enterococci: 'Hydrograph Slope + Flow rate'	66.8 s.e. 0.30	0.001
***L. monocytogenes*:** 'Flow rate'	57.5 s.e. 0.31	0.001

Multiple linear regression model of catchment outflow parameters correlation to E. coli, *Intestinal enterococci and L. monocytogenes* log_{10} *CFU* ml^{-1}. *Coefficient of determination indicated by* r^2, *degrees of freedom total 89 for each model, significance accepted* if $P \leq 0.05$.

5.4. Discussion

This study examined the role of extensive livestock farms in the external dissemination of potentially pathogenic bacteria and AMR *E. coli* by hydrological transfer. This route for off-farm spread of potentially higher risk microbe populations to the wider environment is accepted as a critical control point for the preservation of human and animal health (Craft, 2015, Woolhouse et al, 2015). Identifying the climatic and water conditions associated with increased microbial concentrations being transported from farm land to natural water courses is critical in developing robust control strategies especially in the context of predicted increase in winter storm events.

As indicated in previous Chapters 2 and 3, the capacity of soil matrix to retain significant concentrations of bacteria generates a potential accumulation which may act as a source if released through hydrological transport (Sayah et al, 2005, Hooda et al, 2000). The identification of microbial hazard hotspots was found in the soil (Chapter 3) and correspondingly on grass at pasture (Chapter 4), the catchment scale influence of these increased concentration locations under hydrological action is difficult to predict (Oliver et al, 2018). This Chapter determined the influence at a field management and hydrological catchment scale on FIO in the resulting outflow generated under differing climatic conditions.

There was an initial methodological requirement to compare the accuracy of two automated water sampling techniques relative to manual water sampling. This indicated that applying additional protocol ensured comparable microbial results accuracy of sterilised automated sampling kit to manual sampling techniques. The concentration of faecal indicator organisms and percentage of AMR isolates was assessed across designated climatic event conditions. The ‘winter stormflow’ being a seasonal catchment outflow greater than baseline following a rainfall event was found to present greater risk of increased microbial concentrations and percentage AMR

isolates than annual baseflow conditions. Comprehensive sampling of winter stormflow outflow enabled the correlation of several simultaneously measured parameters. The study indicated correlation between the hydrograph slope, flow rate and the outflow microbe concentration. Presenting a particularly accurate predictive model of flow rate and hydrograph slope for *E. coli* concentration within the North Wyke Farm Platform.

5.4.1. Automated microbial water sampling accuracy

This study identified the need to operate several geographically distinct sampling sites simultaneously. This was required to assess potential differences in measured parameters at field scale across NWFP catchments 4, 5 and 6 on the same farm. To achieve this automated microbial water sampling was deemed necessary. This required a methodological trial of automated sampling compared to manual sampling microbial concentration accuracy. Two differing automated sampling protocols were assessed against concurrent manual sampling FIO concentration results. The automated peristaltic sampler was trialed as *in situ*, with no alteration to the permanently affixed sample tubing or the internal connections contacting the water samples (Orr et al, 2016). This automated protocol was termed ‘existing’ as it was the current automated water sampling protocol used on the NWFP. Identical automated samplers were trialed following fitting of new (but comparable to existing) sample tubing and internal connections. Both tubing and connections were treated by sterilization prior to fitting and were removed following event sampling, this was repeated on all occasions. This sterilized automated protocol was termed ‘treated’ for the purposes of the study. Both automated and the manual sampling methods used sterile sample collection bottles. The trial was completed over SB, WB and WS event conditions with existing and treated results correlated separately to manual by *E. coli* or intestinal enterococci and event type, replicated across the three catchments.

Both SB (Table 5) and WB (Table 6) event types indicated strong correlation between the treated automated and manual sampling *E. coli* and intestinal enterococci concentration results. The correlation between SB existing automated and manual sampling was not as strong but *E. coli* did indicate comparable accuracy to manual, while the intestinal enterococci results did not correlate. The WB existing automated and manual sampling did indicate correlation in *E. coli* and intestinal enterococci concentration results. However, the accuracy relative to the treated protocol was decreased. The relatively low concentrations observed during both baseflow event types made conclusive comment on existing and treated automated protocol accuracy difficult. The issues in reliability of automated sampling principally relate to sample contamination or carry over between sampling points (Richards et al, 2018). These effects are more readily identifiable when recording increased concentrations or periods when there is greater variation between sample points.

The WS event type presented greater variation in sample results than either baseflow events (Table 8). As the *E. coli* and intestinal enterococci concentrations were increased (Table 8) and concentration changed between sample points on the hydrograph this enabled conclusive judgement on the accuracy of existing and treated automated sampling relative to manual. The treated automated sampling strongly correlated with manual sampling results and was considered to demonstrate comparable accuracy (Table 7). The existing automated sampling results indicated high variability and no correlation with manual sampling was suggested. In automated microbial water sampling the impact of biofilms has also been noted as a contributing factor to reduced accuracy (Richards et al, 2018, Watson et al, 2004). The accumulation of biofilms has been associated with periods of wetting and drying (Watson et al., 2004, Martiny et al, 2003). This process as identified in human healthcare devices (Alfa & Howie, 2009) is considered to be similarly replicated in this trial by the periods of sampling and inactivity that the existing

automated samplers underwent. Any potential issues of carryover between sampling points are negligible given the accuracy of the treated automated sampler results to manual sampling.

This trial enabled the application of the treated automated sampling procedure to the subsequent comprehensive simultaneous catchment sampling of events. This benefitted the study design as any variation between catchments 4, 5, and 6 could be assessed under the same meteorological conditions with concurrent sampling. This trial result while a methodological necessity for this study also has external applications in research and industry. Given the sterilisation prerequisites can be engineered within samplers, this automated sampling approach could be applied at a landscape catchment scale at targeted outflow events. This could generate accurate results for microbial contamination of natural water courses with concurrent sampling points enabling identification of regions of greatest risk. This could inform targeted mitigation strategies at regions of increased transmission risk.

5.4.2. Potential microbial risk varies with event type

Different climatic and seasonal events are known to present variations in the corresponding microbial concentration and percentage AMR isolates (Ahmed et al, 2018) in catchment outflows. This is particularly challenging in targeting large scale sampling regimes to periods and conditions where the results will provide the greatest value (Bueno et al, 2017). The target for microbial water sampling will vary with the system and the previously recorded data. Previous studies indicate high outflow rates present the most significant risk of increased microbial contamination of outflow from agricultural land (Ahmed et al, 2018).

This study utilised the treated automated water sampling data for *E. coli* and intestinal enterococci from the automated and manual sampling trial to compare differences in the microbial

concentration of SB, WB and WS. The results indicated intestinal enterococci was present at higher concentrations than *E. coli* in all event types tested. This compares with previous research and industry application which suggests that intestinal enterococci survive longer than *E. coli* in aquatic conditions (Anderson et al, 2005). The period of time which microbes in this study were water borne for is not known. However, particularly during the winter category NWFP soil is regularly saturated (Liu et al, 2017). This may drive the resulting differences between FIO species, *E. coli* and intestinal enterococci, concentrations. It should be noted that the variation in intestinal enterococci and *E. coli* concentration is opposed to that found in soil and at pasture in Chapter 3 and 4, so may support the impact being driven by survival in water. A comparison between event types indicated that WS presented a significantly increased risk of hydrological transmission of higher concentrations of *E. coli* and intestinal enterococci. This result also compares with previous literature which has identified high flow rates as associated with increased microbial concentrations relative to baseflow conditions (Ahmed et al, 2018, Bueno et al, 2017). While supporting existing findings, this confirmation was required given the unique hydrological infrastructure of the NWFP (Orr et al, 2016).

In addition to the FIO analysis, AMR testing was completed on randomly selected samples from all event types. The results indicated that percentage isolate erythromycin resistance was the highest throughout all event types tested. This compares with findings in soil and at pasture in Chapter 3 and 4. Supporting the hypothesis that the resistance patterns on-farm with predominate when transferred to the external environment (Craft, 2015, Woolhouse et al, 2015). The drug types doripenem, enrofloxacin, florfenicol and sulphisoxazole were effective against all isolates tested with no resistance found. This finding is also comparable to soil and pasture (Chapter 3 and 4) with the exception of florfenicol where resistant isolates were identified in soil and pasture. A comparison of

percentage AMR isolates between event types indicated that erythromycin and tetracycline resistance was increased in WS conditions relative to SB and WB. While this result presents WS as possessing an increased risk of AMR transmission to the wider environment it is at reduced prevelance relative to comparable studies (Ahmed et al, 2018, Luo et al, 2011). This finding supports those in Chapters 3 and 4, of a responsible use antimicrobial management strategy (Takahashi et al, 2018) within a system supportive of low input production.

The lack of available replication of SS events (see Section 5.2.2) prevented inclusion in the comparison across event types. Initial data was considered comparable to WS. The stormflow category and the potential increased microbial risk it poses is of particularly importance given the likely impacts of climate change (DEFRA, 2011). Greater frequency of storm events, and the infiltration excess overland flow across grazed fields they generate, could result in the increased transfer of microbes from pasture to the wider environment (Ahmed et al, 2018, Luo et al, 2011).
The comparison of potentially pathogenic bacteria concentration and the percentage AMR resistant isolates identified that WS event type presented an increased microbial risk. While these findings agree with previous research their future application will be critical in benchmarking the holistic sustainability of the NWFP and assessing the success of on-farm microbial control strategies (Takahashi et al, 2018, Orr et al, 2016).

5.4.3. Catchment outflow hydrograph slope and flow rate: predictors of microbial concentration

The correlation between increased concentrations of potentially pathogenic bacterial species, and other outflow conditions is crucial in developing accurate alternative methods to assess microbial hazard. This study and industry monitoring of multiple outflow parameters from agricultural catchments has the potential to provide multiple predictions. The study of conditions

such as Nitrogen (Worral et al, 2009, Jordan & Smith, 2005) and Phosphorus leaching (Vinten et al, 2017) is long established and forms part of regulatory policy (Worral et al, 2009, Jordan & Smith, 2005). Other measures such as outflow rate, turbidity, temperature and dissolved Oxygen could offer more rapid and economical estimates. The simultaneous study of multiple outflow parameters is unfeasible or beyond the scope of most previous research (Orr et al, 2016).

This study applied a comprehensive assessment of microbial concentrations alongside simultaneous measures of other outflow parameters. This followed the demonstration of the treated automated sampling protocol accuracy and identification of the WS event category as indicating the greatest microbial risk to external transmission. In addition to the established use of the FIOs *E. coli* and intestinal enterococci, *L. monocytogenes* concentration was also applied as an additional measure of potential microbial hazard. This followed investigation of increased *L. monocytogenes* concentration in silage samples in Chapter 4 and previous studies indicating the aquatic survival potential of agricultural origin *L. monocytogenes* (Nightingale et al, 2004). The additional WS parameters: flow rate, derived hydrograph slope, Nitrogen concentration, Phosphorus concentration, dissolved Oxygen, temperature and turbidity were all recorded simultaneous to microbial sampling across all catchments. These parameters and the grouping factor of sampling event and catchment were used to develop a potential predictive model for each microbial hazard measure. The parameters of flow rate and hydrograph Slope were found to produce the most accurate predictions of *E. coli* followed by intestinal enterococci concentration. A relatively less accurate but significant correlation was found between flow rate and *L. monocytogenes* concentration.

The finding of correlation between FIO and flow rate or hydrograph slope is supported by previous research (Ahmed et al, 2018, Luo et al, 2011). The accuracy of the *E. coli* concentration

prediction is particularly valuable as this has the potential to provide an estimate of microbial risk by water borne transmission if system inputs are associated. It may be possible to utilise known system parameters of antimicrobial treatment, livestock, management and flow rate to provide estimates of potential risk for microbial transmission at catchment scale. The development of this sort of modelling could enable targeted microbial sampling and control strategies to be applied (McGonigle et al, 2012, Sudol & Ambrose, 2002). The lack of influence of different catchments on the model microbial hazard predictions is also notable as this suggests that despite the rotational management of land for silage production or grazing the accumulated persistence of microbes remains.

5.5. Conclusion

This chapters research highlights the importance of hydrological transmission as the vehicle for transporting potentially pathogenic and AMR bacteria populations off-farm to the wider environment. This route has long been identified as crucial in minimizing the external risk of microbial populations and characteristics generated within agriculture (Craft, 2015, Woolhouse et al, 2015). This contamination of external environments can negatively impact drinking water supply, aquaculture and tourism over significant distances. This study has attempted to contextualise this existing monitoring as a strategy to assess the impact of on-farm mitigation strategies, targeting a reduced accumulation of microbial hazard in fields, such as those described in Chapters 3 and 4. The consistency of outflow microbial analysis across all catchments of different grazed and ungrazed field use as described in section 5.2. strongly supports the findings in chapter 3 of the long term and disproportionate influence which in-field hotspots exert upon catchment scale risk. This study suggests a potential automated sampling protocol whose application within monitoring could better target mitigation strategies. The influence of specific

winter storm events in impacting increased FIO concentrations and AMR isolates within natural watercourses was identified. This has potential in informing future research and stakeholder management strategies during specific high risk periods, such as reducing the influence of livestock generated hotspots on field outflows through optimized grazing strategies. The development of an accurate model of *E. coli* concentration based on flow rate and hydrograph slop suggests there exists a possible alternative method to provide rapid and economical predictions on microbial risk.

Chapter 6

General Conclusion

This research and its ultimate findings provide genuine advancement to knowledge of extensive grazed livestock production systems. Particular emphasis has been placed on contextualising this work's results to inform meaningful and practical intervention strategies. The implications of this thesis has immediate relevance for farmers, veterinary practitioners, environmental consultants and policy makers. This original research describes a comprehensive characterisation of on-farm microbial cycling within an industry representative extensive grazed grassland production system. The work was undertaken with clear development or adaption of existing methodologies to provide high resolution results at a field and farm scale using unique farm scale infrastructure (NWFP; BBSRC National Capability). A multi-disciplinary approach was utilised, combining agricultural management techniques, forage analysis, soil and water chemistry with culture based microbiology and next generation microbial sequencing. Through this strategy complementary public health and system productivity measures combined to provide mutual benefits from recommended control strategies. The approach adopted ensures the greatest possible reductions in perceived microbial risk are supported by tangible economic benefits to the producers who will ultimately implement any research conclusions.

The research findings generated present well defined opportunities throughout the on-farm microbial cycle to effect meaningful improvements to grazed livestock and ultimately human health.

6.1. Recommendations

The findings of this thesis undertaken within a typical extensive, low input, livestock grazing system (sheep and suckler beef) indicate that given resistance patterns and potential disease it is inaccurate to characterise influences upon disease and AMR risk as comparable to

intensive livestock farming. However, there exists specific spatial and temporal instances of increased on-farm microbial risk such as those demonstrated in-field hotspots, silage and winter storm field water outflows. The disease and AMR risk posed from livestock production is not consistent and should not be reported as an amorphous figure. The on-farm microbial hazard varies in time and place on a single farm and within a single production system. Recommendations for mitigating disease and AMR risk should not be limited to the linearity of veterinary drug intervention, but utilise a toolbox of stakeholder scale interventions, modifying everyday management decisions. The initial recommendations derived from this thesis present several points within the farm microbial cycle when beneficial reductions in potential disease and AMR risk can be affected by relatively simple stakeholder interventions.

6.1.1. Pasture management

A diet effect upon livestock faecal and soil microbe populations exists (Neubauer et al, 2018), even within comparable grazing livestock systems (Schmid et al, 2018). This has implications for production and scientific study. The choice of grass species in grazed livestock systems is a fundamental driver of productivity, even in comparably managed farms this difference will result in altered livestock faecal, soil, forage and field water outflow microbe populations. Diversity of soil microbe populations is known to relate with soil quality parameters (Schmid et al, 2018, Luo et al, 2017) and thus any diet which drives species dominance (Neubauer et al, 2018) must be considered carefully.

Intensity of animal activity (Betteridge et al, 2010, Oudshoorn et al, 2008) in given locations resulting from pasture utilisation (Scimone, 2007 et al, Dumont & Boissyenton, 2000, Hodgson et al, 1991) and behavior (Orr et al, 2012) is the principal driver in the generation of

microbial hotspots within fields. Microbial hotspots represent increased concentrations of potentially pathogenic bacteria of livestock origin and increased prevalence of AMR isolates. The influence of these hotspots can be observed within soil and grass at pasture with corresponding influence on microbial hazard to grazing livestock, production of preserved forage and field water outflow. Reducing the intensity of livestock faecal depositions at specific hotspot locations is considered to result in a beneficial reduction in the accumulation of potentially pathogenic and AMR bacteria. The disproportionate intensity of potential disease and AMR exchange which can occur at hotspots should be minimized through effective pasture management. Varying and supplying multiple water sources along with ensuring optimal pasture utilisation through strategies such as strip/rotational grazing are considered beneficial in minimizing the development of field hotspots. This in turn would return nutrients more uniformly to pasture, resulting in improvements to productivity (Bilotta et al, 2007, Clark et al, 1998).

6.1.2. Forage management

The influence of microbial contaminants on preserved forage's nutritional (Naoki and Yuji, 2008) and hygienic (Orsi & Wiedmann, 2016, Wilkinson and Davies, 2012) quality is significant. Previous studies have indicated the importance of minimizing soil incorporation (Cherney et al, 2016, Wagner et al., 2005) in ensuring silage nutritional value is maintained and health risks reduced. This thesis presents aerobic exposure of silage during storage or during prolonged feeding periods, in the absence of soil incorporation, as a significant risk to silage nutritional and hygienic quality. An increased prevalence of AMR was found to correlate to aerobic exposure and was driven by the survival and proliferation of microbial contaminants. Overt mutual benefits to health and production are therefore found to result from ensuring optimal anaerobic conditions are

maintained during silage storage and period of aerobic exposure during feeding is minimized. The increased prevalence of pathogens and AMR isolates in aerobic exposed, and thus reduced nutritional quality, silage suggest that this is a key point in the on-farm microbial cycle. Minimising the risk silage poses as a potential source to livestock and agricultural workers of infection or AMR and improving its nutritional quality as a feed source should be applied through strict stakeholder production practices. The specific infection risk posed by *L. monocytogenes*, or other zoonotic pathogens, and the potential for treatment failure due to AMR can be reduced in silage with adequate bale wrapping, maintaining integrity throughout storage and periodically removing old silage from feeding points.

6.2. Future research

A significant amount is known regarding the influence of agricultural land, and in particular grazed livestock systems, on wider environmental disease and AMR risk. There is also a growing understanding and appreciation of the influence which veterinary interventions, both proactive (such as dry cow therapy) and reactive (such as wound infection) treatments, cause to farms disease and AMR risk. However there remains significant gaps in our knowledge of the on-farm microbial cycle and points which disease and AMR risk can be mitigated. Assessing and addressing specific points of high microbial risk on-farms, benefits a reduction in potential disease and resistance generation within that immediate and the wider environment. A multidisciplinary approach must be applied to offer mutual health and productivity benefits resulting from implementation of any mitigation strategies. Three key areas of future study, as identified within this thesis, are outlined below.

6.2.1. Field hotspots

There is an identified need for improved characterisation of soil and grass at pasture field hotspots. This requires additional scientific study of the locations identified in this thesis and of other increased risk zones, such as the hydrological connectivity of hotspots. Further developing the multidisciplinary approach with microbial analyses complemented by soil chemistry or forage nutritional parameters will ensure the widest possible relevance of results. This approach indentures industry participation in research with direct links to productivity and profitability alongside improvements to system health. Subsequent proofing of suggested intervention strategies is crucial. Demonstrating improvements to pasture productivity derived from greater uniformity in spatial variation of livestock faecal deposition is important. Indicating a direct causality between eliminating hotspot locations from forage harvesting and improved preserved feed quality should also be undertaken. The disproportionate influence of the identified field hotspots has been described in this thesis. The impact extends beyond the identified limits within the soil and pasture to the resulting microbial hazard of water outflows from fields. The impact was found to be strongly correlated to increased outflow rate and this in turn was generated through significant precipitation events. The future study of hotspot influence on water borne microbial burden could focus sampling associated with high risk storm conditions. Investigating whether minimising the occurrence of hotspots in pasture does generate a significant reduction in whole field water outflow microbial hazard. The implementation of water management measures to reduce overland flow particularly from high risk field locations (driven by livestock activity intensity) should also be investigated. It is noted that a residual effect of water borne microbial transport in the soil or pasture was not indicated, such as proximal to flume outlets. However, any

alteration to industry management practices will require demonstrative benefits to system profitability resulting from control of hotspots.

6.2.2. Forage

The representation of forage quality still relies principally on parameters of nutritional characteristics. Forage quality as a measure of potential disease of AMR risk is critical to improving animal health and system productivity. The work described in Chapter 4 demonstrated the association between poor hygienic and nutritional quality silage. Further studies on the resulting impact upon forage nutrition and health from inclusion or elimination of high risk microbial zones in-field from production should be undertaken. This is the foundation of preserved forage both in terms of its ultimate nutritional value and risk to health. Eliminating the inclusion of relatively small zones of high microbial risk from forage production is considered to have a potential impact on resulting nutrition and health quality, even in well produced silage. The role of microbial contaminants, such as FIO, within forage production is not well characterised. While their presence is considered to result from poor production techniques, such as aerobic exposure, they are also known to drive preferential changes in the ensiled environment to benefit their proliferation. Future investigation of the impact of low or high concentration microbial contaminants on anaerobic or aerobic exposed silage is considered extremely valuable to the industry. The critical role of preserved forage as a potential source of disease and AMR to livestock is not fully characterised and would also benefit from wider range study beyond specific pathogen species. The mutual effect of microbial contaminants (potentially pathogenic and AMR) upon forage nutritional and health quality make this a hugely beneficial future study target.

6.2.3. Microbial assessments

The use of culture based methods provides rapid and economically feasible knowledge on potential disease risk and AMR prevalence, as shown in Chapters 3, 4 and 5. This strategy is underutilised at a farm scale, employed principally in disease diagnosis by practitioners or at landscape scale by regulators. As shown in Chapters 3 and 4, culture based microbiology can be used to accurately inform system management particularly on grazing strategy. Chapter 4 also presented the value of proactive culture analysis in identifying sub-optimal production methodologies in preventing the likely occurrence of disease. The value of benchmarking potential disease and AMR risk at various points within the on-farm microbial cycle was presented throughout Chapters 3, 4 and 5. If applied these findings could inform farm specific recommendations on appropriate drug interventions and likely disease risk to livestock. Providing knowledge of farms potential pathogen and AMR status to producers indentures them to further contribute to their control via management strategies beyond veterinary interventions.

The wider application of sequencing based microbial analyses utilised in this thesis may be currently limited by cost and practicality. However, the results provide greater understanding of microbial population differences than is possible using culturing. While culture based AMR testing suggests wider population scale prevalence and FIO counts indicate general disease risk arising from faecal contamination, only sequencing has ability to identify microbe population scale differences at species scale. This information if critical in identifying future study points in-field and within the wider on-farm microbial cycle. The use of sequencing should be considered concurrently to culture based methods, supporting identified key locations of microbial hazard and

identifying potential drivers for species population differences. Sequencing approaches also offer the potential to track differences in microbe hazard, disease and AMR, at a higher resolution than is possible by culture. Undoubtedly as metagenomics is optimised in process and cost it has the potential to not only inform scientific studies but also influence real-time management decisions.

6.3. Concluding statement

This thesis presents targeted recommendations for the mitigation of potentially pathogenic and AMR bacteria through the on-farm cycle. These are principally aimed at producer implementation, with the demonstration of mutual benefits to system productivity and health. The key findings are summarised as:

- Hotspots exist with fields, representing increased accumulation of potential pathogens and AMR prevalence at locations in soil and grass at pasture driven by increased livestock activity (Chapter 3 and 4). Improving pasture management to ensure greater uniformity in livestock grazing behaviour and defecation rates will reduce hotspot development, disease and AMR risk to livestock.
- Aerobic exposure of silage during storage and at point of feeding represents an increased risk of potential disease and AMR (Chapter 4). Ensiling anaerobicity should be ensured at point of production, maintained throughout storage and period of anerobic exposure during feeding reduced. The inclusion of hotspots in preserved forage production should be avoided to improve resulting forage quality, reduce disease and AMR risk from silage.
- Precipitation events resulting in increased field water outflow presents an increased risk of potential pathogen and AMR transfer to external environments (Chapter 5). In-field hotspots should be reduced as disproportionate sources of water borne disease and AMR.

The findings detailed in this thesis also inform the advancement of scientific study of extensive grazed livestock production. These results have direct relevance in current disease/AMR surveillance and land use policy formulation. Subsequent research developing on the key findings of, increased microbe risk at field hotspots and within aerobic exposed silage, has the potential to impact producer and regulator management decisions. This research demonstrates that mitigation strategies targeting the control of potential disease and AMR risk can be applied at stakeholder level on-farm with mutual benefits to system productivity. This approach of linking services to preserving public health with economic benefits increases the likelihood of proactive adoption of research recommendations by farmers and policy makers. This strategy should always be considered for agricultural research, where feasible, as to deliver holistically sustainable livestock systems not only requires new and valuable scientific discoveries but also their adoption by producers.

6.4. Alternative research communication

The delivery of research findings, particularly those in a PhD thesis, are most effective when a variety of communication mediums are employed. The important process of translating original scientific results into policy is often protracted and frustrated by key new findings being overlooked. One strategy to improve this is for researchers to produce summarised documents detailing findings and clearly stating their policy implications. Valuable contributions to scientific knowledge should always inform current policy and stakeholder practice, but this is not always the case. An example policy brief compiling findings in Chapters 3 and 4 is included.

Disease and Antimicrobial Resistance (AMR) Hotspots in Grazed Livestock Fields

Jamie McFadzean[1]

Key Message: Studies of potentially infectious bacteria and AMR on grass and in soil of grazed livestock fields has found significant increases at defined hotspots. This work suggested there may be increased risk of disease and AMR development immediately surrounding field features such as water troughs. Control strategies which reduce the intensity of grazing livestock activity surrounding these hotspots could reduce the risk of disease and AMR accumulation within or wider transfer from fields.

Key Points

- This work explored the increased presence of potentially disease causing (pathogens) and antimicrobial resistant (AMR) microbes at defined hotspots in soil and grass of grazed livestock fields. This is relevant to public health due to microbe transfer by water flow from agricultural fields to the wider natural environment and potential for direct contact with humans.
- The impact of intensive livestock activity and resulting increased faecal deposition surrounding water troughs highlighted these locations as potential hotspots within grazed fields.
- Disease and AMR surveillance at high microbial concentration sites has previously identified current disease/AMR trends and can proactively identify emerging disease/AMR trends.
- Soil and grass sampling demonstrated increased concentrations of potential pathogens at hotspot locations relative to the surrounding fields.
- Soil and grass *Escherichia coli* sampling found higher percentage resistance to antimicrobial agents tested at hotspot locations relative to the surrounding fields.
- The extent of hotspots influence upon increased concentrations of potential pathogens was found to be 10 metres.
- The extent of hotspots influence upon percentage AMR *Escherichia coli* was found to be 10 metres, except for cefpodoxime resistance which extended to 20 meters.
- The findings suggest that intensive livestock faecal contamination associated with hotspots results in an increased accumulation of potential pathogens and AMR bacteria.
- Disease and AMR surveillance at field hotspots could better inform current trends, proactively identify the emergence of new trends, and increase understanding of the role that environmental contamination plays in the spread of disease and AMR.

Introduction

AMR is not a disease but a feature of most bacteria (microscopic organisms). Antimicrobial usage results in the selection of resistant microbes as only those susceptible to the antimicrobial are eliminated, ultimately causing antimicrobial failure [2]. The occurrence of resistant microbes, capable of transmission between animals and humans, may result from direct transfer between species or be acquired from the environment. Faecal indicator organisms (FIO) are microbes (*Escherichia coli* and intestinal enterococci) defined as markers for faecal contamination and are suggested as indicators of potential disease risk. Previous research has indicated increased livestock faecal deposition in areas surrounding field water troughs [3]. These locations have potential to accumulate increased concentrations of potentially pathogenic and AMR microbes. These locations may disproportionately increase the overall microbial risk of grazed fields to livestock, the wider environment and ultimately humans. The influence and extent of these field hotspots is currently not well characterised.

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The accumulation of livestock faeces at hotspots means that disease and AMR surveillance at these locations provides supportive information about identified current trends but can also aid in identifying emerging trends. This research provides a preliminary indication of the presence of potential disease and AMR at hotspots in fields and evaluates the benefits of surveillance at these locations.

Methodology and Findings

Concentration of potentially pathogenic bacteria was assessed by FIO counts. Antimicrobial susceptibility testing was carried out using the disc diffusion test. Samples were not screened using any selective media that would enhance the detection of AMR bacteria. Samples were collected from 10, 20 and 30 metres proximal to hotspot, and from non-hotspot associated field locations. Hotspots were defined as in-field water troughs. Samples were taken from soil and from grass at pasture.

• Soil FIO: FIO concentrations were increased in hotspot relative to non-hotspot soil samples. FIO concentrations were also found to be highest at 10 metres proximal to hotspots.

• Grass FIO: *Escherichia coli* concentrations were increased in hotspots relative to non-hotspot grass samples. FIO concentrations were also found to be highest at 10 metres proximal to hotspots.

• *Escherichia coli* from soil samples: no resistance was found to doripenem, enrofloxacin, florfenicol and sulphisoxazole. However hotspot samples were more resistant to amoxicillin/clavulanate (25.8%), cephalexin (30.7%), erythromycin (77.5%) and neomycin (19.7%) than non-hotspot samples. The highest percentage resistance for amoxicillin/clavulanate (33.3%), cephalexin (29.4%), erythromycin (77.5%), neomycin (24.7%) and tetracycline (29.5%) was found at 10 metres proximal to hotspots.

• *Escherichia coli* from grass samples: no resistance was found to doripenem, enrofloxacin, florfenicol and sulphisoxazole. However hotspot isolates were more resistant to amoxicillin/clavulanate (14.8%), cefpodoxime (7.4%), erythromycin (14.8%) and neomycin (7.4%) than non-hotspot samples. The highest percentage resistance for cephalexin (7.4%), erythromycin (11.1%), neomycin (11.1%) and tetracycline (11.1%) was found at 10 metres proximal to hotspots. The highest percentage resistance for cefpodoxime (7.4%) was found at 20 metres proximal to hotspots.

• Direct comparisons between the soil and grass sample results indicate that increased FIO concentrations are found in hotspot samples, with the highest being at 10 metres proximal to hotspots. Increased percentage resistance of *Escherichia coli* to amoxicillin/clavulanate, erythromycin and neomycin are found in hotspot samples, with the highest being at 10 metres proximal to hotspots for cephalexin, erythromycin, neomycin and tetracycline.

• The disproportionate concentration of potentially disease causing and AMR microbes associated with hotspots is concerning. The increased potential for transfer from hotspots to livestock and the wider environment, compared to non-hotspot field locations, is of public health concern.

Policy and Research Implications

• This work has highlighted the increased concentration of potential pathogens and AMR bacteria in soil and grass surrounding field hotspots. The accumulation of potentially infectious and AMR bacteria at specific locations in grazed fields has the potential to spread resistance and disease to livestock, humans, and the wider environment. The concentration of bacteria present immediately surrounding field hotspots increases the potential for infection and generation of new AMR patterns.

• Disease and AMR control policy can only be effective when policies screen bacterial isolates from a wide range of key potential sources, but this is currently underutilised in existing surveillance schemes.

• Given the disproportionate influence of field hotspots in spreading disease and AMR, enhanced surveillance of these locations could potentially offer increased information about current disease prevalence, AMR trends and identify emerging resistance trends.

• This work suggests that optimising grazing management of fields, to reduce the intensity of livestock interaction with hotspot locations, may have potential health benefits alongside improved animal production. Implementation of this disease and AMR control strategy would be at stakeholder level but proactivity could be supported by recognition in land use policy changes.

Appendices

7.1. Water soluble carbohydrate determination

Extract 200 mg of sample and mix with 20 ml of milli-Q water in a 27 ml vial. Agitate solution on rotary shaker for 1 h at ambient temperature. Filter solution initially with Whatman no. 1 paper and then again with 0.2 µm syringe filter into the high process liquid chromatography (HPLC) vial.

Analysis is completed on duplicate samples by HPLC (Agilent 1260 infinity, California, USA) Sample vials are attached to a Hi-Plex H+ (Agilent, USA) column with the eluent 0.1 % tetrafluoroacetic acid, 0.6ml min^{-1} at 40 ºC. Sample carbohydrates are detected using evaporative light scattering detector. Carbohydrate measurements are quantified against fructan (inulin), sucrose, glucose and fructose standards. The sum of quantified carbohydrates is determined as the total water soluble carbohydrate expressed at % of sample DM.

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