Environmental survival and mobilisation dynamics of *E. coli* and intestinal enterococci associated with common wildlife and wildfowl faecal sources

Emmanuel Olabanji Afolabi

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University of Stirling

Supervisors:

Dr David Oliver

Prof Richard Quilliam

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Declaration of authorship

I, Emmanuel Olabanji Afolabi, declare that this thesis has been composed by me and it embodies the results of my own research. Where appropriate I have acknowledged the nature and extent of work carried out in collaboration with others.

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Emmanuel Olabanji Afolabi

31/08/2022

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Abstract

Faecal pollution of water in rural catchments can lead to downstream impacts associated with water-borne pathogens. However, levels of faecal pollution are most commonly measured by faecal indicator organisms (FIOs) rather than any specific pathogen. While the evidence-base to support our understanding of FIO fate and transfer in the environment is growing, there remain gaps in our understanding of the relative contributions of wildlife versus livestock to microbial impairment of watercourses. The research in this thesis comprises a series of controlled laboratory experiments complemented with an online survey designed to solicit views on the opportunities and challenges of managing microbial pollution in agricultural catchments from different catchment stakeholders. FIO fate and transfer is investigated at three levels: sources of FIOs in the environment; their mobilisation into hydrological pathways; and their delivery to receiving waters and subsequent persistence in streambed sediments. A survival experiment quantifies FIO die-off in dairy cow versus red deer faecal sources exposed to repeated freeze-thaw cycles under controlled laboratory conditions. A laboratory-based approach then investigates whether FIOs are mobilised in different quantities from a typical agricultural, wildlife and wildfowl source, namely dairy, red deer and greylag goose faeces. A final laboratory experiment determines FIO persistence profiles after delivery of dairy, deer and goose faeces into streambed sediment. The online surveys revealed differences in perceptions of livestock versus wildlife contributions to microbial pollution issues at the landscape scale across different catchment stakeholder communities. Characterising how indicators of waterborne pathogens survive and transfer in the environment is of fundamental importance to inform and develop effective strategies for microbial pollution in catchment drainage waters and to reduce associated downstream impacts.

Covid-19 impact statement

On March 23rd, 2020, the University announced that all staff and students should avoid being on campus and the University was closed in terms of on-campus activities. This closure of laboratory facilities and offices was in line with the first UK national lockdown instructed by the UK government. All staff and students were asked to work from home where possible. This approach had significant consequences for my research given that my PhD was largely laboratory based. Over the summer of 2020 a phased return to essential laboratory work was permitted, but access to field site locations to collect field samples for laboratory experiments was impossible. This is because I relied on others for transport, given that I did not have my UK driving licence at the time and social-distancing restrictions associated with COVID prevented me from sharing a vehicle. This further impacted my PhD progress.

At the time of the March 2020 lockdown, I had just completed data collection and laboratory work for my 2nd data chapter (FIO mobilisation). A contingency strategy was devised to ensure data collection could continue outside of the laboratory environment during lockdown to enable a third chapter for my PhD thesis. To do this I opted to pursue the design and distribution of an online survey to solicit views of different catchment stakeholders on their perceived importance of wildlife as contributors to microbial pollution. This alternative study formed a substitute chapter for my PhD thesis (deviating from a potential field-based experiment on campus to complement the laboratory-based chapters); however, the skills and approach necessary for this chapter were very different to undertaking field and laboratory-based analyses. Thus, the process took considerable time to develop while I was also juggling childcare responsibilities at home during the pandemic, further hindering progress. I did manage to spend some of the period at home writing up an earlier data chapter into a manuscript, which I was successful in publishing.

My final PhD data chapter (what became Chapter 4 in the thesis) was also a laboratory experiment, but in the UK we entered 2021 in another lockdown and in Scotland we were again under tighter restrictions. The renewed lockdown once again jeopardised my ability to (i) collect field samples and (ii) carry out essential laboratory experimentation for this fourth and final data chapter. I was able to work around these

challenges with help from my supervisory team and through the gradual easing of university restrictions. However, in total, I lost a significant amount of funded PhD lab-time during the pandemic, which impacted on research and chapter timelines. I secured a 3-month University COVID extension and additional stipend funds from my sponsor, but core laboratory time was lost during a key phase of my PhD and this limited some of the later chapter development. For example, Chapter 4 (streambed sediment) was streamlined to focus on only one FIO (*E. coli*, rather than both *E. coli* and intestinal enterococci) to ease the workload given the reduction in time I had available.

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1.1 Introduction

Protecting water resources from contamination risk by a wide variety of substances is important in order to safeguard human health and ecological status of aquatic receptors. However, water quality is impacted by multiple stressors such as climate and land use change and shifts in population pressure, which challenge environmental decision-makers and those responsible for managing the water environment (Segurado et al., 2021). In particular, pathogen indicators, mainly *Escherichia coli*, are a leading cause of water quality impairment and the number one water quality challenge in the USA (USEPA, 2017). Faecal indicator organisms (FIOs) such as *E. coli* and intestinal enterococci are often used to determine the level of microbial pollution in the environment (Oliver, Porter, *et al.*, 2016). These bacteria are part of the Enterobacteriaceae family and are part of the normal microflora of the gastrointestinal tract of mammals and birds (Bilung *et al.*, 2014). The presence of FIOs in the environment signal faecal pollution and the potential presence of pathogens, hence they are used as regulatory parameters to monitor the hygienic status of designated water bodies worldwide (Porter et al, 2017; Holcomb and Stewart, 2020).

Export of FIOs from catchments via freshwaters can impact on the bathing water environments (Webber *et al.*, 2021). While knowledge of the potential impacts of microbial pollution on bathing waters and shellfish harvesting industries is growing, our understanding of the processes and sources that contribute to FIO export from catchments is far from complete. Therefore, determining and quantifying the full range of possible sources of microbial pollution in catchments is important for helping to devise management approaches to limit contamination of surface waters with pathogens and indicators of their presence.

Table 1.1 Pathogens responsible for particular waterborne illnesses.

Waterborne illness	**Pathogen responsible**
Cryptosporidiosis	*Cryptosporidium parvum*
Cyclosporiasis	*Cyclospora cayetanensis*
Giardiasis	*Giardia duodenalis*
Campylobacteriosis	Campylobacter spp
Legionellosis	*Legionella pneumophila*
Shigellosis	Shigella spp
Typhoid fever	*Salmonella typhi*
Cholera	*Vibrio cholerae*
Dysentery	*Shigella dysenteriae*
Salmonellosis	Salmonella spp

Rainfall events are known to accelerate the transfer of FIOs from land to receiving waters due to entry of storm water runoff from both rural and urban areas (VanWormer et al., 2016); Buckerfield *et al.*, 2019). In urban catchments, sewage discharges and combined sewer overflows represent an important point source of microbial pollution. In more rural catchments, agricultural land will contribute a diffuse source of FIOs to surface waters due to the potential for their mobilisation by rainfall from land applied manures and from faeces deposited on pasture by grazing livestock, but also directly into stream environments (Bu et al., 2014; Stoyanova and Harizanova, 2019). Therefore, identifying various sources of FIOs in catchments and developing effective management strategies to spatially target mitigation and thus limit FIO mobilisation and transfer from land to water is vital in helping to minimise water quality impairment (Reynolds *et al.*, 2021). Some specific pathogens responsible for some particular waterborne illnesses are shown in Table 1.1

Previous studies have shown that transfer of FIOs from agricultural land, especially from livestock farming, is a major contributor to the deterioration of surface water and groundwater quality around the world (Oliver and Page, 2016; Hansen *et al.*, 2020;

Crooks, Harris and Patil, 2021). However, there is growing evidence that wildlife also serve as a reservoir for FIOs in agricultural and forest landscapes, thereby possibly contributing to the microbial load of receiving waters (Kiefer et al., 2012; Guber *et al.*, 2015). One past study, for example, determined that inputs of faecal coliforms (FC) from wildlife accounted for 15–30% of the total FC load for a watershed in north-western Kansas (Parajuli, 2007).

Furthermore, the majority of evolving infectious diseases in humans have been linked to wildlife (Magouras *et al.*, 2020), with a notable significant burden on global economies and public health arising as a result (Crook and Senior, 2017; Smith 2020). Some of the recent reported outbreaks of *E. coli* O157 in the USA were connected to deer; 15 cases of illness, including two deaths in Oregon in early August 2011, were caused by strawberry-transmitted infection of *E. coli* O157:H7 traced back to black-tailed deer and contaminated irrigation water (Laidler *et al.*, 2013).

The majority of studies on microbial pollution of surface waters have focused on *E. coli* from agricultural land use, especially *E. coli* from poultry sources, manure and slurries from dairy facilities and fresh deposits of grazing livestock (Guber et al., 2015). In contrast, very little research documents the contributions of *E. coli* and relative risks of waterborne microbial pollution originating from different wildlife within the agricultural landscape, for example contributions of *E. coli* from deer, geese and other waterfowl. Consequently, researchers in the field of catchment microbial dynamics have made repeated pleas in the published literature for research to plug the gaps in our understanding of data concerning wildlife contributions to catchment FIO dynamics (Oliver et al., 2016). Efforts to investigate wildlife contribution to surface water are increasing (Guber et al., 2015; Muirhead et al., 2011; Kiefer et al., 2012), yet international coverage of useful information (e.g. for understanding magnitudes of source loading or for helping to parameterise models) remains scarce. New knowledge in this area is critical in order to begin to address queries raised among catchment stakeholders (e.g., farmers, farm advisors, regulators) about the uncertainty of *E. coli* loading from wildlife in comparison to the management of livestock and associated agricultural practices.

This chapter will therefore: (i) examine the current state-of-knowledge concerning sources and transfer opportunities of FIOs from wildlife; (ii) identify key challenges and opportunities for developing the empirical evidence base in the area of wildlife-driven risks to microbial water quality; and (iii) outline the thesis aims and objectives along with the thesis structure.

1.2 Wildlife as a source of FIOs in the environment

1.2.1 Wildlife Sources

Agricultural catchments accommodate a variety of wildlife sources in addition to farmed livestock. Populations of deer are known to move across catchment boundaries, populations of resident and migratory geese spend time in farmers' fields, small mammals like badgers, voles and beavers are common in many areas of the world. Therefore, a spatial loading of a variety of faecal sources is distributed across agricultural landscapes, but the relative contribution of microbial pollutants to land (and water) from these sources is relatively unknown. Some studies have shown that wildlife can contribute considerable FIO inputs to both lake and stream sediments, although their contribution might change seasonally (Kiefer *et al.*, 2012; Stocker *et al.*, 2019). An increase in the source contribution of *E. coli* from waterfowl was documented by Ishii et al. (2007) with the 40% observed in spring increasing to approximately 80% in autumn in sediments of Lake Superior. What is less well known is whether those contributions are consistent in other areas of the world or whether land-based contributions from other wildlife, such as deer, can also make such significant contributions to watercourse impairment. The mass of faecal matter produced per day by some selected wildlife/wildfowl are recorded in Table 1.2.

Table 1.2 The mass of faecal matter produced by some selected wildlife/wildfowl.

Source	**Mass of faecal matter (Dry weight g per day)**	**References**
Canada Goose	32.76	(Kear, 1962)
Canada Goose	81.6	(Terres 1987)
Gull	15.6	(Terres 1987)
Red Deer	37.3/ha	(Nugent *et al*., 1997)
Male Rat	6.88	
Female Rat	3.96	(Cavigelli *et al.*, 2005)
Rabbit	28	(Cholis and Nursita, 2022)
Voles	1.27 (daytime)	
	1.57 (night time)	(Liu *et al.*, 2007)

1.2.2. Deer

Previous research has reported the survival of *E. coli* in deer faeces (Kiefer *et al.*, 2012 ; Guber *et al.*, 2015; Pattis *et al.*, 2017; Afolabiet al., 2020) and also the role of deer faeces in providing a reservoir for human pathogens (Kiefer *et al.,* 2012; Dias *et al.*, 2022) The characterisation of enteropathogenic and Shiga toxin-producing *E. coli* in cattle and white-tailed deer (*Odocoileus virginianus*) in a shared agroecosystem in Michigan identified a 40% prevalence of Shiga toxin-producing (STEC), Enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *E. coli* in the faeces of white-tailed deer (Singh et al., 2015). Renter et al., (2001) found white-tailed deer in the USA to harbour *E. coli* O157 and other studies revealed that 83% of roe deer (Mora et al., 2012) and 53% of red and roe deer tested positive for non-O157 STEC (Eggert et al., 2013). Deer were connected to a 2010 *E. coli* O103:H2 outbreak in Minnesota associated with venison (Rounds et al., 2012) as the source of STEC, demonstrating opportunities for faecal contamination of foodstuffs too.

Deer faeces also contain high levels of nutrients, especially phosphate, which can be utilised by FIOs (and pathogens) to enhance their persistence in the environment (Kiefer et al., 2012). High prevalence and concentration of *Campylobacter* spp. and antimicrobial resistance genes (ARGs) have also been recorded in wild white-tailed deer faeces signalling a significant potential (and understudied) risk to the biosecurity of livestock, wild game meat, and wider produce grown in the environment because of possible environment-livestock-deer interactions (Rogers *et al.*, 2018). Other examples of illness traced back to deer include an *E. coli* O157:H7 outbreak via contaminated irrigated water whereby seventy people were infected due to consumption of unpasteurized apple juice in the western United States and British Columbia, Canada, in October 1996 (Guber *et al.*, 2015). Consequently, deer have risen up the agenda in terms of their recognition as being a host of pathogens, most especially where deer share a common catchment area with livestock.

1.2.3 Waterfowl

Waterfowl have been identified as an important reservoir of diffuse sources of faecal pollutants; A New Zealand study determined that *E. coli* was present in 95% of faeces from Canada geese, black swans, ducks and gulls sampled across four different regions (Waldenstrom et al. 2002; Nielsen et al. 2004; Moriarty et al., 2012). They have been referred to as a major FIO contaminant source because of the large numbers of faecal bacteria associated with their faeces, especially gulls and other shore birds (Fogarty *et al.*, 2003; Muirhead et al., 2011). For example, the presence of *E. coli* and enterococci was observed in 95% of samples (n=80) investigated in a survey of microbial concentration in the faeces of waterfowl carried out in New Zealand while *Cryptosporidium* spp. and *Campylobacter* spp were present in 2% and 40% of the samples respectively (Moriarty et al., 2011a), thus posing a potentially important concern for public health.

1.2.4 Rodents and Rabbits

Studies have shown that rodents and rabbits are capable of harbouring different strains of FIOs, even though documented risks from these small mammals are limited (Langholz and Michele, 2013). In an experiment carried out by Bilung *et al.*, (2014) on occurrence of *E. coli* in wildlife from different habitats of Sarawak, Malaysia, it was

evident that rodents had the highest *E. coli* occurrence in the five sampling habitats investigated in Sibu and Nanga Merit, Sarawak, Malaysia. The sampling habitats which included forest park, two recreational parks, oil palm plantation and human settlement recorded 36%, 40% 48% and 82% *E. coli* occurrence in rodent specimens, respectively. An O157 outbreak in eastern England was attributed to contact with the faeces of wild rabbits (Oryctolagus cuniculus) in a play area (Crook & Senior, 2017) thus demonstrating infection pathways via these small mammals. *E. coli* O157 was found in 25% of rabbit faecal samples analysed from a field adjacent to livestock grazing, and 58.6% of samples pooled from a picnic area inside a wildlife park in an experiment to determine the factors affecting the association between cattle known to be *E. coli* O157-positive and individual wild rabbits (Bailey *et al*.,2002). Thus, it is evident that wild rodents and rabbits can harbour FIOs and subsequently contribute to microbial loading of surface water as well as transferring pathogens in the environment. In a recent study, the zoonotic potential of STEC strains sourced from a variety of wildlife sources highlighted the importance of a One Health perspective and monitoring of genomic characteristics in recognising the interconnectivity of environmental health, human health and animal health (Dias et al., 2022)

1.3 **Occurrence, loads and concentrations of FIOs in faeces of wildlife**

Many wild animals are known to roam, graze and thus defecate on, or in close proximity to, agricultural land. Given that wildlife faeces may contain large concentrations of enteric bacteria and potential pathogenic microbes, their faecal contributions may in some circumstances be of public health concern. Approximately 95-99% of FC in gull faeces comprises of *E. coli* (Le´vesque et al. 2000) and concentrations have been reported to range from 1.0 $x10^5$ to 1.9 x 10^9 g^{-1}(Fogarty *et al*., 2003). The average wet weight of faeces excreted by different gull species typically ranges from 11.2 to 24.9 g day^{-1} (Fogarty *et al*., 2003). Using values from this US study, this would result in an average daily load of *E. coli* and enterococci from one gull ranging between 3.5 x 10^8 to 1.2 x 10^{10} for *E. coli* and 4.2 x 10^8 to 1.4 x 10^9 CFU for enterococci on Chicago beach and Traverse City, respectively. It has been estimated that daily faecal excretions by ten ducks is approximately equivalent to the daily *E. coli* excreted by one dairy cow (Zeckoski *et al*. 2005). In Scotland during the summer of 2022, ‘Keep Scotland Beautiful’ ran a campaign at beach environments

warning people to not feed seagulls because their faeces contribute to water pollution (BBC, 2022). However, these signs were challenged by other groups such as conservation charities who commented that seabird droppings provide key sources of nutrients for marine life and their faecal contributions are limited relative to the amount of sewage that human populations discharge into the environment. This highlighted some of the conflicting viewpoints across different stakeholder communities regarding sources of faecal pollution in the environment, and approaches to manage them.

Table 1.3. Reported examples of concentration of E. coli and Enterococci in wildlife/ wildfowl faeces.

Source	***E. coli (CFU/g)***	**Enterococci (CFU/g^{-1})**	**Reference**
Canada Goose	3.5 x 10^6	1.2 x 10^6	(Moriarty *et al.*, 2012)
	1.0 x 10^2	7.3 x10^5	(Meerburg *et al*., 2011a; Middleton & Ambrose 2005)
Gull	1.87 x 10^7	8.90 x 10^6	(Moriarty *et al.*, 2012)
	<1.0 x 10^5– 10^9	1.8 x 10^5	(Fogarty et al. 2003; Wood & Trust 1972)
Deer	2.43 x 10^8	7.32 x 10^5	(Pattis *et al.*, 2017)
	5.9 x 10^7	3.09 x 10^5	(Pattis *et al.*, 2017)
Rabbit	1.48 × 10^7	1.3 x 10^5	(Jeamsripong *et al.*, 2019) (Linaje *et al.*, 2004)

The role of deer in facilitating microbial contamination of the environment and surface waters relative to other sources is unclear, but emerging evidence recognises that they can contribute FIOs. If deer FIO source loading coincides with an opportunity for hydrological transfer (i.e. they form localised critical source areas in the landscape)

then their potential for influencing water quality impairment will increase. Deer have been shown to contaminate the waterways by both direct deposition of faeces into farm waterways and also due to their faecal pellets contaminating runoff that leaves agricultural land via hydrological pathways such as overland flow or drain flow (Pattis *et al.*, 2017). In a study carried out by Pattis *et al.*, (2017), it was discovered that *E. coli* were present in all the deer faeces samples examined, and the concentration ranged between 2.43 x 10^8 cfu g^{-1} dry faeces and 5.9 x 10^7cfu g^{-1} dry faeces. (The mean daily excretion of deer faeces per day was 5.2 kg). In the mean daily excretion of FIOs in the faeces of various livestock and deer estimated, it was discovered that deer had the highest mean daily excretion of *E. coli* compared to all other animals investigated in this study, which included dairy cattle, sheep and Canadian geese (Pattis *et al.*, 2017). Different concentration of FIO reported from different wildlife/wildfowl are summarised in Table 1.3.

A range of other wildlife have potential for contributing microbial pollution to aquatic environments. The reintroduction of beavers in the UK, for example, can bring about environmental benefits by modifying landscape characteristics, which in turn may reduce downstream flooding. However, little is known about their faecal inputs into the complex wetland habitats that they create. In North America, new microbial (Bacteroidales) markers have been designed to detect beaver faecal pollution in an effort to further understand their faecal inputs to wetland systems given the importance of beavers in terms of being a zoonotic reservoir for human pathogens such as *Cryptosporidium* spp. and *Giardia* spp. (Marti et al., 2013). Clearly the importance of different wildlife species depends on the catchment characteristics and environmental context.

1.4 **Survival of wildlife derived FIOs in agricultural catchments**

1.4.1 Survival in faeces

Traditionally, FIOs are believed to survive poorly in the environment, and not grow in secondary habitats, such as water, sediment, and soil because they are primary gut flora of human and warm blooded animals (Winfiel and Groisman, 2003). Once excreted into the environment, FIOs are likely to experience environmental stress such as predation, limited availability of organic matter, high salinity, solar radiation, limited

moisture, temperature differences and challenges linked to pH (Whitman *et al.*, 2004; Evans and Wallenstein 2012; Korajkic et al.2014; Jang *et al.*, 2017). However, concentration of FIOs in fresh animal faeces are often high, and they are released into the environment via faecal deposits where they may persist depending on the environmental conditions. Moriarty *et al.*, (2012) investigated the survival of *E. coli,* enterococci and *Campylobacter jejuni* in Canada goose faeces on pasture in New Zealand. FIOs (*E. coli* and Enterococci) were observed to survive for 77 days, the length of the experiment. While *E. coli* decreased from a peak of 4000% of the initial population on day 2 (i.e., after population growth) to <0.005% by day 77, Enterococci in contrast decreased from 8000% of the initial population on day 2 following a growth phase to 10% by day 77. However, *Campylobacter jejuni o*nly survived for 2 days in summer and 7 days in winter. This is an indication that FIOs can survive in wildfowl faeces for several months in the environment under specific environmental conditions. Also, studies have suggested that warm, wet weather conditions can enhance the growth of *E. coli* in such faeces and also facilitate release of *E. coli* from the wildlife faecal matrix into the wider environment, especially deer (Guber *et al.*, 2015). This represents a growing concern about environmental risks of FIOs released from wildlife sources in the face of climate change. Similarly, FIOs have demonstrated capacity to survive and grow in the soil and become adapted to environmental temperatures which are sub-optimal for their growth while competing for niche space with indigenous soil organisms (Brennan *et al.*, 2010). Furthermore, there is very little research that has investigated the role of temperature cycling on FIO persistence, both with respect to livestock and wildlife/wildfowl derived FIOs. For example, the impact of freeze-thaw cycles on FIO persistence has received little attention and yet is a process that can impact on FIOs contributed from a range of catchment sources.

1.4.2 *Persistence of wildlife-sourced FIOs in different media and sources*

Studies have shown that FIOs can survive and persist in surface water environments (Korajkic *et al.*, 2019; Motlagh and Yang, 2019; Li *et al.*, 2021; Calderon *et al.*, 2022). Indeed, surface water sediment has been demonstrated to be more conducive for FIO survival than the water column because it reduces sunlight inactivation (Curtis and Trapp 2016), protects against predators (Korajkic et al., 2013; Wanjugi and Harwood, 2013) and increases nutrient and organic carbon availability (Craig *et al*., 2004;

Wanjugi et al. 2016). In addition, sandy sediments with large particles have been shown to be a good habitat for FIOs due to porosity, permeability and available nutrient provided by sediment (Cinotto, 2005). However, FIOs persist longer in fine sediments (< 2 µm) due to more surface area and attachment sites which aids cell-particle association and slower inactivation of FIOs in the streambed sediment (Hassard *et al.*, 2016; Wu *et al.*, 2019). Sediment with high silt and clay fractions support higher concentration of FIOs than sandy sediment because of smaller pore spaces of fine particles which provide defence barrier against other bacteria predators (Kunkel *et al.*, 2013), and high organic carbon content for growth (Craig *et al*., 2001). Thus, fine sediment can improve survival of FIOs and may constitute a greater hotspot for FIOs in streambed sediments as well as represent increased risk of microbial pollution to surface water due to high FIO concentration and easy resuspension of these particles during rain events (Kunkel *et al.*, 2013; Wu *et al.*, 2019).

Adhesion efficiency of FIOs has been shown to be dependent on strain > salinity > sediment (Wyness et al., 2018). FIOs have been found to survive in the water column of freshwater as a result of biofilm formation on sediments, which plays a major role in moderating nutrient accessibility through close proximity of organisms, as well as temperature and condition oscillations (Abberton *et al.*, 2016). Far more evidence is available to document sediment-related persistence of FIOs derived from livestock sources relative to those that have been contributed from wildlife. While survival of FIOs from livestock sources in freshwater has been well documented (Muirhead *et al.,* 2004; Ishii *et al*. 2007; Cho et al., 2010), data on the persistence of FIOs from wildlife sources remains scarce. Data about the persistence of FIOs from wildlife source in freshwater sediment reported by Kiefer *et al.*, (2012) remains a reference point to describe persistence of FIO from wildlife; though the die-off period of *E. coli* from wildlife sources in this study only lasted 32 days with *E. coli* concentration within one order of magnitude. Therefore, this highlights the need for further experimental work to determine the die-off period of wildlife-derived FIOs in freshwater environments and the factors that enhance their survival.

1.5 Mobilisation and transfer of FIOs from wildlife sources

Mobilisation of FIOs in agricultural catchments occurs when microbial contaminants from diffuse sources are detached from locations within or on soil, often following raindrop impact. Once mobilised, FIOs are transferred (as both freely suspended cells and attached to soil or organic particles) via hydrological pathways and are either redeposited further downslope or delivered to a watercourse (Oliver *et al.*, 2007). It should be noted that FIO transfer to surface waters is usually a function of organism survival in soil and drainage water, as well as mobility through different hydrological pathways (Oliver *et al.*, 2005).

Transfer of FIOs from wildlife faeces into the wider environment will be controlled by a range of geospatial factors (Weller *et al.*, 2017). For example, a strong negative correlation between the number of splash droplets and the distance from the splash origin, (i.e. faecal pellets) has been recorded using small scale experimentation to investigate *E. coli* transfer from simulated wildlife faeces to lettuce during foliar irrigation (Weller *et al.*, 2017). The likelihood of *E. coli* transfer from faeces to produce was considered negligible past a given distance (25-45 cm from the origin) (Monaghan and Hutchison, 2012, Weller *et al.*, 2017), however local-scale FIO transfer from the point of raindrop impact is more common. *E. coli* strain differences, and age and structure of the faecal pellets, were factors found to influence FIOs transfer from faecal material to produce following splash impact from irrigation. Raindrop impact and associated detachment mechanisms that release FIOs from faecal material will also play a role in initiating the journey of FIOs from source material on grazed pasture. There are risk factors associated by the mobilisation of organisms from mass faeces of different wildlife/ wildfowl, but the catchment risk will be site specific and dependent on the number of the animals (Table 1.2) While data on FIOs mobilisation and transfer from agricultural land have been documented (Oliver *et al.*, 2005; Murphy *et al.*, 2006; Oliver *et al.*, 2007; Muirhead, 2015), data from wildlife source remain scarce. Further study is needed to identify how raindrop impact mobilises *E. coli* from wildlife faeces under natural rainfall, and importantly under a range of different rainfall intensities and cycles of wetting and drying.

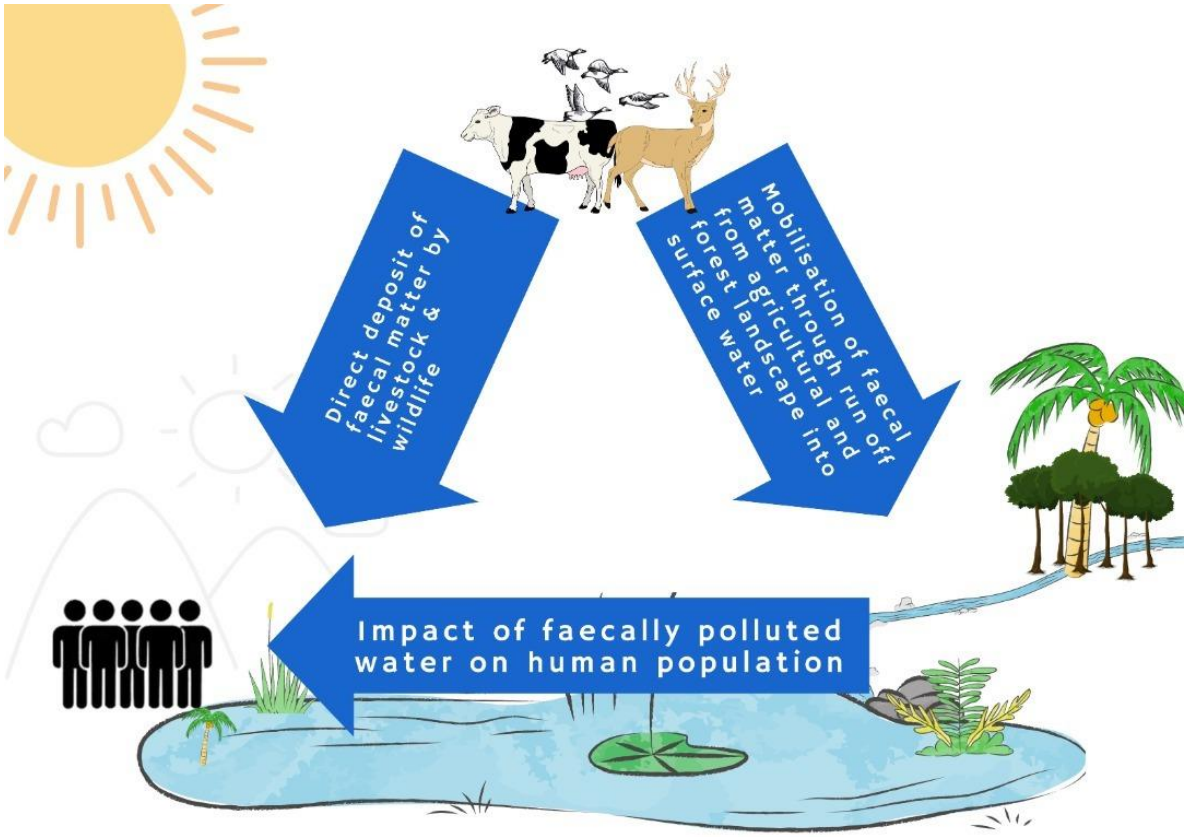

Figure 1.1 *Source-Mobilisation-Delivery-Impact continuum of wildlife faecal sources from agricultural and forest landscape.*

1.6 Wildlife as vectors

Studies have shown that waterfowl harbour FIOs as well as potentially pathogenic microbes (Waldenstrom *et al.* 2002; Nielsen *et al.* 2004). Their ability to migrate means that they can represent highly mobile vectors of FIOs and potential pathogens (Moriarty *et al.*, 2011). Isolation of FIOs from wildfowl faeces by different researchers (Muirhead et al., 2011; Moriarty *et al.*, 2012) as well as isolation of *E. coli* O157 from wild birds in Morecambe Bay and Lancaster, UK is an indication that wild birds can serve as vectors for the dissemination of FIOs and pathogens (Bilung *et al.*, 2014). It has been shown that gulls are an important source of microbial contaminants at the beaches (Converse et al., 2012; Araújo et al., 2014; Staley and Edge, 2016) contributing high densities of FIOs in water (Lu et al., 2011a). More so, gulls can shed bacterial pathogens (Lu et al., 2011b; Ebert et al., 2016) and antibiotic resistant bacteria (Bonnedahl et al., 2009; Dolejská et al., 2009) thus making them relevant to

public health because they have the potential to transport and transfer pathogens to other sites (Alm *et al.*, 2018).

Similarly, deer can acquire and transmit pathogens among livestock and other wild animals (Branham *et al*. 2005). White-tailed deer can be infected by *E. coli* O157:H7 and *Salmonella* spp. through consumption of water from cattle troughs and directly from co-grazing the same pasture and subsequently spread the pathogens to livestock and other wildlife, and *vice versa* (Branham *et al*. 2005). Also, the ability of small mammals (rodents) as a potential vector carrying several strains of *E. coli, Salmonella*, and other pathogenic microorganisms has been documented (Langholz and Michele, 2013).

The territorial nature of wild animals coupled with spatial distribution of individuals across diverse landscapes or complex social systems leads to a heterogeneous contact structure (Craft *et al*., 2011). Their social systems can also vary between populations in different ecosystems as a result of fluctuations in available resource and interactions between wildlife, water, livestock, and human factors, such as management practices (Langholz and Michele, 2013) can play a critical role in microbial cycling on-farm. For example, *E. coli* O157:H7 was found in 15% in feral pig faeces; 4% in surface water samples and 8% in soil and sediment samples in the 2006 *E. coli* O157:H7 spinach outbreak (Jay *et al*. 2007). Feral pigs on the ranch implicated in the outbreak moved freely between the cattle pastures and the crop fields while the cattle had direct access to the major surface water source on the ranch. Therefore, understanding the influence of high population density of wildlife and proximity to human habitation and agricultural farmland on the prevalence of pathogens in these systems is clearly important.

1.7 Tracking different sources of FIOs in rural catchments

The detection of FIOs in environmental samples provides no indication of the pollution source. Microbial source tracking (MST) is an approach that has evolved over recent years to allow catchment scientists and those with a responsibility for landscape decision-making to better understand where in the environment particular microbial pollution signals originate from (Simpson et al., 2002). By targeting host-specific

markers (e.g., Bacteroidales, marker genes etc), MST allows a degree of source identification of microbial pollution (Shrestha et al., 2020) and in turn can offer some opportunities for more spatially targeted management and mitigation. The approach is not without uncertainties and MST is best integrated as part of a toolbox for understanding catchment forensics of microbial water quality pollution, largely because each sample that is analysed using MST will only ever represent a point-in-time and the specific apportionment of source loading may be highly dynamic, particularly under wet weather conditions (Stapleton et al., 2007; Unno *et al.*, 2018).

Deploying FIO sampling in combination with MST analysis offers a complementary approach that can offer advantages for water quality assessment. This was demonstrated in a catchment in Florida that was managed for wildlife conservation, but which historically exceeded the state regulatory guideline for faecal coliforms (Nguyen et al., 2018). Using both bird markers and sewage markers, the study identified bird faecal pollution to be at high levels throughout an annual cycle and highlighted natural bird sources as a key contributor to water quality impairment (Nguyen et al., 2018). Catchment land use composition and characteristics will influence the wildlife contribution of *E. coli* to water, but even in those catchments where high wildlife-derived microbial pollution contributions have been identified using MST approaches there remains a degree of uncertainty as to whether that would convert to higher degrees of risk to human health. A study integrating quantitative microbial risk assessment (QMRA) with MST identified 65% of *E. coli* in a catchment as being sourced from wildlife sources; however, the source of a pathogen (inferred through indicators) can potentially influence their infectivity and link to different levels of human health risk and despite wildlife dominating the catchment pollution burden, the human health risks were estimated to be driven mostly by human faecal sources (Gitter *et al.*, 2020).

1.8 Human exposure to wildlife-derived FIOs and associated impact

The presence of wildlife around recreational parks and water is common (Bailey *et a*l. 2002; Crook and Senior, 2017), thus the potential for interactions between humans and wildlife-sourced FIOs can be facilitated through exposure to contaminated soil and water (Gorham and Lee, 2016). Given the potential for FIOs to persist in different

environmental matrices (Moriarty *et al.*, 2012; Kiefer *et al.*, 2012), and the potential for human interactions with soil and water in recreational environments, it is highly plausible that exposure pathways linking FIOs (and potential pathogens) to the public can arise, and in some cases may result in cases of human infection and resulting illness. Therefore, multiple exposure situations should be considered whenever humans use recreational water and parks for leisure. Little research has explored relationships between the use of recreational space and faecal-oral transmission of wildlife-derived FIOs or other microbial contaminants, though it is an area of research that would offer important exposure pathway data relevant for QMRA.

For example, ingesting pathogens from wildlife contaminated water during swimming may represent a key primary mode of exposure (Gorham and Lee, 2016) because of the direct contact between human and microbially impaired water. On average, 37 ml and 16 ml of water is consumed by children and adults, respectively, during 45 minutes of swimming (Dufour et al*.,* 2006). In countries with warm, dry climates and where recreational swimming in lakes and streams is more common, there will be higher likelihood that FIOs sourced from wildlife may be present in the aquatic environment, due to the lower potential for dilution prior to delivery to coastal waters. Data such as this can be used to inform QMRA for assessing infection risk among swimmers (Gorham and Lee, 2016).

It should be noted that wildlife faeces can affect both water quality but also the quality of terrestrial environments, for example faecal loading of soils and sand (Titchenell and Lynch, 2010). Consequently, their droppings create a potential risk of contact and or infection for children playing in the sand or families picnicking on or near the beach or in recreational parks where direct or indirect contact with wildlife faeces may occur (Gorham and Lee, 2016). Also, sand pore water located in the water-washed swash zone, may serve as a vital reservoir for FIOs from different sources (Heaney et al*.,* 2012; Halliday et al., 2014), including wildlife sources. On freshwater beaches, FIO concentrations were observed to be significantly higher (4–38 and 3–17 times higher) on a per-unit basis in swash zone sand than in nearby freshwater, highlighting that FIOs delivered to this environment can survive in this matrix (Alm et al*.,* 2003; Whitman *et al.*, 2015)

1.9 Wildlife and antimicrobial resistance

With increasing human population and growing fragmentation of natural habitats (Arnold et al., 2016) coupled with the emergence, spread, persistence and evolution of infectious disease in wild animals (Rogers et al., 2018), there is increased risk of infection transmission between and within populations (Jones et al*.,* 2008). Antimicrobial resistance (AMR) has been documented as one of the major challenges to the security of global health (WHO 2014), yet little is known about the movement and fate of AMR in the natural environment (Arnold et al., 2016; Avery et al., 2022), especially in extremely mobile species that might act as effective dispersers of AMR (Greg *et al*., 2015; Huijbers et al., 2015). AMR is a primordial phenomenon that developed in dynamic microbial communities within which antimicrobials are made by environmental organisms (bacteria and fungi) naturally living in soil, water, and other environmental matrices (Davies and Davies, 2010). The phenomenon has been complicated by horizontal gene transfer, a process through which bacteria exchange adaptive genes (Thomas and Nielsen, 2005).

Proximity of wildlife to human activities has been reported as a major factor that influences the carriage of zoonotic pathogens and antimicrobial resistance (AMR) in wild animals (Alonso et al. 2016, 2017; Stedt et al. 2014; Bonnedahl et al. 2009; Rwego et al. 2008). This important interaction, particularly between wildlife and anthropogenic waste streams, usually occurs in agroecosystems (Rogers *et al.*, 2018). Often, selective pressures that favour AMR shed in faeces occur as result of the use of antibiotics for the treatment of human or livestock disease, and for growth promotion and prophylaxis in livestock. This acquired AMR usually results from exposure to antimicrobial drugs, which promotes resistance by selecting bacteria within a population with genetic traits conferring resistance (Arnold et al., 2016). Despite growing global concern about AMR in human medicine and agriculture (Rogers et al., 2018), less attention has been given to the role of wild animals in the ecology and evolution of antimicrobial resistance (Greg et al., 2015; Huijbers *et al*., 2015), even when wildlife shed, and are able to disseminate AMR genes (Greg *et al*., 2015; Wiethoelter et al., 2015). For example, wild small mammal species such as mice and voles have been implicated as carrier of anti-microbial resistant *E. coli* (Furness *et al.*, 2017). The interaction of wildlife with anthropogenic waste as well as application of

manure and bio solid to agricultural land as a fertilizer for pasture and crops have presented a potential pathway for spreading pathogenic microorganisms and AMR to the environment (Rogers et al., 2018). More often than not, runoff from agricultural field and effluent end up flowing into the coastal waters and beaches where they pollute the water body with faecal matter (Graham et al*.,* 2014). These polluted water bodies could serve as a critical point of contact where wildlife and other animals are exposed to AMR (Leonard et al., 2015). Most studies in wildlife suggested that AMR in wildlife is as a result of spill over of resistant bacteria from domestic animals or people (Rwego et al., 2008; Wardyn et al., 2012; Porrero et al., 2013).

1.10 The agricultural wildlife interface and interactions

Understanding the transfer of FIOs across the wildlife-agricultural interface represents an important research challenge. Farm animals are generally known as major reservoirs of FIOs and pathogens (Renter et al. 2003); however, FIOs are commonly sourced from wildlife too (Langholz and Michele, 2013). Ecological factors, concentration and persistence in the shared environment as well as other variables related to local conditions have been mentioned as possible factors that may be responsible for the transfer of FIOs between wildlife and agricultural animals (Langholz and Michele, 2013). FIOs are known to spread through a faecal-oral route (Guber *et al.*, 2016), and both farm and wild animals harbour these bacteria (Renter et al. 2001). Therefore, exposure of livestock, wildlife and wildfowl to contaminated water, soil and foliage could be a possible interface for transmission of FIOs among these different groups of animals (Branham et al*.,* 2005).The role of environmental conditions, such as temperature, moisture content, pH etc, associated with different environmental matrices where livestock, wildfowl and wildlife can coincide is therefore important to understand with regard for their potential to promoting FIO fate and transfer.

Previous studies have highlighted interactions between wildlife and farm animals, especially avian and livestock where the birds fed in cattle yards and areas of concentrated livestock wastes, and the authors have raised concern of incidence of contamination (e.g., Nielsen et al*.,* 2004; Pedersen and Clark 2007; Carlson et al., 2011). Studies recognise co-occurrence of FIOs in wildlife populations sharing close range with cattle, and other domestic ruminants (Nielsen et al., 2004; Foster et al., 2006; Apun et al., 2011; Bilung et al., 2014) as a result of interaction, yet reporting is

inconsistent. For instance, *E. coli* O157 was isolated from one of four deer faecal samples but none was detected from agricultural animals' faecal samples on the same farm in Ireland (Bolton et al. 2011). In another study, *E. coli* O157 was not detected in white-tailed deer's faecal samples in the same rangeland with cattle and sheep that had low prevalence of *E. coli* O157 on grazing land in Texas (Branham et al. 2005). In a further study, *E. coli* O157:H7 was identified in 5/22 faecal samples from white-tailed deer sharing the same pasture with cattle in Kansas, but the cattle were not tested (Sargeant et al. 1999). In an experiment to compare the genetic relatedness of *E. coli* O157:H7 isolates from cattle and deer using pulsed-field gel electrophoresis, Fischer et al. (2001) found different patterns of Shiga toxin genes in the cattle and deer isolates, indicating that there was little relatedness.

The interactions of rodents and rabbits in association with cattle have been documented in a few studies. Wild rabbits were implicated as a potential transport vector of *E. coli* O157 from a cattle pasture to a picnic area during an outbreak investigation in England (Bailey et al. 2002, Scaife et al. 2006; Crook and Senior, 2017). In another experiment, a large number (300) of rodent faecal samples from a farm were tested for *E. coli* O157 – the same farm had tested positive in feedlot and dairy herd faecal samples for O157, but the bacteria were not recovered from the rodent samples. However, *E. coli* O157 was isolated from rats in close proximity to cattle in another two European studies (Cizek et al. 1999, Nielsen et al. 2004).

Mixed reports on wildfowl interactions with livestock have also been documented. In an experiment to clarify the possible role of wild animals in the transmission of verocytotoxin-producing *E. coli*, two wild birds from one pig farm were PCR positive, but VTEC was not isolated from any samples taken on the pig farms (Nielsen et al., 2004). In another study, Cernicchiaro et al. (2012) found that the presence of European starlings was one of multiple factors positively associated with *E. coli* O157 in dairy cattle faecal pats.

1.11 Research Opportunities

Limited data on wildlife/wildfowl FIO sources and their potential public health risks calls for more robust research in agricultural catchment vis-a-vis the microbial contribution of wildlife to freshwater pollution. Wildlife and wildfowl are highly mobile, covering a great distance and making contact with agricultural produce and livestock. Thus, future research is needed to quantify the loading, fate, mobilisation, transfer and cycling of FIOs in the environment, which potentially can be extended to different domestic and wildlife species, and is a clear priority. A clear gap in the current evidence base is good quality data on how wildlife-derived FIOs survive and transfer in the environment, and whether their fate, mobilisation and transfer is any different to that of FIOs derived from common agricultural sources. Fundamental questions remain unanswered, for example: How important are common UK wildlife, e.g. deer and geese, in terms of their contribution to FIO loading of catchments?; does the magnitude of FIOs sourced from common wildlife types vary in space (e.g. in different catchments) and time (e.g. across seasons)?; to what extent does FIO die-off vary within different wildlife faeces under specific environmental conditions?; and what factors control the efficiency of FIO mobilisation from wildlife faeces?

Further research on behavioural observation and electronic tracking devices to measure contact between and movements of wildlife and wildfowl should also be explored. There is potential for technologies to be deployed to enhance tracking and sensing and provide for complementary data to support our understanding of wildlife contributions to faecal pollution. These include, for example, Global Positioning System (GPS) tracking, internet-of-things and farm sensors for better understanding of both the environment and wildlife movement within the sensed environment. Furthermore, future studies could further exploit microbial source tracking (MST) technologies to detect signatures human versus livestock versus wildlife strains to determine the relative contributions of potential pollution sources in complex catchments.

Beyond technology, there are citizen science and stakeholder survey opportunities that can shed further light on wildlife sightings and numbers. Both approaches can help in understanding numbers and movements of different wildlife and wildfowl, which

is an important precursor to understanding the spatial distributions of wildlife FIO sources in catchments and yet this crucial data is difficult to access or inherently uncertain.

1.12 Thesis aims and objectives

In response to some of the research opportunities and questions identified above, this PhD thesis broadly focuses on understanding how the survival and mobilisation dynamics of two faecal indicator organisms, *E. coli* and intestinal enterococci, vary when contributed to the environment from a common livestock (dairy cow), wildlife (red deer) and wildfowl (greylag goose) faecal source. The overarching aim was to provide new quantitative data to support our understanding of the role of wildlife and wildfowl in contributing to FIO pollution of surface waters in rural catchments. The following objectives contribute to the body of research in this thesis:

Objective 1: to characterise FIO die-off in dairy cow versus red deer faecal sources exposed to freeze-thaw cycles representative of environmental conditions during the colder seasons of temperate regions;

Objective 2: to determine whether there are differences in FIO mobilisation dynamics from typical agricultural, wildlife and wildfowl sources;

Objective 3: to quantify the persistence of *E. coli* derived from dairy cow, deer and goose faecal sources introduced to streambed sediment under different temperature regimes;

Objective 4: to investigate how different stakeholders perceive the potential for wildlife to impact on microbial water quality and contribute towards spreading of antimicrobial resistance via the water environment.

1.13 Thesis Structure

To deliver on the thesis aim and objectives the research in this thesis comprises a series of controlled laboratory experiments complemented with an online survey designed to solicit views on the opportunities and challenges of managing microbial pollution in agricultural catchments from different catchment stakeholders. FIO fate

and transfer is investigated at three levels**: sources** of FIOs in the environment; their **mobilisation** into hydrological pathways; and their **delivery** to receiving waters and subsequent persistence in streambed sediments. A survival experiment quantifies FIO die-off in dairy cow versus red deer faecal sources exposed to repeated freeze-thaw cycles under controlled laboratory conditions. A laboratory-based approach then investigates whether FIOs are mobilised in different quantities from a typical agricultural, wildlife and wildfowl source. A final laboratory experiment determines FIO persistence profiles after delivery of dairy, deer and goose faeces into streambed sediment. An online survey then provides a characterisation of stakeholder views on the potential **impacts** of wildlife and wildfowl on microbial quality of surface waters in rural catchments. The structure of the thesis therefore maps each data chapter in sequence to the source-mobilisation-delivery-impact (SMDI) continuum originally developed for diffuse nutrient pollution (Haygarth et al., 2005). This framework is transferable and useful for conceptualising how generic diffuse pollutants, including FIOs, interact with the environment to become a threat to water quality at the catchment scale.

2. Impact of freeze–thaw cycles on die-off of *E. coli* and intestinal enterococci in deer and dairy faeces: Implications for landscape contamination of watercourses

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Abstract

Characterising faecal indicator organism (FIO) survival in the environment is important for informing land management and minimising public health risk to downstream water users. However, key gaps in knowledge include understanding how wildlife contribute to catchment-wide FIO sources and how FIO survival is affected by low environmental temperatures. The aim of this study was to quantify *E. coli* and intestinal enterococci die-off in dairy cow versus red deer faecal sources exposed to repeated freeze–thaw cycles under controlled laboratory conditions. Survival of FIOs in water exposed to freeze–thaw was also investigated to help interpret survival responses. Both *E. coli* and intestinal enterococci were capable of surviving sub-freezing conditions with the faeces from both animals able to sustain relatively high FIO concentrations, as indicated by modelling, and observations revealing persistence in excess of 11 days and in some cases confirmed beyond 22 days. Die-off responses of deer-derived FIOs in both faeces and water exposed to low temperatures provide much needed information to enable better accounting of the varied catchment sources of faecal pollution and results from this study help constrain the parameterisation of die-off coefficients to better inform more integrated modelling and decision-making for microbial water quality management.

2.1 Introduction

Agricultural landscapes can harbour a large burden of faecal indicator organisms (FIOs), such as *Escherichia coli* and intestinal enterococci. Major contributors to this burden include grazing livestock and land applications of both solid and liquid manures (Oliver et al., 2018; Sharma et al., 2019). Microbial water quality is inferred via FIO concentration but the detection of FIOs in water samples does not necessarily imply that pathogens are present; rather, increased concentrations of FIOs signal a higher level of faecal pollution (García-Aljaro et al., 2019). Knowledge of how FIOs survive in the environment is therefore important for informing land management and understanding wider aspects of public health risk to downstream water users, e.g., those exposed to contaminated recreational water (Kay et al., 2008). However, there is now a growing recognition that wildlife, e.g., deer and geese, can further contribute to the FIO burden in rural and agricultural landscapes (Cho et al., 2020); the importance of this contribution to downstream impacts on microbial water quality is relatively unknown (Jeong et al., 2019).

FIO survival outside of the host gut is strongly influenced by temperature (Cho et al., 2016). Previous research has focused on FIO persistence under constant temperature conditions and how diurnal temperature fluctuations can impact on FIO survival (Oliver et al., 2016a; Smith et al., 2019), with particular attention given to the likely effects of climate change and warming temperature cycles on the persistence profiles of faecal bacteria (e.g., Hellberg & Chu, 2016; Porter et al., 2019). There are, however, relatively few studies of FIO survival at low environmental temperatures, including subfreezing conditions, or through freeze–thaw (F–T) cycles, and those that do exist have focused on FIOs in soil and water matrices. Findings from those studies have identified reduced *E. coli* survival times in river water undergoing repeated F–T stress, with a more pronounced reduction in cell numbers during the first F–T cycle (Wang et al., 2019). Similarly, there are reports of repeated F–T cycles in soil accelerating die-off rates of enteric bacteria relative to constant cold temperature conditions (Asadishad et al., 2013; Rocard et al., 2018) and total coliforms have been found to persist in excess of six months in subfreezing soil temperatures (Adhikari et al, 2007). Bacterial cells that enter the soil pore architecture after mobilisation from faeces are likely to be more susceptible to freezing conditions than those that remain in the protective

insulation and nutrient rich matrix of a faecal deposit, but data to confirm or refute this are lacking.

Overprediction of modelled versus observed *E. coli* burden at the landscape scale during winter has been hypothesised to be a consequence of non-conducive conditions of sub-freezing temperatures for *E. coli* survival (Oliver et al., 2012). Indeed, many catchment scale models of FIO fate and transfer are highly parameterised to account for typical seasonal temperature effects on cell persistence (e.g. Jeon et al., 2019) and yet the impact of sub-zero temperatures and F–T on FIO concentrations in faeces remains largely unquantified and missing from such models (Oliver et al., 2016b; Guber et al., 2015). This is in parallel to the lack of inclusion of relevant die-off coefficients for FIOs derived from wildlife faeces in general. Understanding whether FIOs are insulated from F–T processes by a protective faecal matrix and quantifying the impacts, if any, on FIO die-off rates for different F–T temperature regimes is therefore important to more fully account for the temporal dynamics of FIO burden in the landscape. In addition to the effects of varying F–T temperatures on FIO survival, there is also likely to be differential protection of the FIO population attributed to the characteristics of the faecal source, e.g., faecal pats versus faecal pellets.

Opportunities for livestock and wildlife faecal deposits to undergo F–T stress are not uncommon. While cattle may be offered some protection from cold weather in the form of housing, European temperate grassland management sometimes favours early turnout of cows to pasture in spring when overnight temperatures can promote F–T, and in other areas of the world, e.g., New Zealand, the use of cattle housing, and in turn protection from F–T, is much less common than in the UK or the USA (Wilkinson *et al.*, 2020). Extensive sheep grazing in remote uplands is also typical in many regions of the world where both livestock and wildlife faeces will be frequently exposed to regular F–T processes during colder seasons of the year and indeed other seasons depending on altitude.

The overarching aim of this study, therefore, was to characterise *E. coli* and intestinal enterococci die-off in dairy cow versus red deer faecal sources exposed to F–T cycles representative of environmental conditions during the colder seasons of temperate regions. The specific objectives of the experiment were to: (i) quantify differences in

die-off of FIOs exposed to varying degrees of F–T cycling relative to faeces held at constant low temperatures; (ii) evaluate whether the nature of the faecal source influenced the rate of die-off observed during F–T cycles relative to freely suspended cells in water; and (iii) provide parameter values to represent new understanding of the importance of F–T processes influencing the environmental persistence of FIOs to better inform more integrated modelling and decision-making for microbial water quality management.

2.2 Materials and Methods

2.2.1 Provenance of Faeces Used in All Experiments

Fresh dairy faeces were collected from the livestock housing of a conventional dairy farm in Stirlingshire, Scotland. Cows were permanently housed and a mechanical barn floor scraper was in operation meaning that any faeces collected was guaranteed to have been deposited within the previous 30 min. Fresh faeces of red deer were collected from the Scottish Deer Centre, Fife, Scotland. Fields containing deer were harrowed prior to faecal collection, which ensured that all faeces collected were fresh (<12 h old). After collection, all faeces were transferred immediately (<1 h) to the laboratory for use in the experiment and thus no interim storage was required.

2.2.2 Experiment Design

2.2.2.1 Faecal Mesocosms

A laboratory-controlled experiment was used to mimic the effect of F–T temperature cycles on the survival of FIOs indigenous in red deer and dairy cow faeces. All experiments were carried out in temperature-programmable incubators (Sanyo Incubator MIR−153, Japan). Two temperature treatments cycling over 24 h periods were used: (i) 4 ◦C, 0 ◦C, −4 ◦C (herein −4 ◦C F–T); and (ii) 0 ◦C, −4 ◦C, −8 ◦C (herein −8 ◦C F–T). Both treatments spanned an 8 ◦C temperature range to focus investigation on different temperature extremes rather than rates of temperature change. Faeces were held for 8 h at each of the three temperatures during the 24 h cycle to ensure a rapid thaw and a gradual freeze. Figure 2.1 shows the experimental set-up within the incubator. Two constant temperature control treatments were used (4 ◦C for comparison with the −4 ◦C F–T treatment and 0 ◦C for comparison with the −8 ◦C treatment). Both treatments ran for a minimum of 11 days and maximum of 22 days.

Each F–T and control temperature treatment consisted of five and three replicate faecal pat/pellet deposits per sampling day, respectively. The use of full-size faecal deposits was impractical for a replicated laboratory experiment; therefore, faecal samples were bulked and homogenised in a sterile plastic container and then distributed into shallow circular 70 mm diameter foil trays as either 100 g dairy faecal deposits or 90 g piles of deer faecal pellets. Replicates were randomly divided into each treatment and each replicate was destructively sampled on days 0, 1, 2, 4, 7, 11 and (where relevant) day 22. All treatments included two additional faecal samples that were used to measure the internal temperature of faeces over the course of the experiment, i.e., a DS1921G Thermochron i-button temperature logger (iButtonLink; Whitewater, WI, USA) was placed within the core of the faecal matrix. The same loggers were used as a quality control indicator of the incubator air temperature; in some treatments a drift in the F–T temperature regime was observed after ~ 14 days and in those cases, any data derived after sampling day 11 were not used. Every two days all faecal deposits were misted with sterile distilled water at a rate of 1 mL/100 cm^2 to avoid complete dehydration of the faeces under incubator conditions. Prior to sampling, each replicate faecal deposit was weighed to determine fresh weight change over time. Next, approximately 30 g of faeces (6 × 5 g subsamples) was randomly sampled from each replicate tray using a sterile spatula and transferred to a sterile 50 mL collection tube. Faecal samples were extracted from the core of the deposits to avoid sampling surface crust. Microbial analysis to determine concentrations of colony forming units (CFU) was initiated immediately after obtaining the samples.

***Figure 2.1**: Deer faecal sample undergoing freeze-thaw cycles in a temperature programmable incubator.*

2.2.2.2 Water Mesocosms

In order to investigate whether a faecal matrix offered protection from F–T impacts, waterborne FIOs were subjected to the same F–T conditions. Plastic tubes contained 40 mL of sterile distilled water and were inoculated with 1 mL of a mixed inoculant of *E. coli* and intestinal enterococci with an initial concentration of ~8.0 and 7.2 $\log_{10}$ CFU/mL, respectively. The inoculant was prepared from *E. coli* and intestinal enterococci strains isolated from either the dairy or deer faeces from the same herds. Cells from an overnight culture of LB broth (Fisher Bioreagents, UK) were harvested following centrifugation at 3600 rpm for 3 min. The pelleted cells were resuspended in 10 mL phosphate buffered saline (PBS, Fisher Bioreagents, NJ, USA) and washed three times through resuspension and centrifugation before final suspension in 40 mL PBS. Three replicates per sampling day were randomly divided into each F–T treatment and replicates was destructively sampled on the same days as the respective faecal treatments.

2.3 FIO Enumeration

At each time point, approximately one gram of faeces was transferred to 9 mL of sterile PBS in a 15 mL centrifuge tube and homogenised using an orbital shaker (160 rpm for 60 min at ambient temperature). Each tube was vortex mixed for 30 s prior to subsequent 1:10 serial dilution in PBS. For water samples, 1 mL of water from each replicate mesocosm was transferred to 9 mL of sterile PBS and vortex mixed for 30 s prior to subsequent 1:10 serial dilution in PBS.

Briefly, 1 mL of each serially diluted sample was pipetted onto a sterile 0.45 µm cellulose acetate membrane and washed through a vacuum-filtration unit (Sartorius Stedim Biotech., Goettingen, Germany) with ~20 mL of sterile PBS. To determine presumptive *E. coli*, membranes were aseptically transferred to a Petri dish containing Membrane Lactose Glucuronide Agar (MLGA) (CM1031, Oxoid, Basingstoke, UK) and incubated inverted at 37 ◦C (±0.2 ◦C) for 18–24 h. To quantify intestinal enterococci, membranes were aseptically transferred to Slanetz and Bartley medium (CM0377, Oxoid) and incubated inverted at 44 ◦C (±0.2 ◦C) for 48 h. A spread plate method was also used where necessary to complement filtration techniques, e.g., at low serial dilutions to avoid interference of faecal particles with CFU growth on membrane filters. Intestinal enterococci isolates were aseptically transferred to Kanamycin Sulphate Supplement agar and incubated for 6 h at 37 ◦C to confirm that they were of faecal rather than environmental origin: all isolates were confirmed as faecal. Method blanks (i.e., sterile PBS) were used to confirm aseptic technique and the flame sterilisation procedure between samples. The limit of detection was 50 CFU per g fresh weight faeces. All sample analysis was performed in duplicate. The remaining faecal sample (~29 g) was used to determine the gravimetric water content by drying at 105 ◦C for 48 h (until constant mass) and weighing the residual to allow all FIO concentrations to be expressed as CFU g^{-1} dry weight of faeces.

2.4 Statistical Analysis

2.4.1 Faecal Samples

Plate counts of *E. coli* and intestinal enterococci were normalised by transforming to $\log_{10}$ CFU g^{-1} dry weight faeces. For both FIOs, non-linear regression analysis was

used to establish the relationship that best described the pattern of decline in each faecal source (dairy, deer) and temperature (−8 ◦C F–T, −4 ◦C F–T, 0 ◦C, 4 ◦C) treatment. An exponential model was fitted to each resulting time-series of FIO die-off associated with the five replicates of deer and dairy faecal samples exposed to two contrasting F–T temperature regimes. The exponential model fitted to the $\log_{10}$ transformed FIO data is described by Equation (1):

$$\mathrm{Log}_{10}(C) = A + Be^{-\lambda t}$$

where C is the cell concentration (CFU g^{-1}), λ is the exponential rate of decline (d^{-1}), governing the decay of the die-off rate constant over time, B is the difference in cell numbers between experiment start and finish ($\log_{10}$ CFU g^{-1}), A is the final level of bacterial population stability ($\log_{10}$ CFU g^{-1}) and t is time (d). The % decrease in FIO concentration per unit time is not constant and instead decays with time. A three-way analysis of variance (ANOVA) and a Tukey multiple comparison test were used to test for differences in λ, A and B associated with the fitted models as a function of FIO type, faecal source and F–T temperature cycle and to test for any interactions between these factors (Minitab 18.0 software, Minitab Inc.; State College, PA, USA). The same exponential model was fitted to the control treatments and one-way ANOVA and Mann–Whitney U tests were used to test for differences in λ, A and B associated with the models of die-off for FIOs in the control versus F–T treatments. Differences at the $p < 0.05$ level (95% confidence interval) were considered statistically significant.

2.4.2 Water Samples

Plate counts of *E. coli* and intestinal enterococci were normalised by transforming to $\log_{10}$ CFU mL^{-1}. No single model was suitable for all die-off data and so linear and non-linear regression were used as appropriate to model FIO decline for each faecal source and temperature treatment. The log-linear model fitted to the $\log_{10}$ transformed FIO data is described by Equation (2):

$$\mathrm{Log}_{10}(C) = \mathrm{Log}_{10}(C_0) - kt$$

where C_0 is the cell concentration at t = 0 and *k* is a die-off rate constant (d^{-1}). This model describes die-off based on first-order kinetics whereby the % decrease in FIO concentration per unit time is constant. *D*-values, which represent decimal reduction times, were calculated based on the average rate of decline for those populations

following a log-linear die-off profile. For those water treatments where a non-linear model was fitted to the data, a one-way ANOVA was used to test for differences in their die-off characteristics relative to the faecal treatments exposed to the same F–T temperature cycle.

2.5 Interpreting Parameter Values of Log—Linear and Exponential Models

The % decrease in FIO population per unit time associated with the exponential model fitted to the $\log_{10}$ transformed FIO data jointly reflects λ and the difference in cell numbers over the experiment (parameter B). In this model λ is the decay rate constant of the die-off rate constant, describing how quickly the die-off rate constant decreases in time. Thus, higher λ equates to a more rapid decay of the rate constant, i.e., the % decrease (in FIO concentration) per unit time is initially high but rapidly declines. In contrast, *k* represents a die-off rate constant in the log-linear model, and it alone directly sets the % decrease in concentration per unit time; i.e., higher *k* equates to a higher % decrease in population per unit time.

2.6 Results

2.6.1 FIO Die-Off in Faeces

No *E. coli* populations showed any growth immediately post-defecation (Figure 2.2). However, there was evidence of short-term cell growth in two of the intestinal enterococci treatments. In deer faeces held at 0 ◦C, there was a small increase of 0.15 $\log_{10}$ CFU g^{-1} dry weight faeces in the initial 24 h but this was followed by a drop of 1.34 $\log_{10}$ CFU g^{-1} dry weight faeces. In dairy faeces held at a constant 4 ◦C, an increase of 0.35 $\log_{10}$ CFU g^{-1} dry weight faeces was recorded and cell numbers were sustained longer than in the deer faeces (Figure 2.3 A, B)

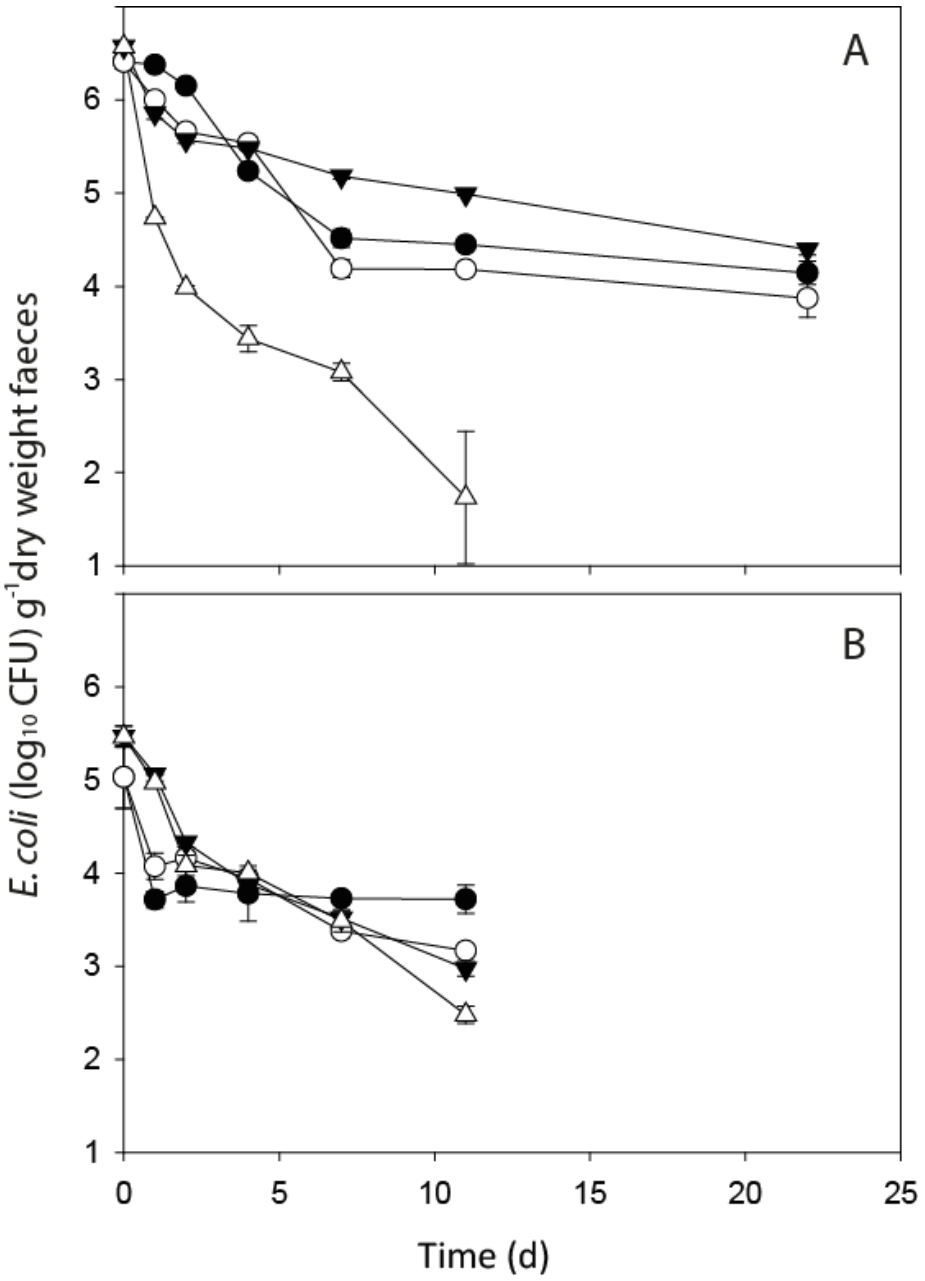

***Figure 2.2**: E. coli die-off profile in dairy faeces (A) and deer faeces (B) held at: constant 4◦C (solid black circle); −4 ◦C freeze-thaw (open circles); constant 0 ◦C (solid triangle); −8 ◦C freeze-thaw (open triangles). Data points are the mean of five replicate ± standard error (constant).*

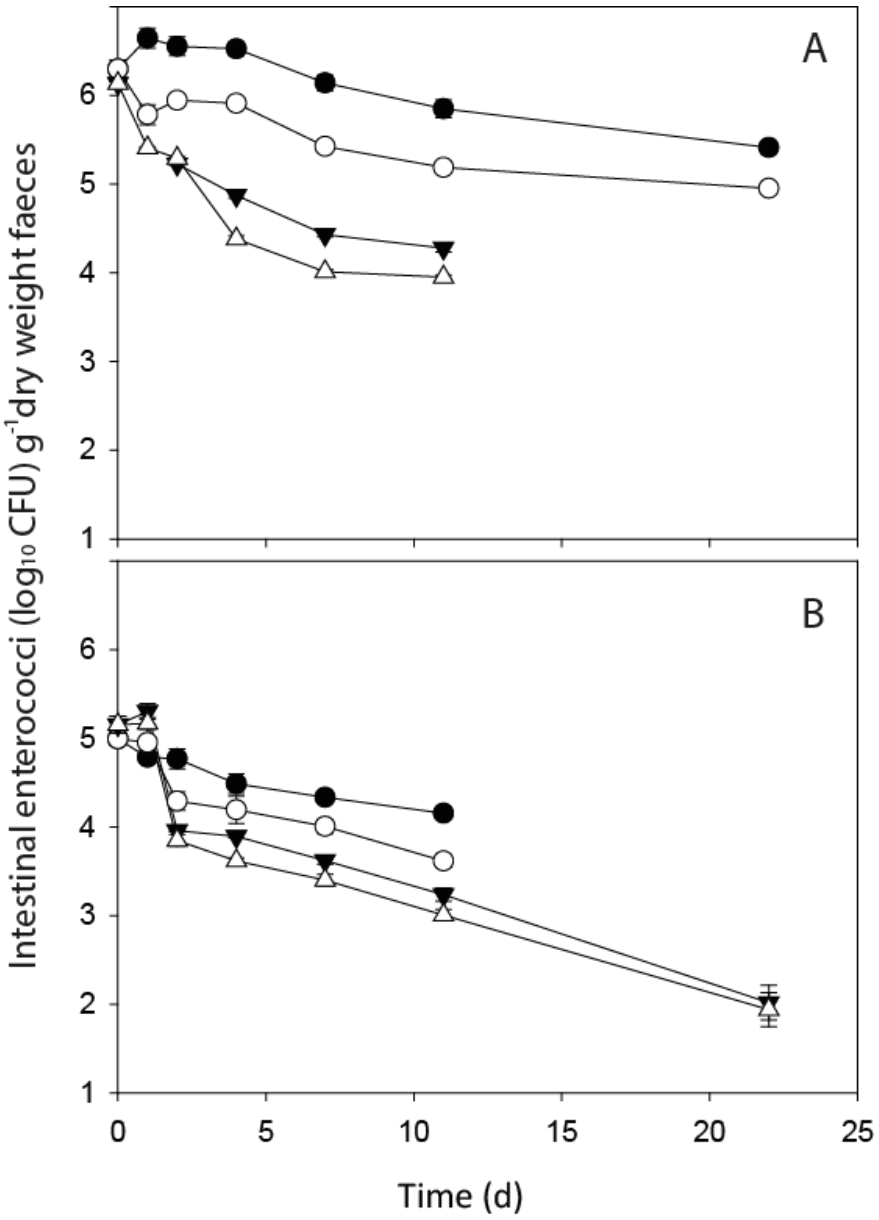

Figure 2.3: *Intestinal enterococci die-off profiles in dairy faeces (A) and deer faeces (B) held at: constant 4 ◦C (solid black circle); −4 ◦C freeze–thaw (open circles); constant 0 ◦C (solid triangle); −8 ◦C freeze-thaw (open triangles). Data points are the mean of five replicates ± standard error (freeze-thaw) and three replicates ± standard error (constant).*

Mean FIO concentrations in fresh faeces from the dairy cow and red deer sources are shown in Table 2.1. The persistence profiles of *E. coli* and intestinal enterococci in deer and dairy faeces under different temperature regimes were recorded over a minimum of 11 days and over the timeframe of sampling all FIO concentrations decreased, with changes most pronounced during the initial 24 h (Figures 2.2 and 2.3). Parameter results from the fitting of the non-linear

model to all faecal treatments are shown in Table 2.2 (*E. coli*) and Table 2.3 (intestinal enterococci). Overall, no significant difference was recorded between the values of λ, the exponential rate constant, for *E. coli* or intestinal enterococci in dairy and deer faeces held at the two different F–T cycles. However, there was a significant interactive effect of faecal source and temperature on FIO exponential rate constants ($p < 0.01$). While there was a clear visual difference in the pattern of *E. coli* decline for the dairy faeces exposed to the −8 ◦C F–T, the high variability in *E. coli* numbers at day 11 resulted in no statistically significant difference in λ values across treatments (Figure 2.2A). On day 11, two of the five dairy replicates exposed to the −8 ◦C F–T treatment dropped below detection limits and there is clear variability in *E. coli* concentrations in the replicates as time increases. There was no significant difference between λ values determined for both FIOs exposed to −4 ◦C and −8 ◦C F–T cycles in both faecal types relative to the equivalent FIOs held at constant temperatures of 4 ◦C and 0 ◦C, respectively.

Table 2.1. FIO concentrations in fresh faeces from the dairy cow and red deer sources used in this experiment.

FIO Concentration in Fresh Faeces (CFU g^{-1} Dry Weight)				
	Escherichia coli		**Intestinal Enterococci**	
FIO Source	Mean	SE	Mean	SE
Dairy cow	6.49	0.04	6.20	0.05
Red deer	5.30	0.15	5.08	0.06

FIO = faecal indicator organism; CFU = colony forming unit; SE = standard error.

Table 2.2. Parameter values for E. coli die-off associated with non-linear models.

Treatment	Exponential rate constant λ (Day^{-1})		Level of population stability A (log_{10} CFU g^{-1})		Magnitude of population decline B (log_{10} CFU g^{-1})	
	Mean	SE	Mean	SE	Mean	SE
Dairy, Freeze-thaw (4, 0, -4 °C)	0.166	0.028	3.757	0.164	2.692	0.172
Dairy, Freeze-thaw (0, -4, -8 °C)	0.410	0.093	2.415	0.266	3.957	0.344
Dairy, static (4 °C)	0.157	0.023	3.995	0.144	2.572	0.146
Dairy, static (0 °C)	0.166	0.034	4.504	0.144	1.877	0.149
Deer, Freeze-thaw (4, 0, -4 °C)	0.215	0.089	3.035	0.274	1.696	0.249
Deer, Freeze-thaw (0, -4, -8 °C)	0.122	0.042	1.658	0.701	3.661	0.653
Deer, static (4 °C)	n/a	n/a	n/a	n/a	n/a	n/a
Deer, static (0 °C)	0.075	0.019	0.688	0.615	4.630	0.580

n/a = inappropriate model fit

Table 2.3. Parameter values for intestinal enterococci die-off associated with non-linear models.

Treatment	Exponential rate constant λ (Day^{-1})		Level of population stability A (log_{10} CFU g^{-1})		Magnitude of population decline B (log_{10} CFU g^{-1})	
	Mean	**SE**	**Mean**	**SE**	**Mean**	**SE**
Dairy, Freeze-thaw (4, 0, -4 °C)	0.091	0.029	4.738	0.208	1.402	0.189
Dairy, Freeze-thaw (0, -4, -8 °C)	0.314	0.067	2.993	0.165	2.316	0.181
Dairy, static (4 °C)	n/a	n/a	n/a	n/a	n/a	n/a
Dairy, static (0 °C)	0.360	0.040	3.981	0.064	2.171	0.086
Deer, Freeze-thaw (4, 0, -4 °C)	0.206	0.093	3.525	0.280	1.512	0.256
Deer, Freeze-thaw (0, -4, -8 °C)	0.121	0.026	1.864	0.282	3.232	0.269
Deer, static (4 °C)	0.155	0.073	3.980	0.233	1.002	0.216
Deer, static (0 °C)	0.085	0.027	1.586	0.557	3.527	0.525

n/a = inappropriate model fit

Three-way ANOVA did, however, identify significant differences in the non-linear model parameters A and B between treatments. For parameter A (the final concentration of population stability) significant differences were identified between faecal source ($p < 0.01$) and F–T temperature cycle ($p < 0.001$). Dairy faeces supported higher modelled final FIO concentrations than deer faeces and lower modelled levels of population stability were recorded for cells exposed to the lower temperature (−8 °C) F–T cycle. There were no significant interactions between factors. Parameter B represents the magnitude of population decline, removing the potential influence of differences in the initial concentration. For parameter B, a significant difference was identified between FIO type ($p < 0.001$) and F–T temperature cycle ($p < 0.001$), but not between faecal source ($p > 0.05$). Cells exposed to the −8 °C F–T cycle recorded the higher magnitude decline in cell numbers and *E. coli* recorded a higher decline relative to intestinal enterococci, consistent with findings for parameter A. In addition, a significant interaction occurred between FIO type and faecal source

($p < 0.001$) and FIO type and F–T temperature cycle ($p < 0.001$), but not between faecal source and F–T temperature cycle ($p > 0.05$).

In dairy faeces, no significant difference was determined for A and B values of survival curves for *E. coli* monitored at 4 °C versus a −4 °C F–T cycle. However, there was a significant difference for both parameters when comparing the 0 °C and −8 °C F–T cycle, with the final concentration of modelled *E. coli* population stability significantly lower for the F–T treatment relative to the constant 0 °C treatment, and so a larger population decline was also recorded for the F–T treatment ($p < 0.05$). The final concentration of population stability was significantly lower for intestinal enterococci exposed to the −8 °C F–T treatment relative to the 0 °C treatment. Differences in parameters A and B were not evident in the modelled survival curves for *E. coli* in deer faeces.

2.6.2. FIO Die-Off in Water

The change in FIO concentration (normalised to 100% of the inoculum concentration), for all water treatments is shown in Figure 2.4. The persistence of FIOs in water across all F–T treatments did not follow a consistent die-off pattern and so both non-linear and log-linear models of population decline were fitted to the data.

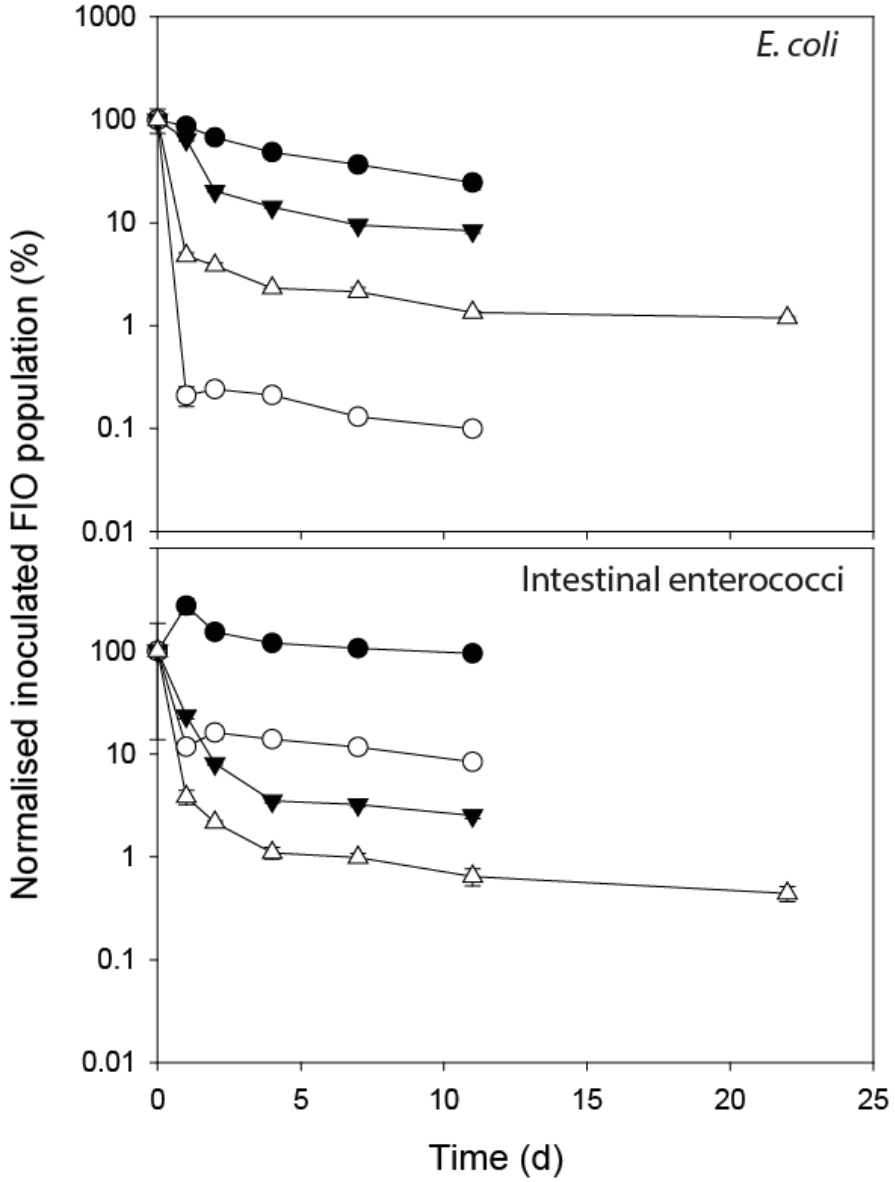

Figure 2.4 *Normalised die-off profiles of faecal indicator organisms (FIOs) inoculated into water undergoing freeze–thaw (F–T) cycles: isolated from dairy cow faeces held at −4 ◦C F–T (solid black circles) and −8 ◦C F–T (solid black triangle); isolated from red faeces held at −4 ˚C F–T (open circles) and −8 ˚C F–T (open triangles). Data points show mean of 3 replicates ± standard error.*

2.6.3. E. coli

Linear regression models were applied to all deer replicates (r^2 ranged from 0.519 to 0.957) to determine modelled linear decline rate constants and decimal reduction times (*D*-values, Table 2.4). Both F–T treatments for deer faeces clearly displayed a two phase die-off, with an immediate rapid decline shifting to a slower decline phase after 24 h; however, the non-linear model was a poor fit and so a two-phase log-linear

model was applied. The data for both dairy treatments mapped well to the previously described non-linear model: dairy −4 ◦C F–T (λ = 0.094 day^{-1}, A = 6.399 log$_{10}$ CFU mL^{-1}, B = 0.954 log$_{10}$ CFU mL^{-1}); dairy -8 ◦C F–T (λ = 0.390 day^{-1}, A = 5.694 log$_{10}$ CFU mL^{-1}, B = 1.143 log$_{10}$ CFU mL^{-1}).

Table 2.4*. Linear decline parameters and decimal reduction times for E. coli isolated from deer faeces inoculated into water undergoing freeze-thaw cycling.*

Treatment	Modelled Linear Decline Rate (Day^{-1}) [a]	*D*-Values (Days)	R2
Deer, Freeze-thaw (4, 0, −4 ◦C) [b]	6.209 & 0.086	26.8 [c]	0.714 [c]
Deer, Freeze-thaw (0, −4, −8 ◦C) [b]	2.957 & 0.06	37.2 [c]	0.749 [c]

[a] = Linear decline rate constant = (2.303 × Figure 3 slope gradient) [b] = treatment split into a 2-stage linear decline (rapid and slow) [c] = values for “stage 2” slow decline

There was no significant difference in λ for *E. coli* in water versus faecal treatments exposed to the same F–T temperature regime. The modelled drop in population numbers was not significantly different for water and dairy faecal treatments exposed to the −4 ◦C F–T cycle but for the −8 ◦C F–T cycle the *E. coli* in the dairy faeces experienced the larger population decline (p = 0.01). It was not possible to directly compare the deer treatments due to the different model profiles, but the pattern of decline in water versus faecal treatments was similar.

2.6.4 Intestinal Enterococci

A non-linear model (λ = 0.89 day^{-1}, A = 7.390 log$_{10}$ CFU mL^{-1}, B = 2.122 log$_{10}$ CFU mL^{-1}) mapped best to the intestinal enterococci sourced from deer faeces exposed to the -8 ◦C F–T cycle and likewise the dairy faeces exposed to the -8 ◦C F–T cycle (λ = 0.57 day^{-1}, A = 4.460 log$_{10}$ CFU mL^{-1}, B = 1.593 log$_{10}$ CFU mL^{-1}). A linear model was more fitting to the cells sourced from deer faeces exposed to the −4 ◦C F–T cycle (*D* = 25.5 days) while the cells isolated from the dairy faeces and held in water at the −4

°C F–T cycle showed a small increase in concentration, affecting the model fit. The intestinal enterococci sourced from deer and exposed to the −8 °C F–T showed no significant difference in non-linear die-off characteristics between faecal and water treatments, but those sourced from dairy cows and exposed to the −8 °C F–T were found to exhibit a significantly higher λ in water relative to faeces ($p < 0.05$) but maintained an overall modelled population level that was higher than cells in the faecal treatment ($p < 0.01$).

2.7 Temperature Fluctuations Within the Faeces

The faecal matrix (and therefore associated cells) within the deer faeces appear to be marginally less well insulated from the F–T cycle relative to the dairy faeces, with minimum internal temperatures more frequently approaching that of the external air temperature than observed for dairy faeces (Figure 2.5). Similar diurnal patterns and trends were observed for the −8 °C F–T cycle.

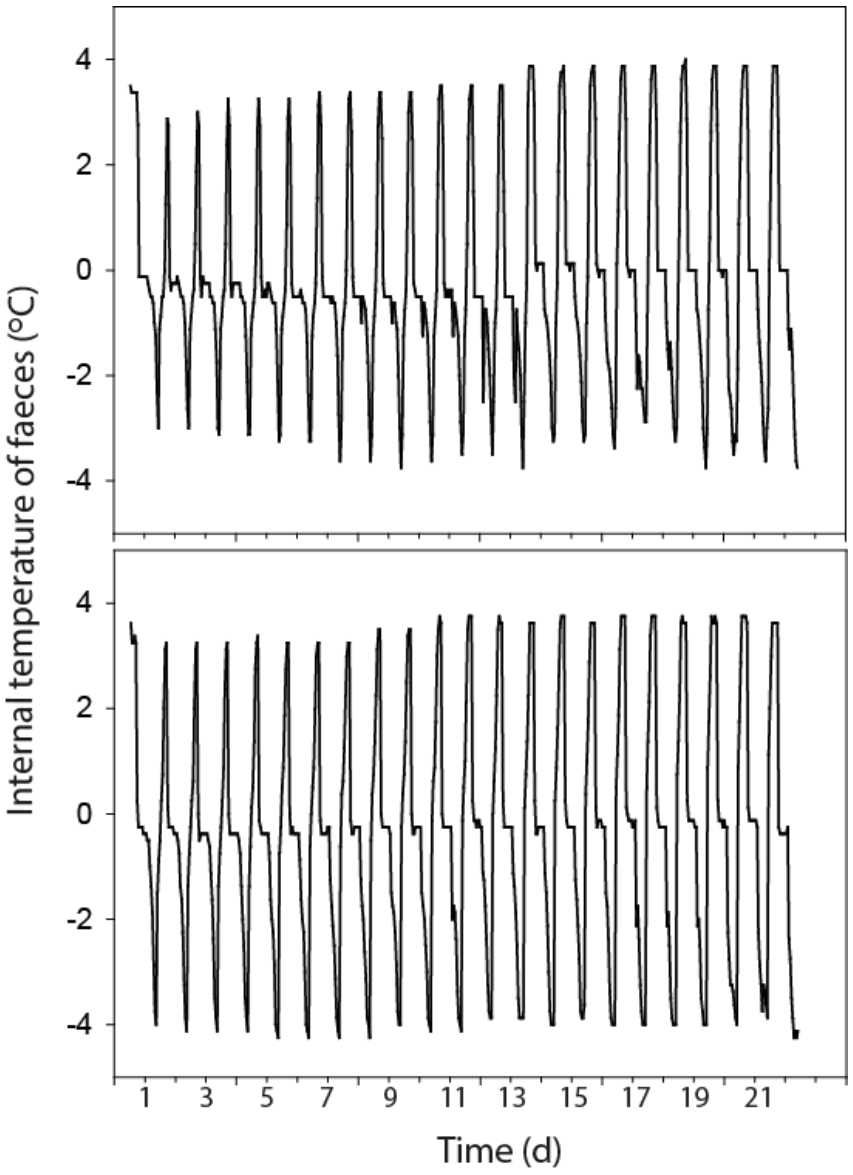

Figure 2.5: *Diurnal patterns of internal temperature of the dairy (top) and deer (bottom) faecal matrix exposed to the −4 ◦C freeze–thaw cycle.*

2.8 Changes in Moisture Content

In dairy faeces, the rate of moisture content loss was more rapid under constant temperature conditions than under F–T cycles (Figure 2.6A). After 22 days, dairy faeces reduced to 22.7% and 28.0% of the original faecal weight (4 ◦C and 0 ◦C, respectively), compared to 43.4% and 53.8% (−4 ◦C F–T and −8 ◦C F–T cycles, respectively). Fresh weight reduction over time in deer faeces was initially more rapid at 4 ◦C relative to the −4 ◦C F–T, but after 11 days both treatments converged to similar fresh weight values (Figure 2.6B). Changes in moisture content followed similar rates

of change in both the −8 ◦C F–T cycle and the constant 0 ◦C treatments for deer faeces, but after 22 days the 0 ◦C treatment had dropped to 28.5% of the original weight compared with 38.2% for the −8 ◦C F–T treatment.

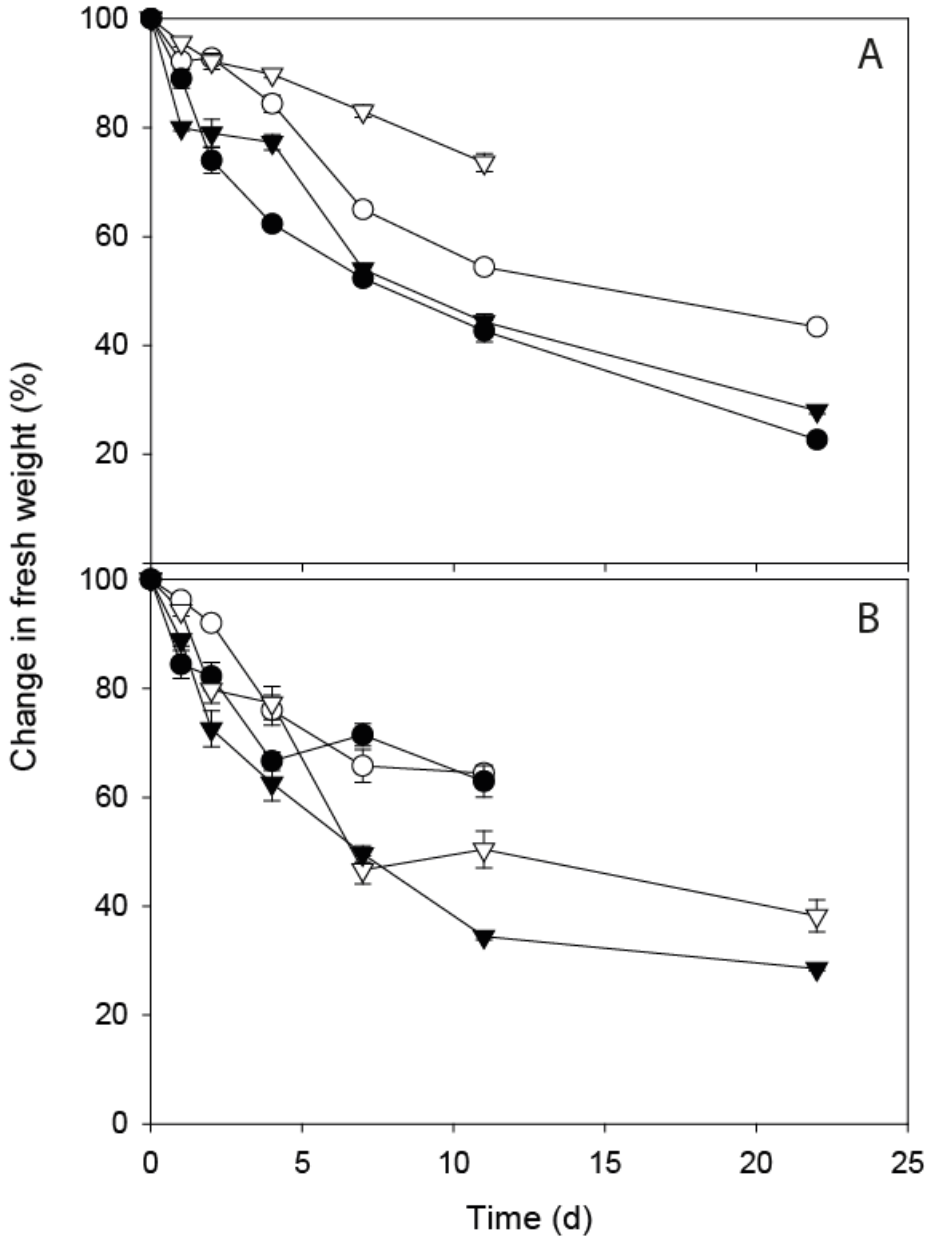

Figure 2.6: *Changes in fresh weight (%) over time dairy faeces (A) and deer faeces (B) held at: constant 4 ◦C (solid black circle); −4 ◦C freeze–thaw (open circles); constant 0 ◦C (solid triangle); −8 ◦C freeze–thaw (open triangles). Data points are the mean of five replicates ± standard error.*

2.9 Discussion

Sub-freezing conditions are often considered hostile to gut-derived bacteria and detrimental to FIO persistence, but quantitative evidence to support our understanding of FIO persistence in the environment in response to different cold temperature regimes remains limited. This study provides novel FIO die-off data in dairy cow and red deer faeces exposed to low temperatures, including freezing conditions and through repeated F–T cycles. A key result is that both *E. coli* and intestinal enterococci are capable of surviving these harsh temperature conditions with the faeces from both animals able to sustain relatively high FIO concentrations, as indicated by modelling, and observations revealing persistence in excess of 11 days and in some cases confirmed beyond 22 days. Faecal contamination of surface waters can pose a public health risk to downstream users, e.g., via recreational exposure (Russo et al., 2020). To predict the risk associated with different receiving waters we first need to understand how microbial pollutants, e.g., FIOs, survive in the environment and improve our knowledge of FIO contributions from sources other than just humans and livestock, i.e., quantify FIO risk from wildlife sources. The findings from this series of experiments therefore contribute to an improved understanding of how catchment FIO burden can vary over time, which is fundamentally important for accurate assessment of the risk of microbial contamination of watercourses at the catchment scale (Neill et al., 2020a). In particular, these data help constrain the parameterisation of die-off coefficients used in the modelling of fate and transfer of FIOs in landscapes where F–T cycling is common (e.g., Neill et al., 2019). The inclusion of laboratory-derived process representation into catchment-scale models needs careful assessment but, given that temperature is such a well-recognised driver of FIO survival, any additional understanding regarding more nuanced temperature-driven FIO responses in both agricultural and wildlife sources is likely to be important for advancing both our predictive capability and appreciation of uncertainty in catchment-scale modelling (Cho et al., 2016).

The two F–T temperature cycles used in this experiment did not result in different exponential rate constants (λ), which govern the decay of the die-off rate constant over time. FIOs in both treatments would have experienced mechanical disruption to cells, e.g., dehydration and shrinking, as a result of extracellular ice crystal formation typical

of the temperature regimes used in this study (Gao et al., 2009). The growth characteristics of ice crystals are influenced by the rate of cooling (Marcellini et al, 2016), and this rate of temperature change in turn influences cell die-off (Gao et al., 2006). In our study, different minimum and maximum temperatures were associated with the two F–T cycles, but the range in temperature was the same for both treatments (8 °C). Cooling rates would therefore have been similar in both treatments, potentially explaining the lack of difference in λ associated with the models of FIO die-off. However, further research on how the magnitude of the F–T fluctuation impacts on FIO persistence in faeces in relation to differential cooling rates is warranted, and sample size in terms of faecal load or water volume is likely to play a role in the rate of cooling and hence population die-off characteristics (e.g., Neill et al., 2019). Although exponential rate constants did not vary between F–T treatments other survival curve characteristics such as the final level of FIO concentration and the magnitude of drop in FIO population were significantly different. This is important because the % decrease in FIO population per unit time observed in the exponential model jointly reflects λ and B. Dairy faeces sustained FIOs at higher concentrations over the course of repeated F–T cycles relative to deer faeces, highlighting a need for distinct parameter combinations in models of FIO die-off in dairy versus deer faecal sources in the environment. Biphasic decay of FIO populations is increasingly recognised as a consequence of rapid initial die-off of labile cells followed by slower die-off of resistant cells (Brouwer et al., 2017); it may be that the strains of *E. coli* and intestinal enterococci derived from dairy faecal sources were more resistant to temperature stress than strains sourced from deer, with heterogeneity in FIO strain behaviour commonly reported (Guber at al., 2015). However, the internal temperature profiles obtained from the faeces provided evidence that the deer faecal pellets were less insulated to the outside air temperature relative to dairy faeces and so a greater proportion of FIOs in deer pellets are likely to have either experienced temperature-induced cell structural damage or entered a viable-but-non-culturable (VBNC) state, both reducing culturable counts (Wang et al., 2019; Orruño et al, 2017). The significant interaction between faecal source and temperature further reinforces the assumption that the structure of a faecal pellet versus a faecal pat is influential on FIO persistence. Moisture loss from faeces via sublimation would become more common with increasing time exposed to sub-zero temperatures and, in general, water content was more efficiently lost from the deer faecal pellets, with this rapid loss of moisture

potentially exacerbating FIO die-off further (Porter et al., 2019). Of these two FIOs, *E. coli* was more susceptible to die-off when exposed to the F–T cycle with the lower temperature, consistent with enterococci being recognised as a more robust indicator when exposed to freezing stress (Neill et al., 2019). Across all treatments, there was very little FIO growth, with temperatures <4 ◦C known to retard metabolic processes in *E. coli* (Guber et al., 2015). A proportion of both FIO cells may have entered into a VBNC state and it may be that some of the differences between the concentrations of E. coli and enterococci were attributable to VBNC cells (Rocard et al, 2018; Wei & Zhao, 2018). While there is uncertainty over whether cells are therefore truly dead or just metabolically inactive, the 'die-off' parameters derived from studies such as ours remain crucially important given that models used to inform on landscape FIO fate and transfer, and guide environmental decision-making, are largely built on data derived from culturable counts in order to align with culture-based standards used by environmental regulators (Oliver et al., 2010).

Our results suggest that die-off responses to just-above-freezing, sub-freezing and F–T temperatures within the range of +4 ◦C to −8 ◦C lead to a clear decline in FIO populations. Given that the % decrease in FIO population per unit time jointly reflects λ and B, the differences we determined in parameter B across treatments are crucially important for describing different FIO die-off responses despite values of λ not varying substantially. For example, the magnitude of population decline experienced by *E. coli* in dairy faeces exposed to the −8 ◦C F–T cycle was more pronounced than when exposed to a constant 0 ◦C. This difference was only evident for the F–T regime at the lower temperature and likely reflects the greater damage inflicted to cell membranes and walls given that repeated F–T cycles would encourage recrystallisation and therefore promote the growth of larger ice crystals relative to those developing at a constant 0 ◦C (Moon et al., 2020). The difference observed between control and F–T treatments was not apparent in the data for deer faeces, suggesting that the dairy faeces offered greater protection to FIOs at a constant of 0 ◦C, whereas the detrimental impacts of ice crystal formation appeared to have occurred to a similar extent under both control and F–T treatments for deer faecal pellets. However, at larger scales, many processes will be influenced by a wider set of interacting environmental variables unaccounted for under controlled conditions; those interactions may

accelerate or dampen FIO die-off responses to F–T at the range of temperatures investigated and recognising this remains an ever-present challenge of parameterising landscape models with laboratory-derived data (Oliver et al., 2016). Future investigation of how the specific number of F–T cycles or how varying length of F–T cycles influences survival responses would provide further insight into FIO behaviour under F–T conditions, as would studies of field-relevant die-off under F–T cycles.

Overall, patterns of cell decline were inconsistent in the water mesocosms and required the fitting of different model forms; however, FIO die-off in water relative to faeces exposed to F–T cycles provides useful information for interpreting survival responses, as does the implication of fitting different model forms. The water used in our study was distilled and therefore of a purified form rather than sourced from the environment. When the water mesocosms were exposed to the −4 °C F–T cycle, the water did not freeze, but pure water forms are able to be undercooled by several degrees before ice crystal nucleation initiates (Akyurt et al., 2002). Had an environmental source of water been used, accommodating impurities, particulates and an indigenous microbial community, the nucleation phenomenon may have occurred more quickly due to a more plentiful availability of nucleating centres and potentially impacted on FIO persistence to a greater degree. At −8 °C F–T, the water did freeze but the decline in *E. coli* concentration was more marked in the dairy faeces. This may be due to the fact that the water mesocosms remained frozen and did not thaw under the F–T cycle, whereas the faecal matrix, assumed to offer protection, likely experienced recrystalisation during the F–T cycling, resulting in a more detrimental effect on *E. coli* physiology. This would be consistent with suggestions that cell viability under freezing conditions is influenced more by repeated freeze–thawing than the duration of exposure to freezing conditions (Sleight et al., 2006). Survival curves of intestinal enterococci sourced from dairy cows and exposed to the lower F–T regime accommodated higher λ values in water relative to faeces but, as with *E. coli*, their final concentration in water was maintained at a higher level than the population held within the faeces. One explanation is that the enterococci, similar to *E. coli*, coped with being encapsulated in ice better than they did in a partially frozen faecal matrix, with population numbers supported by the resistant enterococci subpopulation following the rapid demise of the more labile subpopulation (Gao et al., 2009; Brouwer et al., 2017). However, the fitting of a log-linear model to relevant data associated with deer-

derived FIOs in water highlights an important point. This model indicates that over time FIO concentrations will decay towards zero, whereas the non-linear model used to describe die-off in deer faeces stabilizes above zero, at a value represented by parameter A. If those fitted models held beyond the timespan covered by the experiments, the deer faeces would actually provide protection to the FIOs such that a residual population can be maintained. There is a degree of uncertainty in extrapolating beyond the timespan of the experiment, but the models at least indicate this to be a possibility.

While the deer faeces used in our research was not from truly 'wild' deer, or deer farmed over extensive landscape systems, their diet was unlikely to have differed considerably from wild deer. However, one difference might be attributed to a supplementary feed given to the deer through the winter. This feed (palm kernel, wheat feed, beans, barley oat feed cane molasses, calcium carbonate and sodium chloride) amounts to ~0.6 Kg per animal per day; however, wild deer may also supplement their diet by scavenging livestock feeds distributed in farmlands, for example, from feeding troughs (Walter et al., 2012). A major gap in our understanding is the behaviour of FIOs sourced from wildlife faeces. Therefore, die-off responses of deer-derived FIOs in both faeces and water exposed to low temperatures provide much needed information to enable better accounting of the varied catchment sources of faecal pollution, and opportunities for model refinement through the inclusion of wildlife sources have been acknowledged (e.g. Neill et al., 2020b). Typical values for initial concentrations of FIOs in fresh deer faeces are also valuable for modelling, with source attribution reliant on understanding the relative FIO contributions of wildlife and wildfowl to the landscape (Muirhead et al., 2011).

2.10 Conclusions

Understanding *E. coli* and intestinal enterococci die-off in environmental matrices has important implications for managing microbial pollution of wider landscapes. Survival characteristics of FIOs in response to environmental variables such as temperature, UV radiation and moisture availability are used to inform many microbial fate and transfer models and provide evidence to underpin on-farm management practices designed to reduce the risk of microbial transfer from land to aquatic receptors and

thus wider contamination of water resources. However, FIO die-off in response to sub-freezing temperatures and F–T cycles in common faecal sources of rural landscapes is not well reported and although investigations of microbial persistence during frozen storage via food microbiology research provide a basis for understanding, it lacks an environmental context. Our results highlight that exposure of fresh faeces to freezing conditions does not completely eradicate the FIO population present and that, in some cases, substantial concentrations of FIOs remain culturable despite lengthy and severe sub-freezing temperatures. Those hardy cells that survive the F–T and sub-freezing temperatures clearly represent a resistant subpopulation, suggesting that if subsequently mobilised from a faecal source and transferred to the wider landscape, their proven ability to persist in the environment may lead to longer-term faecal contamination and greater challenges for environmental regulators. So, while landscape burden of FIOs, whether on-farm or in rural landscapes where deer are common, is likely to be reduced by sub-freezing temperatures it is not an environmental scenario that delivers risk-free and effective removal of FIO source at the landscape-scale. Further, if warmer weather follows soon after F–T cycling, there is potential for a resuscitation of a VBNC population too. Future research should determine how F–T and freezing temperatures affect FIO mobilisation from different faecal sources, including that of wildlife, and whether prior exposure to F–T stress can enhance future resistance and survivability of FIOs as they transfer into different environments.

3. Time since faecal deposition influences mobilisation of *E. coli* and intestinal enterococci from deer, goose and dairy cow faeces

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Abstract

Mobilisation is a term used to describe the supply of a pollutant from its environmental source, e.g., soil or faeces, into a hydrological transfer pathway. The overarching aim of this chapter was to determine, using a laboratory-based approach, whether FIOs are hydrologically mobilised in different quantities from a typical agricultural, wildlife and wildfowl source, namely dairy cattle, red deer and greylag goose faeces. The mobilisation of FIOs from fresh and ageing faeces under two contrasting temperatures was determined, with significant differences in the concentrations of both *E. coli* and intestinal enterococci lost from all faecal sources. FIO mobilisation from these faecal matrices followed the order of dairy cow > goose > deer (greatest to least, expressed as a proportion of the total FIOs present). Significant changes in mobilisation rates from faecal sources over time were also recorded and this was influenced by the temperature at which the faecal material had aged over the course of the 12-day study. Characterising how indicators of waterborne pathogens are mobilised in the environment is of fundamental importance to inform models and risk assessments and develop effective strategies for reducing microbial pollution in catchment drainage waters and associated downstream impacts. Findings from this chapter add quantitative evidence to support the understanding of FIO mobilisation potential from three important faecal sources in the environment.

3.1 Introduction

Faecal pollution of surface waters, commonly measured by the presence of faecal indicator organisms (FIOs), can be linked to a variety of catchment sources. These include wastewater discharge points and combined sewer overflows, runoff from agricultural land and contributions from wildlife and wildfowl (Afolabi et al., 2020; Alegbeleye & Sant'Aba, 2020; Pascual-Benito et al., 2020). FIOs delivered to receiving waters via point sources such as effluent pipes are mobilised and transferred through a combination of managed water flows and engineered infrastructure. In contrast, FIOs contributed from agricultural and wildlife/wildfowl sources are mobilised and transferred largely as a function of rainfall-runoff responses in the environment (García-Aljaro et al., 2017). The latter represent a supply of FIOs that are distributed across the landscape as diffuse sources associated with direct faecal deposits or applications of manures and slurries to land.

The level of faecal pollution contributed from diffuse sources is related to a variety of factors, e.g., the burden of FIOs in the environment (source factors; Oliver et al 2018), landscape and environmental characteristics that influence the generation of runoff (transfer factors; Gray et al, 2021) and the extent of hydrological connectivity linking hillslope to stream which provides a pathway for FIOs being transferred in runoff (delivery factors; Neill et al., 2019). The crucial step that supplies a pollutant from its source into the hydrological transfer pathway is termed mobilisation (Haygarth et al., 2005). The mobilisation of FIOs from source material distributed across a landscape is largely driven by detachment processes. For example, the physical disruption of faeces by raindrop impact can dislodge faecal particles and FIOs, whilst the resulting overland flow following rainfall also has the potential to slough FIOs from faecal sources (Blaustein et al., 2015a; Kim et al., 2016; Wang et al., 2018). Mobilisation reflects a combination of FIO survival in the faeces and the erodibility of the faecal source and subsequent detachment rate of the FIO population, which both vary as a function of faecal ageing and desiccation (Kim et al., 2016).

The occurrence of a rainfall event therefore provides an energy source to physically disrupt the faecal deposit and initiate mobilisation, and rainfall characteristics such as intensity and volume have been investigated as determinants of microbial water quality

(Blaustein et al., 2016). The importance of factors such as angle of sloping land and how this varies at different scales, e.g., plot to hillslope, in influencing rainfall-induced release of FIOs and their subsequent loss from land to water have also been explored (Blaustein et al., 2015b; Kim et al., 2016). However, those studies have focused on quantifying FIOs after their transfer across a soil surface and subsequent delivery to a receptacle or receiving water but, have not specifically quantified all FIOs mobilised from the faecal matrix. There can be significant increases in post-rainfall FIO numbers in soil, which highlights that although FIOs can be released from faeces during rainfall, not all of these FIOs are necessarily transferred to a receiving water in a single rainfall event (e.g., Stocker et al., 2018). In one study where mobilisation was quantified directly, a laboratory assay was used to determine relative differences in FIO release rates from sheep and beef cow faeces, in addition to dairy slurry and beef cow manure (Hodgson et al., 2009). The methodology of Hodgson et al. (2009) modified a protocol originally used to measure phosphorus mobilisation from soil under highly controlled conditions and mimicked the impact of a rainfall event, providing important data on differences in FIO mobilisation attributed to faecal substrate and FIO type. Data concerning the mobilisation potential of FIOs from a range of faecal types, and not just livestock sources, can be used to better parameterise process-based models of FIO fate and transfer in environmental systems (e.g., Jeong et al., 2019) or to support and inform more simple risk-based approaches to mapping FIO pollution (e.g., Oliver et al., 2010).

Although detachment processes have been included in some existing FIO model structures, a current lack of information prevents good quality detachment representation across the spectrum of different faecal sources, especially non-agricultural sources, that often exist within a catchment (Alegbeleye et al., 2020; Neill et al., 2020). For example, different livestock, wildlife and wildfowl excrete faecal material of varying physio-chemical characteristics, which in turn are likely to influence FIO mobilisation. Differences in faecal characteristics might include typical initial FIO concentrations, dry matter content and physical structure, all of which will change over time as a result of faecal ageing and, in the absence of rainfall, desiccation. Such changes will vary as a function of temperature and the combined effects of temperature and desiccation will impact on FIO survival (Oliver & Page, 2016). Thus,

temperature is likely to play a key role in influencing changes in FIO mobilisation potential of different faecal matrices over time.

FIO mobilisation from faecal sources is an important process but is often unaccounted for in tools and models designed to assess FIO risk in the environment (Fish et al., 2009). There is also a lack of quantitative understanding of how FIO mobilisation from wildlife or wildfowl faeces may differ relative to common agricultural sources of faeces, which can lead landowners to query the FIO contribution from different sources (Muirhead et al., 2011). The overarching aim of this study, therefore, was to determine whether there are differences in FIO mobilisation dynamics from a typical agricultural, wildlife and wildfowl source, namely Holstein-Friesian dairy cattle (*Bos taurus*), red deer (*Cervus elaphus*) and greylag goose (*Anser anser*) faeces. Specifically, our objectives were to: (i) quantify culturable FIO mobilisation from faecal sources following deposition; (ii) evaluate how two contrasting temperature conditions influence the temporal dynamics of culturable FIO mobilisation from faeces; and (iii) determine whether *E. coli* and intestinal enterococci (IE) exhibit differential mobilisation potential.

3.2 Materials and Methods

3.2.1 Provenance of faeces used in all experiments

Fresh dairy faeces were collected from the livestock housing of a conventional dairy farm in Stirlingshire, Scotland. Cows were permanently housed and a mechanical barn floor scraper was in operation meaning that any faeces collected was guaranteed to have been deposited within the previous 30 minutes (see Fig 3.1). In total, ~12 dairy cow faecal deposits were collected and pooled. Fresh faeces of red deer were collected from the Scottish Deer Centre, Fife, Scotland. Red deer were selected as a representative wildlife species because they are widely distributed across much of Scotland and occupy a range of habitat that span moorlands through to woodlands (SNH, 2017). Fields containing deer were harrowed prior to faecal collection, which ensured that all faeces collected were fresh (<12 h old). The diet of the deer used in this study was unlikely to have differed considerably from wild deer (Afolabi et at., 2020). In total, ~30 faecal deposits from red deer were collected and pooled. Fresh faeces from greylag geese were collected from the Royal Society for Protection of

Birds (RSPB) reservation located on the shores of Loch Leven, Fife Scotland. Greylag geese were selected as a representative wildfowl species because they are present year-round and are currently breeding successfully in several regions of Scotland (Mitchell, 2012). The diet of the geese reflects that of a wild population. In total, ~50 faecal deposits from greylag geese were collected and pooled. After collection, all faeces were transferred immediately (< 1 h) to the laboratory for use in the experiment and thus no interim storage was required. No permits were required for collection of faecal samples because sampling was done with permission and assistance from the relevant landowners described above. Ethical approval for the project was granted by the University of Stirling General University Ethics Panel.

3.2.2 Sampling of faecal material

A controlled laboratory experiment was carried out to mimic how rainfall mobilises FIOs from faecal matter into the watercourses. Incubators (Sanyo Incubator MIR-153, Japan) were used to allow for two constant temperature treatments (0 °C and 15 °C) that represented two environmental scenarios under which faeces can be deposited: one a freezing scenario typical of winter conditions and the other typical of average summer temperature conditions in the UK (Kendon et al., 2019). The experiments were conducted over a duration of 12 days, allowing faecal material to age and dry. Figure 3.1 shows the experimental set-up in a tray at the dairy farm before the laboratory procedure. Each treatment consisted of five replicates of faecal pat/pellet deposits that were destructively harvested per sampling day. Every two days all faecal deposits were misted with sterile distilled water at a rate of 1 mL/100 cm^2 of faecal surface area, as measured by the area of trays on which the samples were situated. This was done to mimic a ‘morning dew’ effect and avoid complete dehydration of the faeces under incubator conditions. The use of full-size dairy faecal deposits was impractical for a replicated laboratory experiment; therefore, faecal samples were bulked and homogenised in a sterile plastic container and then distributed into shallow circular 70 mm diameter foil trays as either 50 g fresh weight dairy faecal deposits (84% moisture content). Likewise, deer faecal samples from several deer were pooled to form 50 g fresh weight piles of deer faecal pellets (78% moisture content) and goose faeces from several birds were pooled to form 10 g fresh weight goose faecal deposits (79% moisture content). The difference in mass used for geese faeces was due to the

small amount of geese faeces excreted per day in the field relative to dairy cows and deer. Furthermore, using 50 g of goose faeces per experimental replicate was impractical because: (i) it was more important to guarantee freshness of faecal sample than volume of faecal sample, the latter being constrained by the size of the flock; and (ii) goose faeces are naturally much smaller, and such a volume was therefore not representative of typical goose faecal depositions. While the red deer and dairy cow samples used in the experiment were smaller than typical depositions in the field, such deposition piles are plausible if defecations occur while the animal is moving. In contrast, artificially increasing the size of a goose faecal deposit was considered unrealistic. Experimental replicates were randomly divided into each treatment and sampled on days 0, 3, 7 and 12 for use in the mobilisation experiment. This allowed for investigation of FIB release from freshly deposited faeces but also ageing faecal material held at either 0 °C or 15 °C. On sampling days, a total of 3 g was randomly sampled from each replicate of dairy and deer faeces using a sterile spatula. For goose faeces, the reduced starting faecal mass necessitated a smaller subsample of 1 g for use in the mobilisation experiment.

Figure 3.1 *Dairy faeces sample prepared in aluminium plates at dairy farm ready to be transported to the laboratory to be subjected to two different temperature regimes (0 °C and 15 °C) for mobilisation experiment.*

3.2.3 Artificial sterile rainwater preparation

A standardised rainwater was prepared following the method described by Hodgson et al (2009). The resulting rainfall (pH 5.64) had the following composition (g L^{-1}): CaCl, 2.465; MgCl, 1.919; FeCl, 0.0445; NH_4NO_3, 0.430; K_2SO_4,0.617; NaCl, 3.317. The artificial rainwater was sterilised using an autoclave (15 min at 121 °C).

3.2.4 Simulating rainfall-initiated mobilisation

The DESPRAL test is a laboratory-based protocol originally developed to quantify phosphorus mobilisation from soil, with test results correlating well (r^2 = 0.7-0.8) with amounts of suspended sediment and total phosphorus generated in overland flow using rainfall simulators (intensity 60 mm h^{-1} for 30 min) (Withers et al., 2017). The DESPRAL approach was modified by Hodgson et al. (2009) to evaluate FIO mobilisation from agricultural faecal matrices, and we have used this modified approach to quantify FIO mobilisation from an extended range of faecal sources.

Figure 3.2 shows the adapted experimental set-up to initiate mobilisation of FIOs from faecal sources. Briefly, the 3 g deer and dairy faecal subsamples were transferred to a 50 mL sterile centrifuge tube in replicate (*n* = 5) to which 27 mL of sterile standardised rainwater was pipetted slowly down the side of the tube to avoid disturbing the faeces. For goose faeces, 1 g was taken from each of the 5 replicate faecal samples for each time point and added to a 15 mL centrifuge tube (*n* = 5) to which 9 mL of sterile rainwater was added as described above. Different sized tubes were used to maintain as close a ratio as possible of liquid to air given the different mass of faeces; importantly, the faeces: rainwater ratios were consistent across all treatments (representing 1:10 dilutions). The tubes were mounted on a tabletop rotator, and rotated vertically (i.e., perpendicular to the benchtop) through 360^{o} for one minute at a speed of 35 revolutions per minute (rpm) – see figure 3.2 for experimental apparatus. This simulated a standardised interaction between faeces and rainfall, mimicking raindrop impact and subsequent faecal disruption. This approach provides an assay of FIO mobilisation potential under controlled laboratory conditions.

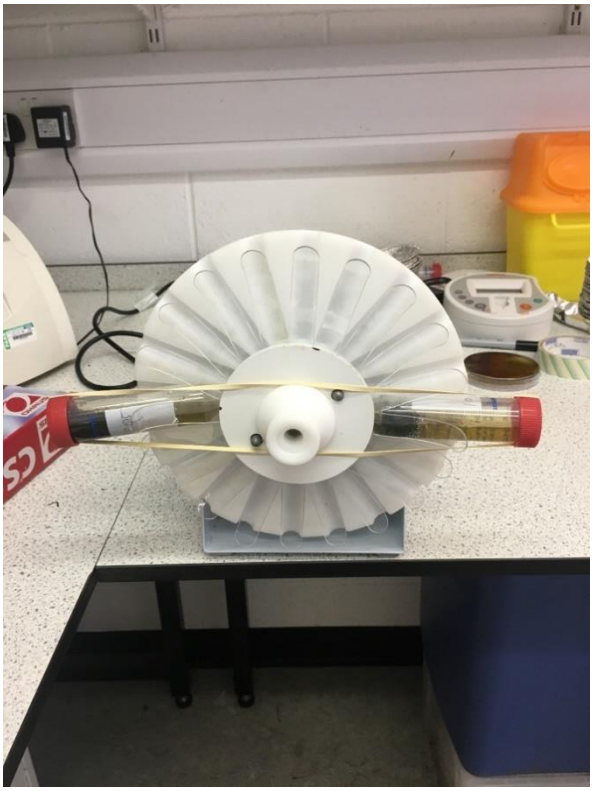

Figure 3.2*: Tubes containing mixture of faecal samples and artificial rainwater undergoing rotation on a tabletop rotator.*

3.3 FIO Enumeration

To determine culturable counts of FIOs, at each time point, 1 mL of eluent (i.e., 'wash-off') was transferred to 9 mL of sterile PBS and a series of serial 10-fold dilutions were made using PBS. Briefly, 1 mL of each serially diluted sample was pipetted on to a 0.45 µm cellulose acetate membrane and washed through a vacuum-filtration unit (Sartorius Stedim Biotech., Goettingen, Germany) with ~20 mL of sterile PBS. To determine presumptive *E. coli*, membranes were aseptically transferred to a Petri dish containing Membrane Lactose Glucuronide Agar (MLGA) (CM1031, Oxoid, Basingstoke, UK), inverted and incubated at 37 °C (± 0.2 °C) for 18 – 24 h. To quantify IE, membranes filters were aseptically transferred to Slanetz & Bartley medium (CM0377, Oxoid), inverted and incubated at 44 °C (± 0.2 °C) for 48 h. Following the method of Hodgson et al. (2009), the remaining rainwater-faecal mix was homogenised by vortex mixing for 60 s and appropriate serial dilutions prepared again in sterile PBS. Duplicate FIO concentrations were determined for the faecal component, as described above for the eluent. This provided the basis for determining the mobilised fraction of FIOs given that the total colony forming units (CFU) of FIOs in the original sample could now be calculated. Method blanks (i.e., sterile PBS) were used to confirm aseptic technique and the flame sterilisation procedure between samples. The remaining faecal material from each replicate was used to determine the gravimetric water content by drying at 105 °C for 24 h (until constant mass) and weighing the residual.

3.4 Data Analysis

All FIO counts underwent $\log_{10}$ transformation prior to statistical analysis, and distributions of CFU were log normally distributed as determined using the Kolmogorov−Smirnov goodness of fit test. Differences at the $p < 0.05$ level (95% confidence interval) were considered statistically significant. Proportions of cells mobilised from each faecal type were determined, although statistical analysis was performed on the normalised CFU released per treatment and not on the proportion (%) of FIOs mobilised. The proportion of CFU mobilised was calculated to account for the changes in source concentrations of FIOs in faeces as a function of die-off and provided complementary data. A three-way analysis of variance (ANOVA) and a Tukey multiple comparison test were used to test for differences in FIO concentrations (over

time at different temperatures from three faecal types). ANOVA was also used to test for differences in mobilisation of the two FIOs and to determine whether there were differences in FIO concentrations in the different faecal types as the faeces aged. Student's *t*-test was used to identify any difference between *E. coli* and IE concentrations within each faecal source. Pearson correlation coefficients were used to measure the strength and direction of relationships between moisture content of faeces and the percentage of FIB mobilised. All statistical analysis was performed using Minitab (Minitab 18.0 software, Minitab Inc.; State College, PA).

3.5 Results

All method blanks were negative for FIOs indicating that no cross contamination occurred during sample processing. The persistence profiles of both *E. coli* and IE in all three faecal types, reported over time as CFU g^{-1} dry weight faeces, provide important contextual information to help understand proportions of FIOs mobilised from the different faecal matrices (Fig 3.3). When excreted (day 0), concentrations of *E. coli* and IE in dairy faeces were significantly higher that concentrations found in deer faeces, which in turn were significantly higher than concentrations in goose faeces ($P < 0.001$; Fig 3.3). For *E. coli*, this pattern was consistent at all time points and for both temperatures apart from day 12 under 15 °C, when cell concentrations were highest in deer faeces. The deer faeces incubated at 15 °C and monitored for *E. coli* was the only scenario whereby the final concentration of FIOs was higher than the starting concentration ($P < 0.001$). For IE, concentrations in deer faeces were lowest on day 12 when incubated at 15 °C ($P < 0.001$). Earlier, on day 3, IE concentrations in deer and dairy were of a similar magnitude before concentrations in deer faeces dropped to levels consistent with goose faeces on day 7 (Fig 3.3). There were differences in relative proportions of the two FIOs in fresh faeces: *E. coli* concentrations were significantly higher than IE concentrations in dairy faeces ($P < 0.001$); IE concentrations were significantly higher that *E. coli* concentrations in deer faeces ($P < 0.01$); and there was no significant difference between FIO concentrations in goose faeces ($P > 0.05$).

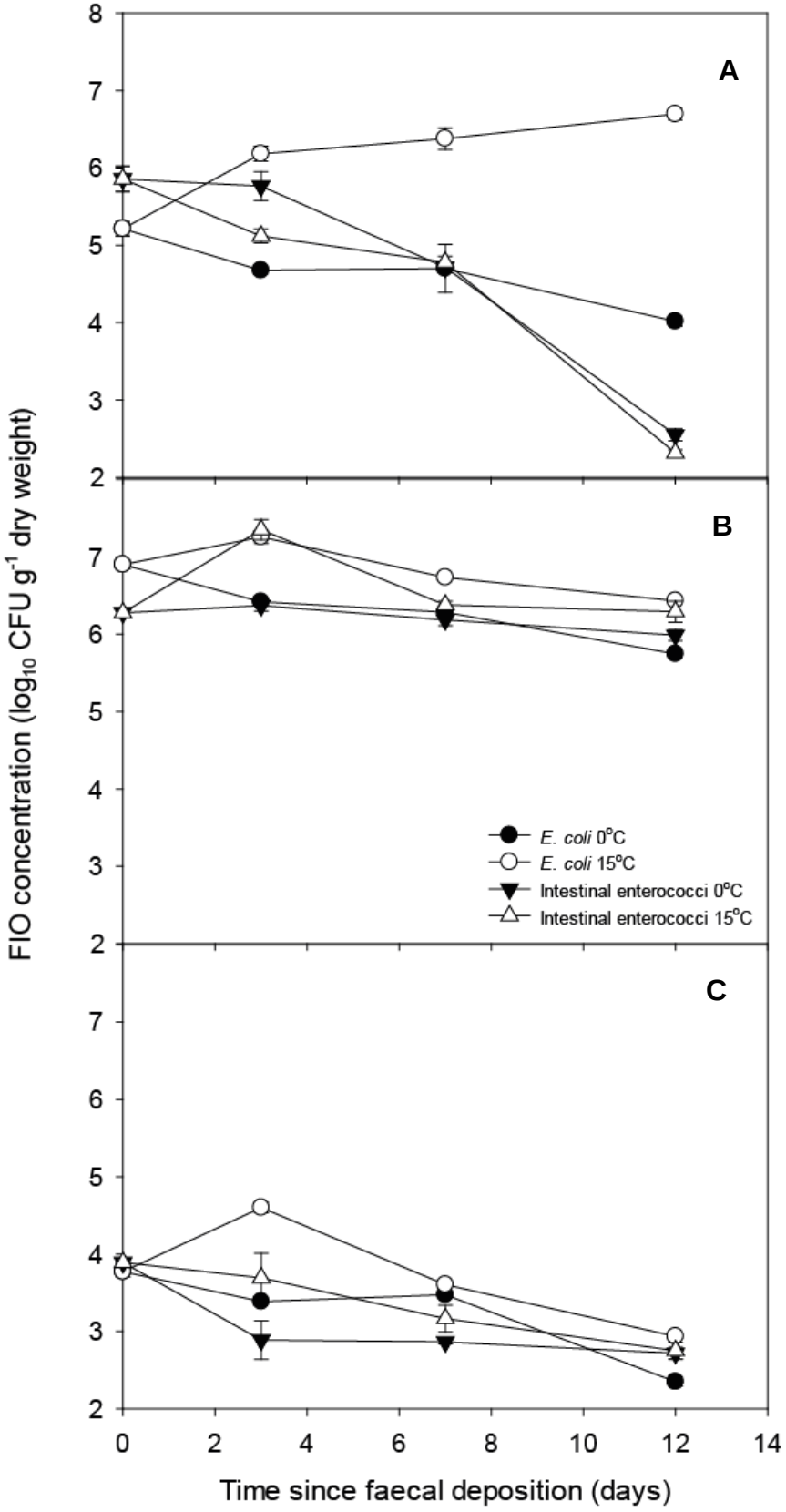

Figure 3.3*: Persistence profiles of FIOs in A) red deer faeces; B) dairy faeces; and C) greylag goose faeces. Data points are the mean of five replicates ± standard error.*

There were significant differences in the concentrations of both of the FIOs mobilised from all three faecal types ($P < 0.001$). The order of mobilisation potential for both *E. coli* and IE from the faecal matrices (greatest to least, expressed as a proportion of the total present) was dairy cow > goose > deer. The proportion of *E. coli* mobilised relative to the source concentration was also determined (Figure 3.4). The mean *E. coli* concentration mobilised from dairy, deer and goose faeces was 6.01, 2.96 and 1.66 $\log_{10}$ CFU mL^{-1}, respectively. For IE, the concentrations were 4.27, 1.96 and 1.24 $\log_{10}$ CFU mL^{-1}, respectively. There were significant interactions between all factors, thus day and temperature had an interactive effect on FIO mobilisation as did day and faecal type and temperature and faecal type. Significantly higher concentrations of *E. coli* compared to IE were mobilised from dairy cow faeces ($P < 0.05$), deer faeces ($P < 0.001$) and goose faeces ($P = 0.01$).

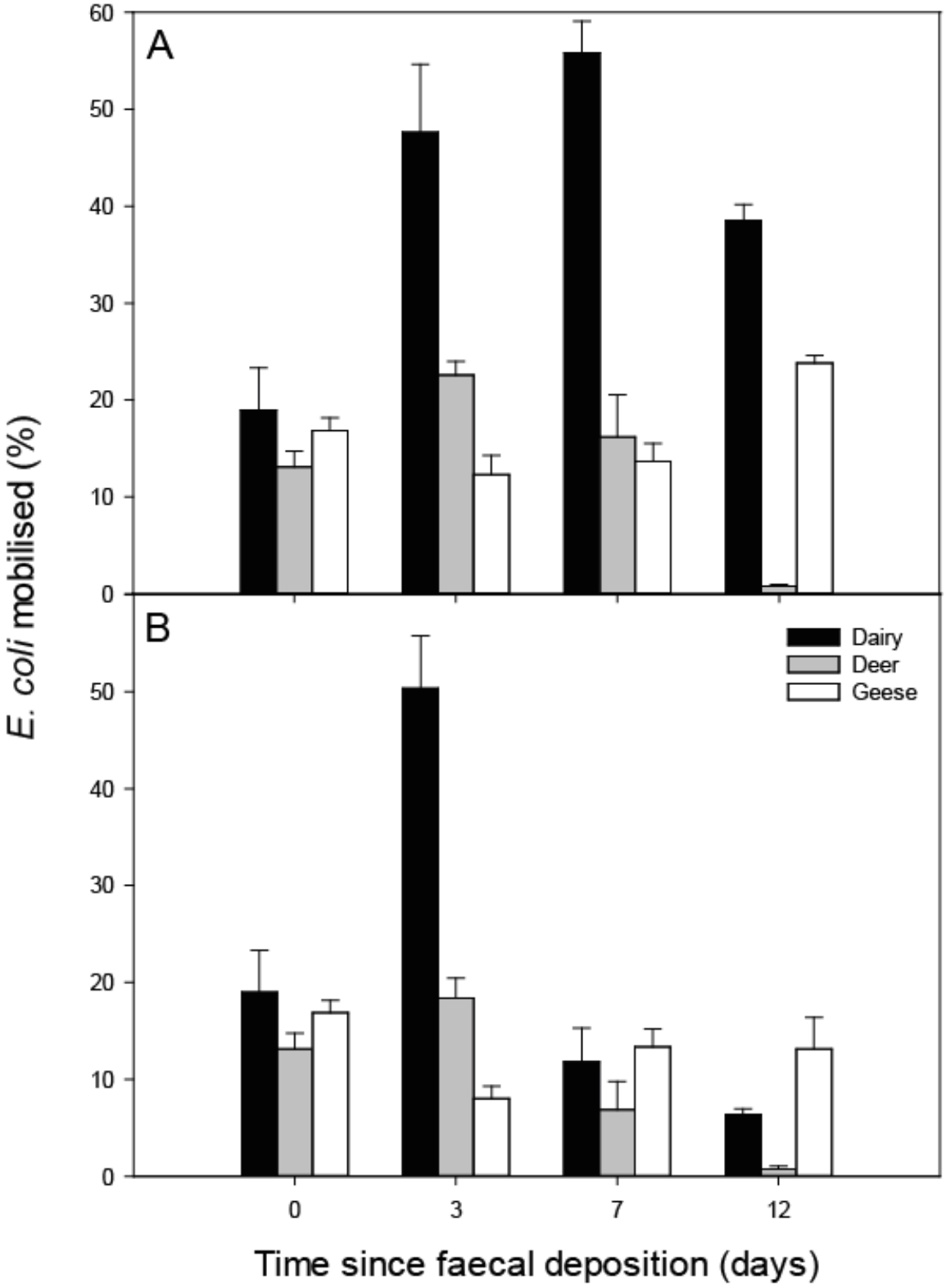

***Figure 3.4**: The proportion of E. coli mobilised from faeces over time under constant temperature conditions of A) 15 °C and B) 0 °C. Data points are the mean of five replicates ± standard error.*

E. coli concentrations recovered in the rainwater eluent identified significant changes in mobilisation potential over time from all faecal types. Mobilisation potential decreased significantly with time, with 12 days after deposition resulting in significantly less *E. coli* CFU per mL ($P < 0.001$). Mobilisation potential of *E. coli* was also influenced by the temperature at which the faecal material had aged over the course of the 12-day study ($P < 0.001$). In all cases, mobilised *E. coli* concentrations were greater when faeces were incubated at 15 °C (Table 3.1).

***Table 3.1**. Total number of E. coli CFU in faecal and eluent samples, associated E. coli concentrations in eluent samples and percentage of cells mobilised for each time-point. Data are the mean of five replicates ± standard error.*

Faecal matrix	**Day**	**Total *E. coli* in faecal sample (Log_{10} CFU)**		**Total *E. coli* in eluent (Log_{10} CFU)**		***E. coli* concentration in eluent (Log_{10} CFU mL^{-1})**		***E. coli* mobilised at point in time (%)**	
		15 °C	0 °C	15 °C	0 °C	15 °C	0 °C	15 °C	0 °C
Dairy	0	6.58 (0.07)	6.58 (0.07)	5.82 (0.13)	5.82 (0.13)	4.39 (0.13)	4.39 (0.13)	18.96 (4.34)	18.96 (4.34)
	3	7.26 (0.10)	6.30 (0.05)	6.92 (0.12)	5.99 (0.04)	5.49 (0.12)	4.56 (0.04)	47.63 (7.01)	50.34 (5.44)
	7	7.03 (0.02)	6.46 (0.09)	6.77 (0.04)	5.41 (0.13)	5.34 (0.04)	3.97 (0.13)	55.83 (3.29)	11.79 (3.50)
	12	6.83 (0.01)	6.15 (0.03)	6.41 (0.03)	4.94 (0.04)	4.98 (0.03)	3.51 (0.04)	38.55 (1.62)	6.35 (0.56)
Deer	0	5.14 (0.09)	5.14 (0.09)	4.24 (0.11)	4.24 (0.11)	2.82 (0.11)	2.82 (0.11)	13.12 (1.60)	13.12 (1.60)
	3	6.30 (0.09)	4.86 (0.03)	5.65 (0.11)	4.11 (0.08)	4.23 (0.11)	2.69 (0.08)	22.60 (1.38)	18.35 (2.10)
	7	6.82 (0.12)	5.17 (0.31)	5.96 (0.20)	3.78 (0.09)	4.53 (0.20)	2.36 (0.09)	16.17 (4.40)	6.86 (2.92)
	12	7.21 (0.07)	4.52 (0.07)	5.03 (0.19)	2.01 (0.29)	3.61 (0.19)	0.58 (0.29)	0.79 (0.17)	0.70 (0.31)
Goose	0	3.77 (0.07)	3.77 (0.07)	2.99 (0.09)	2.99 (0.09)	2.04 (0.10)	2.04 (0.09)	16.84 (1.33)	16.84 (1.33)
	3	4.60 (0.07)	3.39 (0.02)	3.67 (0.09)	2.26 (0.06)	2.71 (0.09)	1.31 (0.06)	12.32 (1.98)	7.98 (1.30)
	7	3.60 (0.02)	3.47 (0.02)	2.72 (0.04)	2.58 (0.05)	1.77 (0.04)	1.63 (0.05)	13.68 (1.86)	13.34 (1.86)
	12	2.94 (0.02)	2.35 (0.06)	2.31 (0.01)	1.36 (0.21)	1.36 (0.01)	0.41 (0.21)	23.81 (0.80)	13.10 (3.28)

Temporal patterns of IE mobilisation varied from that of *E. coli*; concentrations of IE recovered in rainwater from dairy faeces were not significantly different over time ($P > 0.05$). There were, however, significant differences in IE concentrations lost from deer (day 0 and 3 > day 7 > day 12) and goose (day 0 > day 3, 7 and 12) faeces over time. The warmer temperature treatment promoted significantly higher mobilisation of IE concentrations from dairy faeces ($P < 0.001$) but no temperature-driven mobilisation effect was observed for IE from deer or goose faeces ($P > 0.05$) (Table 3.2). Despite no difference in mobilised IE concentrations in dairy faeces over time, Figure 3.3 shows that IE concentrations in all three faecal sources fluctuated over the course of the experiment and in turn influenced the proportion of IE that was mobilised (Figure 3.5). The concentration and the proportion of mobilised FIOs therefore represent two different measures of mobilisation. No clear relationship between *E. coli* or IE mobilisation as a function of the FIO source load (normalised to reflect the FIO population relative to day 0) was observed across all faecal sources and timepoints (Fig 3.6a/b).

***Table 3.2**. Total number of intestinal enterococci CFU in faecal and eluent samples, associated intestinal enterococci concentrations in eluent samples and percentage of cells mobilised for each time-point. Data are the mean of five replicates (± standard).*

Faecal matrix	Day	Total intestinal enterococci in faecal sample (Log_{10} CFU)		Total intestinal enterococci eluent (Log_{10} CFU)		Intestinal enterococci concentration in eluent (Log_{10} CFU mL^{-1})		Intestinal enterococci mobilised at point in time (%)	
		15 °C	0 °C	15 °C	0 °C	15 °C	0 °C	15 °C	0 °C
Dairy	0	5.96 (0.04)	5.96 (0.04)	5.50 (0.06)	5.50 (0.06)	4.07 (0.06)	4.07 (0.06)	35.29 (2.52)	35.29 (2.52)
	3	7.35 (0.15	6.25 (0.05)	6.54 (0.19)	5.41 (0.08)	5.11 (0.19)	3.98 (0.08)	31.99 (17.16)	15.63 (2.96)
	7	6.67 (0.06)	6.36 (0.08)	6.34 (0.03)	5.36 (0.08)	4.91 (0.03)	3.92 (0.08)	49.52 (8.44)	11.80 (2.74)
	12	6.69 (0.14)	6.39 (0.07)	5.53 (0.18)	5.44 (0.13)	4.10 (0.18)	4.01 (0.13)	7.99 (1.95)	14.30 (4.67)
Deer	0	5.78 (0.16)	5.78 (0.16)	3.97 (0.11)	3.97 (0.11)	2.55 (0.11)	2.55 (0.11)	2.29 (1.19)	2.29 (1.19)
	3	5.23 (0.09)	5.95 (0.19)	3.86 (0.03)	4.19 (0.05)	2.44 (0.03)	2.77 (0.05)	4.57 (0.99)	2.92 (1.77)
	7	5.22 (0.06)	5.18 (0.07)	3.56 (0.04)	3.60 (0.07)	2.13 (0.04)	2.18 (0.07)	2.28 (0.40)	2.65 (0.16)
	12	2.83 (0.04)	3.06 (0.06)	2.09 (0.11)	1.81 (0.07)	0.67 (0.11)	0.39 (0.07)	20.94 (4.57)	6.50 (1.48)
Goose	0	3.89 (0.01)	3.89 (0.01)	3.11 (0.03)	3.11 (0.03)	2.15 (0.03)	2.15 (0.03)	16.52 (1.07)	16.52 (1.07)
	3	3.69 (0.32)	2.89 (0.25)	2.43 (0.34)	1.39 (0.20)	1.47 (0.34)	0.44 (0.20)	5.47 (0.39)	3.27 (0.38)
	7	3.17 (0.17)	2.87 (0.03)	2.12 (0.13)	2.04 (0.01)	1.167 (0.13)	1.08 (0.01)	10.96 (2.51)	14.89 (0.62)
	12	2.75 (0.11)	2.72 (0.08)	1.85 (0.08)	1.53 (0.10)	0.90 (0.08)	0.57 (0.10)	8.89 (0.55)	6.54 (0.57)

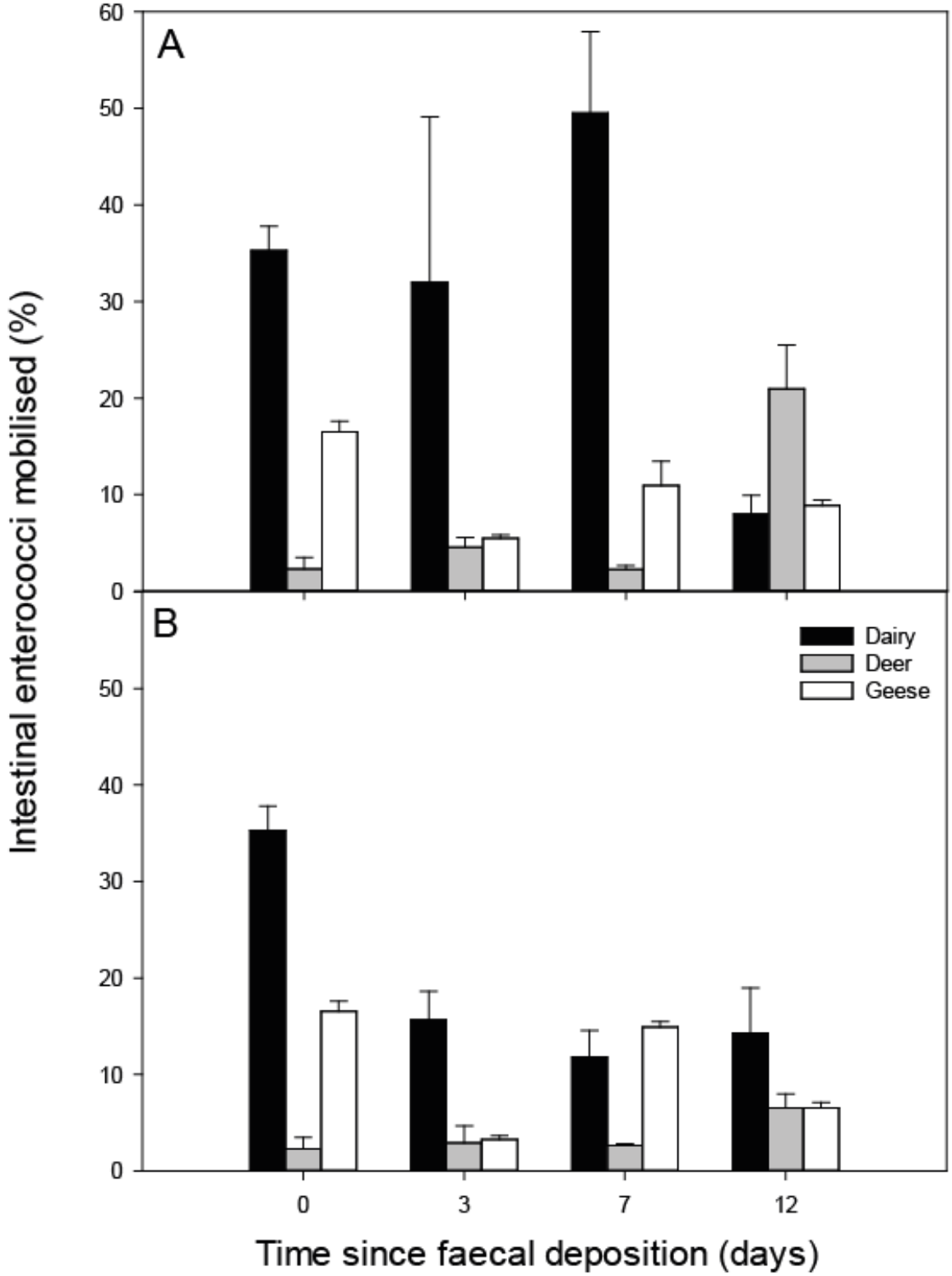

Figure 3.5*: The proportion of intestinal enterococci mobilised from faeces over time under constant temperature conditions of A) 15 °C and B) 0 °C. Data points are the mean of five replicates ± standard error.*

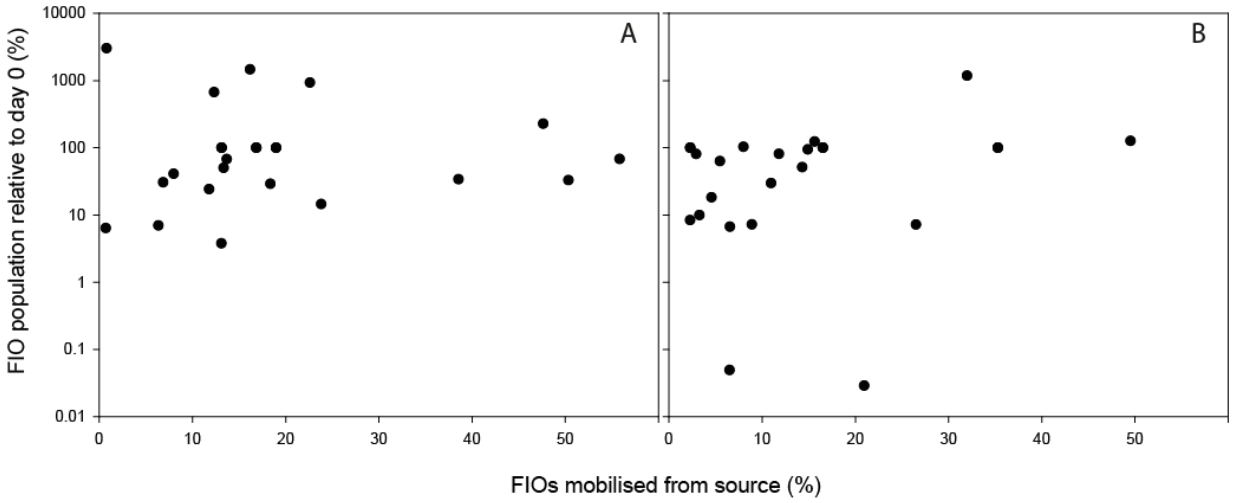

Figure 3.6*: The proportion of E. coli (A) and intestinal enterococci (B) mobilised as a function of the FIO source load for all three faecal sources combined (normalised to reflect the FIO population relative to day 0)*

Changes in moisture content of the three faecal sources were evident over the duration of the experiment (Fig. 3.6). Deer and goose faeces showed similar patterns of moisture loss under both temperature treatments. Dairy faeces retained more moisture over the duration of the experiment, with the difference in moisture content in dairy faeces relative to deer and goose faeces most prominent on day 7 under both temperature treatments. At a temperature of 15 °C, the change in moisture content over the experiment represented a decrease of 71.4%, 68.0% and 66.5% for deer, goose and dairy faeces, respectively. The overall change in moisture content when faeces were held at 0 °C was similar, with a decrease of 69.6%, 67.9% and 67.8% moisture recorded for deer, goose and dairy faeces, respectively. No significant correlation was observed between faecal moisture content and FIB mobilisation from dairy cow or goose faeces. In deer faeces, a significant positive correlation ($r=0.66$, $P < 0.001$) and significant negative correlation ($r=0.41$, $P < 0.05$) was recorded between moisture content of the faeces and the percentage of *E. coli* and intestinal enterococci mobilised, respectively.

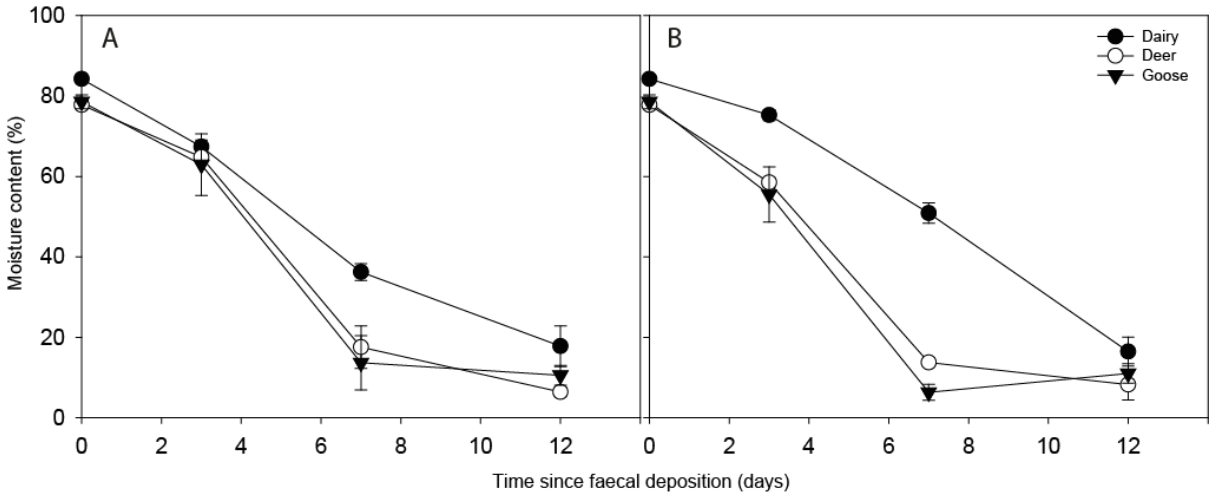

Figure 3.7*: Change in moisture content of faeces over time when held at a constant 15 °C (A) and 0 °C (B). Data points are the mean of five replicates ± standard error.*

3.6 Discussion

The potential for FIO mobilisation from faecal material varies with time, temperature and the source of faecal material. Patterns of mobilisation are different for the two most common FIOs, *E. coli* and IE; however, quantifying mobilisation of microorganisms from faecal matrices is complex because of the dynamic nature of microbial persistence patterns, which again can differ for *E. coli* and IE. Mobilisation therefore reflects a combination of FIO survival in the faeces and the erodibility of the faecal source and subsequent detachment rate of the FIO population, which both vary as a function of faecal ageing and desiccation (Kim et al., 2016). Detachment processes are the main driver of FIO mobilisation from faeces, although mechanistic understanding of how different faecal sources influence FIO retention and mobilisation is limited (Sepehrnia et al., 2018). In our study we specifically quantified FIO mobilisation from three different faecal matrices and contribute important process-based information concerning how FIO mobilisation changes over time and under different temperature scenarios.

The FIO concentrations mobilised from dairy faeces after 12 days, was substantially higher than from red deer and greylag goose faeces and reflects the varying levels of FIOs present in the source of the faeces, with goose faeces containing much lower

concentrations of FIOs overall in our study. The one exception was the concentration of *E. coli* mobilised from deer faeces after 12 days; however, the *E. coli* population in deer faeces at 15 °C underwent considerable regrowth and deviated substantially from the persistence profiles of other faecal sources leading to a greater availability of *E. coli* for mobilisation. Regrowth of FIOs in faecal material is not uncommon, and over an order of magnitude increase in *E. coli* numbers post excretion has been reported in deer faecal pellets incubated at lower temperatures than those under which regrowth was observed in our study (4 °C versus 15 °C) (Guber et al., 2015). The magnitude of decline in concentration of mobilised FIO was greatest for goose faeces, whereas in general the other faecal sources maintained similar levels to day 0, suggesting a more consistent risk of FIO release to the environment from dairy cow and deer faeces over the study duration. Mobilisation potential of FIOs will vary depending on the starting concentration of FIOs at source, but more research is needed to fully characterise FIO mobilisation at the point of excretion, over time, and through different seasons, which is important given the potential for large variability in FIO shedding rates from different animals (Porter et al., 2019). Prior research has acknowledged the challenges that a dynamic FIO population can introduce as part of quantifying mobilisation, both in terms of FIO regrowth and via FIO decay (Stocker et al., 2020a).

When considering the proportion of FIOs mobilised relative to the source load, rather than mobilised FIO concentrations, dairy cow faeces still consistently generated greater *E. coli* mobilisation from the faecal matrix relative to the other faecal sources under the warmer temperature treatment. The dominance of the livestock faecal source in generating greater proportions of mobilised *E. coli* relative to the wildlife / wildfowl faecal sources was more short-lived at 0 °C; a pattern repeated in the IE mobilisation data too. To some extent this will be due to variations in survival of FIOs at the two temperatures investigated, with freezing temperatures known to be less conducive to FIO persistence (Afolabi et al., 2020). However, changes in mobilisation will also be influenced by how quickly the outer layers of the faecal matrix develop a crust in the absence of rainfall. As faecal matter aged under constant temperatures, the moisture content of the faecal matrix also decreased. This was more rapid for the red deer and greylag goose faeces than for dairy cow faeces, which would have retained a moist interior for longer due to differences in surface area to volume ratio

and offers further explanation for the more readily available supply of cells in the dairy faeces for mobilisation. Beef cow faeces monitored previously using the same experimental approach also demonstrated greater mobilisation potential relative to other manure sources, suggesting that the physical make-up of cowpats is conducive for releasing substantial numbers of FIOs even with lengthy lag times between faecal deposition and the onset of rainfall (Hodgson et al., 2009).

Concentrations of mobilised *E. coli* were substantially less after 12 days relative to earlier in the experiment in all faecal sources, while for IE this was only the case for goose and deer faeces. Under the colder temperature regime, FIO concentrations declined but remained orders of magnitude above detection limits in all faecal sources; however, mobilised concentrations were further reduced, and this may reflect limited mobilisation potential arising from the formation of a crust rather than reduced FIO survival alone (Stocker et al., 2020b). Under field conditions, a crust forms when faeces are exposed to sunlight (Sinton et al., 2007), but under laboratory conditions the constant temperature and lack of rehydration from rainfall would help to promote more rapid drying in the faecal boundary layers, and thus a dry 'skin' on the faeces surface likely developed that acted as a form of crust. The misting step done to mimic morning dew formation would not compensate for the level of rehydration provided by rainfall, nor was this the intention.

The deer, dairy and goose faeces represent different faecal matrix structures and the differences in the rate of FIO mobilisation from them are probably related to the differences in physical structure and make-up of the faecal sources. Differences in physical structure of deer and dairy faeces can also impact FIO survival rates (Afolabi et al., 2020), while the soluble/solid faecal composition can govern the release rates of FIOs to the wider environment (Blaustein et al., 2015a; Sepehrnia et al., 2018). The faecal structure will also dictate the speed at which infiltrating water can make contact with resident cells, in turn facilitating their wash-out (Kim et al., 2016). The proportion of finer and larger fractions of organic matter that characterises each faecal matrix may also explain differences in mobilisation potential, as has been reported in studies exploring *E. coli* release from specific fractions of faeces (0.25, 0.5, 1.0, 2.0 mm faecal components) (Sepehrnia et al., 2021). FIO mobilisation will be influenced by the

degree of erodibility associated with the faecal source (Kim et al., 2016) and observations of faecal disruption during the DESPRAL test identified that dairy faeces more readily disaggregated relative to the goose and deer faeces. The pellet-like structure of deer and, to a lesser extent, goose faeces is similar to sheep faecal pellets, with the latter also demonstrating an ability to maintain its physical composition when subjected to the same experimental approach (Hodgson et al., 2009). This suggests that FIO mobilisation patterns from wildlife and wildfowl faeces are not distinct from a number of other livestock FIO sources, e.g. sheep faeces and farmyard manures, but that larger faecal pats associated with cattle present greater risk of contributing FIOs to hydrological pathways with the onset of rainfall. Information on the chemo-physical (e.g., fibre content) and main organic composition of samples was not collected in this study. Such analysis could provide useful data to further interpretate the results and aid comparisons with studies of other animal faecal sources. Therefore, future research should include such supporting information if possible.

The results of our study highlight changes in FIO mobilisation as a function of increasing lag time between faecal excretion and rainwater contact with the faecal source. The role of subsequent rewetting episodes and how consecutive rainfall events influence mobilisation was not considered. Wetting and drying cycles are likely to be influential in altering mobilisation potential and requires further research. Recent studies have highlighted the importance of freeze-thaw processes on FIO survival (Afolabi et al., 2020) and like wetting and drying cycles the physical changes to faecal structure resulting from freeze-thaw cycles will probably lead to changes in mobilisation potential of FIOs from faecal sources. Few studies are available that specifically quantify how rainfall recurrence impacts on FIO mobilisation from a suite of faecal sources. There are examples of larger scale monitoring campaigns that consider the impacts of repeated rainfall events across multiple landscape sources on FIO export via drainage networks, but again studies such as this are specifically quantifying FIO transfer and delivery and not mobilisation *per se* (e.g., Buckerfield et al., 2019).

Under field conditions, the duration of a rainfall event will influence FIO mobilisation dynamics and the two key detachment processes operating over the ‘event’ are likely to be raindrop impact and subsequent sloughing of the faecal surface in response to

resulting surface runoff, in turn leading to the gradual disintegration of the faecal matrix. A number of factors will influence the intensity of raindrop impact and subsequent cell detachment, and these include the kinetic energy of the falling precipitation, the angle of raindrop contact and the moisture content of the material being impacted by the raindrops (Mügler et al., 2021). In our study the 'event' was limited in duration to 60 s and the disruption to the faecal source was constant over that timeframe because we used a controlled laboratory assay specifically developed to investigate FIO mobilisation (Hodgson et al., 2009). We did not determine how FIO mobilisation varied over the duration of the 'event'. Over longer experimental timeframes and using different measures of mobilisation there are reports of a faster, or in some cases more irregular, initial FIO release from faeces followed by a slower steady-state FIO release (Sepehrnia et al., 2021; 2017). Despite a smaller mass of faeces being used in assessing FIO mobilisation from goose faeces, the ratio of faecal mass to rainwater was consistent with that used to determine FIO concentrations released from dairy cow and deer faeces, and thus the difference in faecal mass should not impact on comparing the concentrations of FIO mobilised across different treatments.

Differences in mobilisation characteristics of *E. coli* and IE probably reflected varying properties of the different bacterial cells, e.g., physiological properties, surface structure, that can influence mechanisms associated with their release from liquid versus solid fractions of the faecal matrix (Blaustein et al, 2015b). For example, it has been suggested that *E. coli* resides in the more liquid fraction of manure whereas IE associates with more strongly with particles and this can impact the relative release dynamics following rainfall detachment (Guber et al., 2007). The proportions of FIOs mobilised from the three faecal sources were of a similar magnitude to those reported by Hodgson et al., (2009) who used the same methodology (but at a slower, less intense DESPRAL test rpm) to mimic rainfall driven mobilisation. In general, faecal sources with higher moisture content (beef cow faeces, dairy cattle slurry (Hodgson et al., 2009); dairy cow faeces; (this study)) were found to promote mobilisation of up to ~ 50% of the FIOs in the faeces at times of peak mobilisation, whereas faecal sources with lower moisture content (sheep faeces, farmyard manure (Hodgson et al., 2009); deer and goose faeces (this study)) tended to promote up to ~ 20% mobilisation. However, the dynamic relationship between changes in moisture content of faeces

and mobilisation of FIO is more complicated, as evidenced by no clear correlation between these variables in dairy cow or goose faeces and with inverse relationships observed between moisture content and *E. coli* versus intestinal enterococci mobilisation from deer faeces over the experiment duration. This complication arises because of the combined influence of both die-off and regrowth on FIO persistence. It is worth highlighting that the DESPRAL test, originally developed to estimate the intrinsic risk of sediment and phosphorus mobilisation from a wide range of bare European soils (Withers et al., 2017), is a laboratory-based simulation that provides a surrogate for assessing rainfall-driven mobilisation rather than a direct measure of, for example, rain-drop impact detachment processes. Our application of the DESPRAL test for measuring FIO mobilisation from faecal sources provides an assay for the relative likelihood of FIO detachment following interaction with rainwater. The approach has been used widely as a proxy for pollutant mobilisation [e.g., Hodgson et al., 2009; Villa et al., 2014; Martin et al., 2015; Djodjic et al, 2018).

Data reported in our study can help to constrain the parameterisation of mobilisation coefficients in models, which are often ignored in the modelling of FIO fate and transfer because of a lack of such information (Alegbeleye and Sant'Ana, 2020a; Oliver and Page, 2016). Understanding mobilisation potential is important because it provides an indication of the magnitude of a pollutant load that may subsequently be transferred through the environment. The inclusion of laboratory-derived mobilisation coefficients into a landscape model would, however, require careful assessment but this would be the case for any laboratory-derived process representation (Afolabi et al., 2020). While the mobilisation data generated from the DESPRAL experiments does not include molecular analyses it is important to highlight that the majority of models used to understand FIB pollution are developed on data derived from culture-based studies (Dorevitch *et al.*, 2017).

Published studies report on FIO mobilisation, but also FIO release and/or removal, and there is some ambiguity in the use of terminology in studies reporting on FIO loss from faeces. Blaustein et al (2015a) recognise two meanings associated with the term release: in some studies, this is used to represent FIOs leaving a faecal matrix, whereby release is a boundary condition, but in other studies release is taken to mean cell concentrations found within runoff and leachate at plot scales or coarser. The

latter, in our view, is not truly a measure of mobilisation because some cells that are mobilised from the faecal source may become trapped on the soil surface or in the soil pore architecture prior to sample collection. FIO concentrations measured in runoff and leachate represent FIOs in a state of transfer through the environment. They have already been mobilised from the faeces by detachment processes such as raindrop impact and sloughing. The data reported in our study therefore provides new information relating specifically to mobilisation rates of FIOs from three common faecal sources in rural catchments. The findings will enable refinement of existing models and decision support tools that recognise detachment processes as an important step in understanding FIO risk to the wider environment.

3.7 Conclusion

The loss of FIOs from land to water from diffuse sources represents a continuum whereby cells are mobilised from faeces following rainfall and transferred in hydrological pathways before being delivered to a receiving water. Our findings add quantitative evidence to support our understanding of FIO mobilisation potential from three important faecal sources in the environment and demonstrate that dairy cow faeces represent a greater risk of contributing FIOs to the wider environment than goose or deer faeces following rainfall events. As faeces age, deer and geese contributions can become more important contributors of mobilised FIOs, highlighting a complex and nuanced pattern to FIO mobilisation from different sources typical of rural catchments. While our study focused on mimicking hydrological mobilisation from the terrestrial environment, such processes can be circumvented if faecal deposition by livestock, wildlife or wildfowl occurs direct to a receiving water and the rate of faecal breakdown and dissolution as drivers of FIO mobilisation following submergence would then require investigation. Our findings provide novel data to help characterise this important, yet often underrepresented process associated with FIO fate and transfer; however, further laboratory and field quantification of mobilisation processes is needed to support the parameterisation of modelling efforts designed to predict FIO impairment of receiving waters in catchments that contain complex mixes of faecal sources.

4. Persistence of *E. coli* in streambed sediment contaminated with dairy cow, goose and deer faeces

Abstract

Streambed sediments play an important role in persistence of FIOs in surface water by protecting them from solar radiation and providing necessary nutrients for their survival. The concentration of FIOs is often high in streambed sediments relative to overlying water. This is due to settling of faecal matter and associated FIOs into the streambed sediments which provide a potential legacy store of microbial pollution. There is growing recognition that streambed sediments can cause delay in further subsequent impairment of water quality due to resuspension. However, key gaps in knowledge include understanding how temperature regimes affect the growth and survival of FIOs in streambed sediment and settling of faecal matter from different sources (dairy and wildlife/wildfowl) in streambed sediment. The overarching aim of this study was to determine, using laboratory-based approach, the persistence of *E. coli* derived from dairy cow, deer and goose faecal sources introduced to streambed sediment under different temperature regimes. The difference in settling rate of solid constituents of dairy cow, deer and goose faeces once delivered to an aquatic environment was also determined. The greater reduction in percent survival of *E. coli* in sediment contaminated with goose faeces relative to dairy cow and deer faces provides empirical data on persistence of *E. coli* and how it varies between faeces from different sources. Characterising how *E. coli* from different catchment sources survive in streambed sediment under varying temperature regimes is key to understanding legacy stores of FIO and potential faecal pollution in catchment following resuspension.

4.1 Introduction

Streambed sediments can harbour a range of terrestrially sourced pollutants (Parkes et al., 2014; Abia et al., 2015). Faecal contamination of the water environment following agricultural runoff and sewage overflow delivers faecal indicator organisms (FIOs) into suspension in river drainage networks and, depending on river flow rates, cell-particle associations and sedimentation rates, a proportion of FIOs will become entrained in streambed sediment (Abia et al., 2017). The settling of faecal material and associated FIOs into the streambed sediment provides a potential legacy store of microbial pollution, which can result in further subsequent delayed impairment to water quality following resuspension. There is a growing body of research that has documented the delayed impacts that legacy phosphorus can have on water quality and associated management (Withers et al., 2017; Doydora et al., 2020), but legacy risks associated with environmental stores of FIOs require further investigation.

Recently there has been increased recognition and public awareness of the risks posed to water quality from sewage pollution and spills from combined sewer overflows (CSOs) (Ahmed et al., 2018; Devane et al., 2020). However, those debates have focused on the immediate impacts to the hygienic status of the receiving water, but a secondary issue is that faecal pollution will contribute FIOs to the streambed environment too, where they may be stored for longer periods in the sediment (Padilla and Vesper, 2018; Buckerfield et al., 2019). Contaminated streambed sediments downstream of CSOs represent an easily identifiable hotspot of potential legacy FIO pollution because of their proximity to point source discharges. By contrast, diffuse agricultural pollution to surface waters represents a much more challenging delivery of FIOs to identify and manage. This is because there is no single identifiable source in the landscape and therefore the loading of stream and river sediments with FIOs from agricultural practices, although potentially not as intense as a sewage discharge spill, may be more chronic and represent a long-term hindrance to effectively managing microbial water quality.

In addition to agricultural and sewage sources, FIO loading of watercourses and streambed sediments can also originate from wildlife and wildfowl. Populations of deer, geese and other wildlife may defecate directly into an aquatic environment or

their faecal depositions to land can be disrupted following rainfall events, with a proportion of FIOs subsequently mobilised and transferred to receiving waters (Moriarty et al., 2012; Stocker et al., 2020a). Studies have reported on black-tailed deer and Canadian goose as contributors of FIOs in environmental matrices (Moriarty et al., 2012; Guber et al., 2015), and there are reports of increased FIO inputs to lake and streambed sediment that can result from wildlife activity (Parajuli 2007; Smith et al., 2020). While the environmental persistence of sewage and livestock derived FIOs has been well studied, with key factors recognised as being influential in promoting or hindering survival, less is known about FIOs contributed by wildlife and wildfowl or whether there are any important differences in their survival characteristics. Temperature is recognised as one of the most important environmental variables that controls FIO persistence once excreted from the human or livestock gut environment into the wider landscape (Smith et al., 2019; Brandão et al., 2022); however, in comparison empirical data and the associated evidence base of how wildlife and wildfowl derived FIOs respond to different temperatures when associated with a range of environmental matrices is limited. With respect to streambed sediment survival, *E. coli* from goose, deer and bovine faeces introduced into sediments versus survival of indigenous strains was studied by Kiefer et al (2012), but only at ambient temperatures. Final concentrations of *E. coli* across all faecal types were comparable after 32 days; however, *E. coli* die-off rates in sediments contaminated by different faecal sources were variable. Smith et al., (2019) evaluated the effect of temperatures oscillations from 17 °C to 28 °C, typical of a diurnal summer temperature range for the location of study (Maryland, USA) on populations of *E. coli* and enterococci in sediments and in the water column. Again, lower temperature regimes were not considered. Both of these studies simulated stream conditions using a flow chamber, which provided a steady stream of water above the sediment via a closed-circuit water reservoir.

The persistence of FIOs in streambed sediments has been linked to other factors such as availability of nutrients (Wanjugi et al., 2016), sediment characteristics (Fluke et al., 2019) and protection from ultraviolet radiation and predative organisms (Korajkic et al., 2013; Wanjugi and Harwood, 2013). FIOs in stream sediment can also regrow under some favourable conditions (Zimmer-Faust et al., 2017), thus providing a potentially long-term input into the overlying water column (Jang et al., 2017).

Association of FIOs with organic and/or mineral matter can play an important role in their delivery to streambed sediment relative to freely suspended cells because of the impact on increased settling speeds (Karbasdehi et al., 2017). Extracellular polymeric substances of bacteria, an important protein that play a major role in cell-sediment flocculation, can aid delivery of bacteria to the bottom sediment as a result of an increase in the downward flux associated with higher floc mass (Karbasdehi *et al.*, 2017). The differential settling rates of faecal material associated with varying faecal types through the water column is therefore another factor that can influence the magnitude of FIOs stored within the streambed sediment, although there is little data available that reports on rates of faecal sedimentation and how this varies between different sources, e.g., livestock, wildlife or wildfowl, and their associated differences in faecal characteristics.

It is therefore important to study the survival pattern of *E. coli* from different faecal types, beyond the well-recognised human and livestock sources, at different temperatures to improve our knowledge on the persistence of *E. coli* in streambed sediment. The overarching aim of this chapter was to determine the persistence of *E. coli* derived from dairy cow, deer and goose faecal sources introduced to streambed sediment under different temperature regimes. The specific objectives of the experiment were to: (i) determine how die-off rates of *E. coli* vary in sediment contaminated with dairy cow, deer and geese faeces; (ii) quantify how temperature influences concentrations of *E. coli* in faecally contaminated streambed sediments; and (iii) evaluate how the solid constituents of dairy cow, deer and goose faeces vary with respect to their settling rate once delivered to an aquatic environment.

4.2 Materials and Methods

4.2.1 Provenance of faeces used in all experiments

Please refer to the methodology described in Section 3.1.1 of this thesis for details of provenance of faecal sources. Field sampling of goose and deer faeces is shown in figures 4.1 and 4.2, respectively.

4.2.2 Experiment design

A controlled laboratory experiment was carried out to determine the persistence of *E. coli* in streambed sediment under two constant temperature regimes (4°C and 18°C), which represent two typical UK streambed temperatures in winter and summer seasons (Klaar et al., 2020). The temperature treatment of 18°C was higher than 15°C used in the mobilisation chapter to reflect differences in typical temperatures of terrestrial and aquatic environments. All experiments were carried out using incubators over a duration of 22 days to monitor the difference in the persistence of *E. coli* derived from different common rural faecal sources (dairy cow, red deer, greylag goose) once integrated into streambed sediment. Sediment samples were collected more frequently in the early stages of the experiment and after 22 days it was assumed that changes in *E. coli* population would be minimal relative to earlier in the experiment.

4.2.3. Preparation of mesocosms

Each treatment consisted of four replicates of a faecal/sediment mix per sampling day that were destructively harvested. The treatments were prepared as a mix of sterile sediment (dry) and fresh faeces at a ratio of 8:2, respectively. The sediment and faecal mix were homogenised in a sterile tray to ensure even distribution of cells. A total of 15 g of this contaminated mix was added to a 50 mL centrifuge tube (n = 4) and the tube tapped to allow the sediment to settle evenly. Next, 30 mL of sterile river water (see 4.2.4) was slowly pipetted down the side of each tube to prevent agitation of the contaminated mix. The river water delivered moisture to the faecal/sediment mix. The overlying water was not flowing and thus provided a standing water scenario. The tubes were randomly divided into each treatment and arranged in plastic racks and stored in incubators at either 18°C or 4°C for 22 days. A destructive sampling approach was used whereby each treatment was sampled on day 0, 2, 6, 9, 15 and 22 to monitor the persistence of *E. coli* in streambed sediment. Samples were collected more frequently in the early stages of the experiment to capture the more dynamic phase of population change.

Figure 4.1: *Field sampling of greylag goose droppings at RSBP loch Lomond with support from RSBP staff.*

4.2.4. Artificial sterile river water preparation

A standardised river water (soft water) was made from three stock solutions, which were prepared in advance following the method described by Smith et al., (2002). Stock 1 was composed of $MgCl_2 \cdot 6H_2O$, 12.168 g/l, (0.06mM), $CaCl_2 \cdot 2H_2O$, 11.76 g/l (0.08mM), and $Ca(NO_3)_2 \cdot 4H_2O$, 3.542 g/l (0.015mM). Stock 2 was composed of $CaCO_3$, 0.01872 g/l (0.170mM) while stock 3 was composed of Na_2SO_4, 16.334 g/l (0.115mM), K_2CO_3, 1.725 g/l (0.0125mM) and Na_2CO_3, 1.06 g/l (0.01mM). All stock solutions were prepared in mg L^{-1} and vigorously stirred throughout the preparation, and sub samples of the final matrix were taken to verify the actual final concentration of cations and anions in the solution. A total of 2727 mL of stock 2 was added to a 5-litre beaker, while 3 mL of stock 1 and stock 3 were added to the beaker, respectively, and the solution was vigorously stirred to ensure that the solutes completely dissolved with final pH of 8.41. The artificial river water was sterilised in Duran bottles using an autoclave (15 min at 121 °C).

4.2.5. Preparation of streambed sediment

The streambed sediment was sourced from a local first order agricultural stream and transported to the laboratory in a sterile polyethene bag. About 5 kg of wet weight sediment (see 4.2.6) was sterilised in an autoclave at 121°C for 15 minutes to remove background microorganisms from the sediment. The sediment was then distributed into three clean foil trays with surface area of 324 cm^2 each and oven dried at temperature 100°C for 72 hours until the moisture content was completely removed, and sediment measurements recorded constant mass. The sediment was allowed to cool to room temperature and sieved using a sterile 2 mm sand sieve to remove debris, stones, and large particles before the preparation of mesocosms. The absence of opportunistic faecal indicator microbes (*E. coli* and enterococci) in the sterilised sediment was confirmed by streaking a suspension of sediment onto Membrane Lactose Glucuronide Agar (MLGA) (CM1031, Oxoid, Basingstoke, UK) and Slanetz and Bartley medium and recording zero growth after incubation.

4.2.6. Analysis of streambed sediment particle texture

The streambed sediment was analysed using a coulter counter (Beckman Coulter L5230). A sub-sample of the oven-dried, well-mixed and sieved streambed sediment was divided into three replicates. To do this, 50 mL plastic sample bottles were filled with sediment to a depth of approximately 0.5 cm and topped up to 1.5 cm with distilled water. Next, 2 mL of dispersant sodium hexametaphosphate (Calgon) was added to the mixture to aid deflocculation. Then, the samples were agitated using a table shaker overnight to ensure homogeneity of the mixture. The samples were then prepared for analysis by stirring the mixtures using a magnetic stirrer for a minimum of 30 minutes and the samples were run through coulter counter machine to determine particle size distribution.

***Figure 4.2**: Field sampling for red deer faeces at Scottish deer centre, Fife.*

4.3 *E. coli* Enumeration in Streambed Sediment

On sampling days, approximately 3g of contaminated mix (faecal streambed sediment) was randomly sampled from all replicates of each treatment using a sterile spatula after the removal of the overlying water using a pipette. To enumerate the *E. coli* present in the sediment, each 3 g sample was transferred to 27 mL of sterile river water in a 50mL centrifuge tube and vortex mixed for 30 seconds to ensure homogeneity prior to subsequent 1:10 serial dilution in PBS. Subsequently, 1 mL of each serially diluted sample was pipetted on to a 0.45 µm cellulose acetate membrane and washed through a vacuum-filtration unit (Sartorius Stedim Biotech., Goettingen, Germany) with ~20 mL of sterile PBS. To determine presumptive *E. coli*, the membranes were aseptically transferred to a Petri dish containing Membrane Lactose Glucuronide Agar (MLGA) (CM1031, Oxoid, Basingstoke, UK), inverted and incubated

at 37°C (± 0.2°C) for 18 – 24 h. The remaining sediment (~12 g) was used to determine gravimetric water content by drying at 100°C for 48 h.

4.4 Rate of Faecal Material Sedimentation in Water

An experiment to infer the rate of sedimentation of faecal material delivered to water was conducted to complement the investigation of *E. coli* persistence in streambed sediment. Briefly, 10 g of faecal matter (dairy cow, red deer, greylag goose) was weighed into a 50mL centrifuge tube in replicate (n = 3) and 30 mL of distilled water added. The mixture was vigorously shaken until the faecal matter disaggregated in the water and the tubes were then left to stand as the faecal material settled. The rate of sedimentation was inferred by measuring the change in water turbidity at 0, 1, 2, 3, 4, 5, 10, 20, 30, 60, 120, 180, 240, 300, 360, 420, 480, 540, 600, 660 and 720 minutes. Approximately 1 mL of the mixture was sampled at each time point and diluted with 9 mL of distilled water in a cuvette and the sample shaken to mix. All samples were then analysed for turbidity using a Hannah LP2000 benchtop turbidity metre (Hanna Instruments, Bedfordshire, UK).

4.5 Statistical Analysis

All statistical analysis was performed using Minitab (Minitab 18.0 software, Minitab Inc.: State College, PA). Differences at the $p < 0.05$ level (95% confidence interval) were considered statistically significant. Plate counts of *E. coli* were normalised by transforming to $\log_{10}$ CFU g^{-1} dry weight sediment. A two-way analysis of variance (ANOVA) and follow-up Tukey multiple comparison tests were used to test for differences in *E. coli* concentration between temperature treatments and faecal sources and to test for any interactions between these factors. Linear regression was used to estimate the rate of *E. coli* decline (k) in the streambed sediment. If any treatment recorded an initial period of growth, the linear model was fitted once the *E. coli* population began to decline (i.e., from the timepoint of peak concentration). ANOVA was used to test for differences in *E*. *coli* die-off characteristics (e.g., k values) relative to the source of faecal contamination and temperature treatment. D-values, which represent decimal reduction times, were calculated based on the average rate of decline of *E. coli* following a log-linear die-off profile. Given that concentrations of *E. coli* in the different sources of fresh faeces varied, initial populations were normalised

to represent 100% to allow for a more comparable plot of persistence over time (i.e. percentage survival curves were plotted). The statistical analysis was performed on normalised CFU data and not on the proportion (%) of *E. coli* remaining over time.

4.6 Results

4.6.1 Persistence of *E. coli* in streambed sediments

The initial concentration of *E. coli* and dry matter characteristics associated with all faecal types prior to their mixing with sediment is recorded in Table 4.1. Goose faeces recorded the highest *E. coli* concentration, several orders of magnitude greater than concentrations in dairy and deer faeces.

Table 4.1. Characteristics of fresh faecal material used in the experiment (Day 0).

	Faecal source		
	Dairy cow	**Red deer**	**Greylag goose**
Mean *E. coli* concentration ± SE (Log_{10} CFU g^{-1} dry weight)	4.51 ± 0.10	4.92 ± 0.03	7.57 ± 0.03
Dry matter %	70.02	75.90	69.78

The percent survival curves of *E. coli* in streambed sediment held at different temperatures (18°C and 4°C) show a greater proportional reduction in cells after 22 days in sediment contaminated with goose faeces relative to dairy cow or deer faces, irrespective of temperature (Fig 4.3). *E. coli* from dairy and deer faecal matter exposed to 18°C exhibited initial *E. coli* growth followed by a slow decline to the end of the experiment but remained at levels far greater than the initial population of *E. coli* recorded on day 0 (Fig 4.1A). By contrast, concentrations of *E. coli* in sediment contaminated with goose faeces and held at 18°C showed no evidence of an initial growth period, instead declining in population size from day 0 and reaching 0.6% of the initial population by day 22 (c.f. 1000% and 640% of initial population for sediments contaminated with dairy cow and deer faeces, respectively). The changes in percent

survival of *E. coli* populations at 4°C were less distinct, with sediments contaminated with all three faecal sources showing a degree of fluctuation up to day 9, after which patterns of persistence diverged (Fig 4.3B). At 4°C, the proportion of the initial *E. coli* population remaining after 22 days in the sediment contaminated with goose, dairy, and deer faeces were each separated by an order of magnitude, with 0.3%, 4% and 38% remaining, respectively.

The concentration of *E. coli* detected in the sediment contaminated by goose faeces was significantly higher than in sediment contaminated with dairy cow or deer faeces ($P < 0.001$). The 18°C temperature treatment promoted significantly higher concentrations of *E. coli* relative to the 4°C temperature treatment ($P < 0.001$). In addition, a significant interaction occurred between temperature and faecal source ($P < 0.001$); higher concentrations of *E. coli* were observed in sediment contaminated with dairy cow and deer faeces at 18°C relative to 4°C, whereas sediments contaminated with goose faeces showed no distinction in *E. coli* concentrations between temperature treatments over the period of study (Fig 4.4).

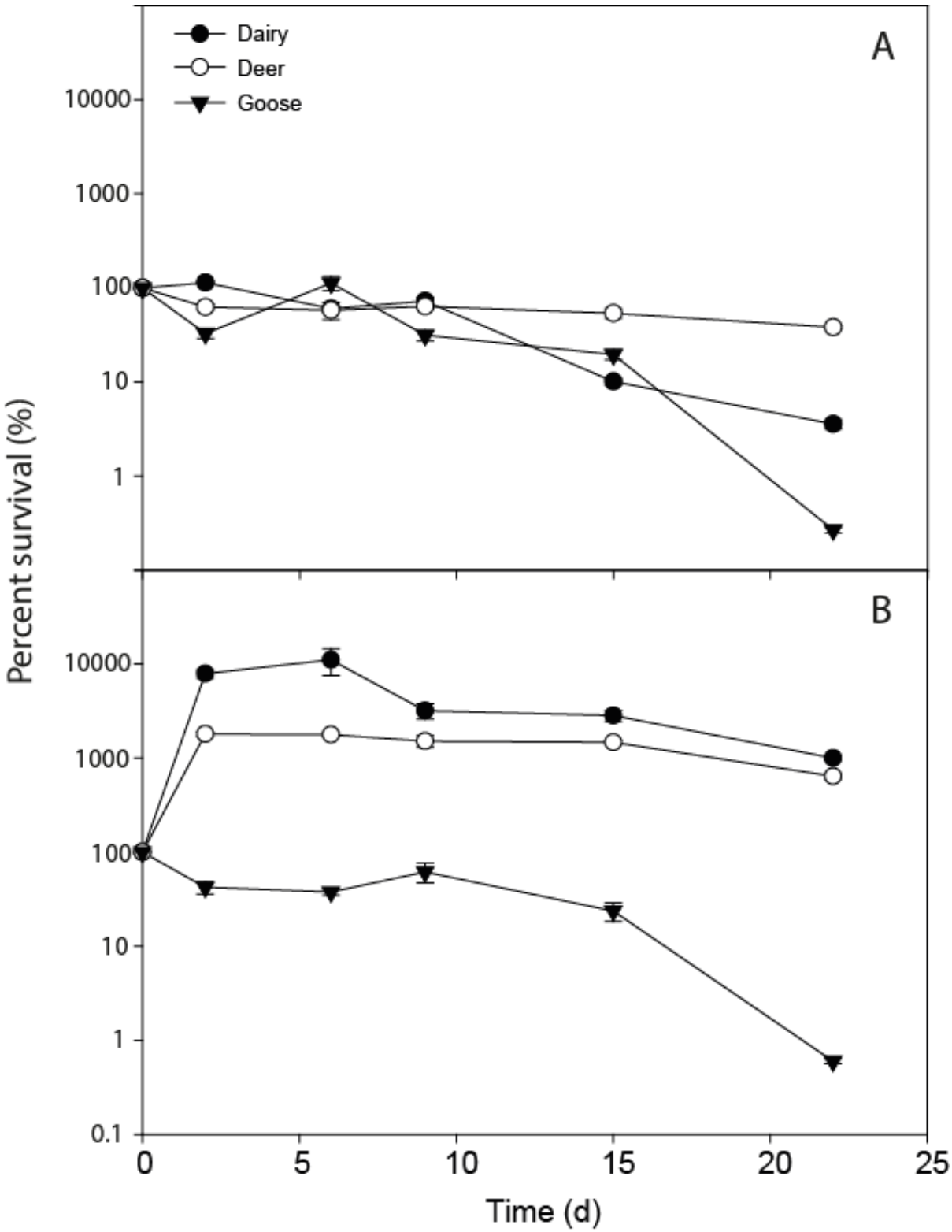

Figure 4.3: *Percent survival curves of E. coli in streambed sediment contaminated with dairy cow, red deer and greylag goose faeces and incubated at 4°C (A) and 18°C (B). Data points are the mean of four replicates ± standard error.*

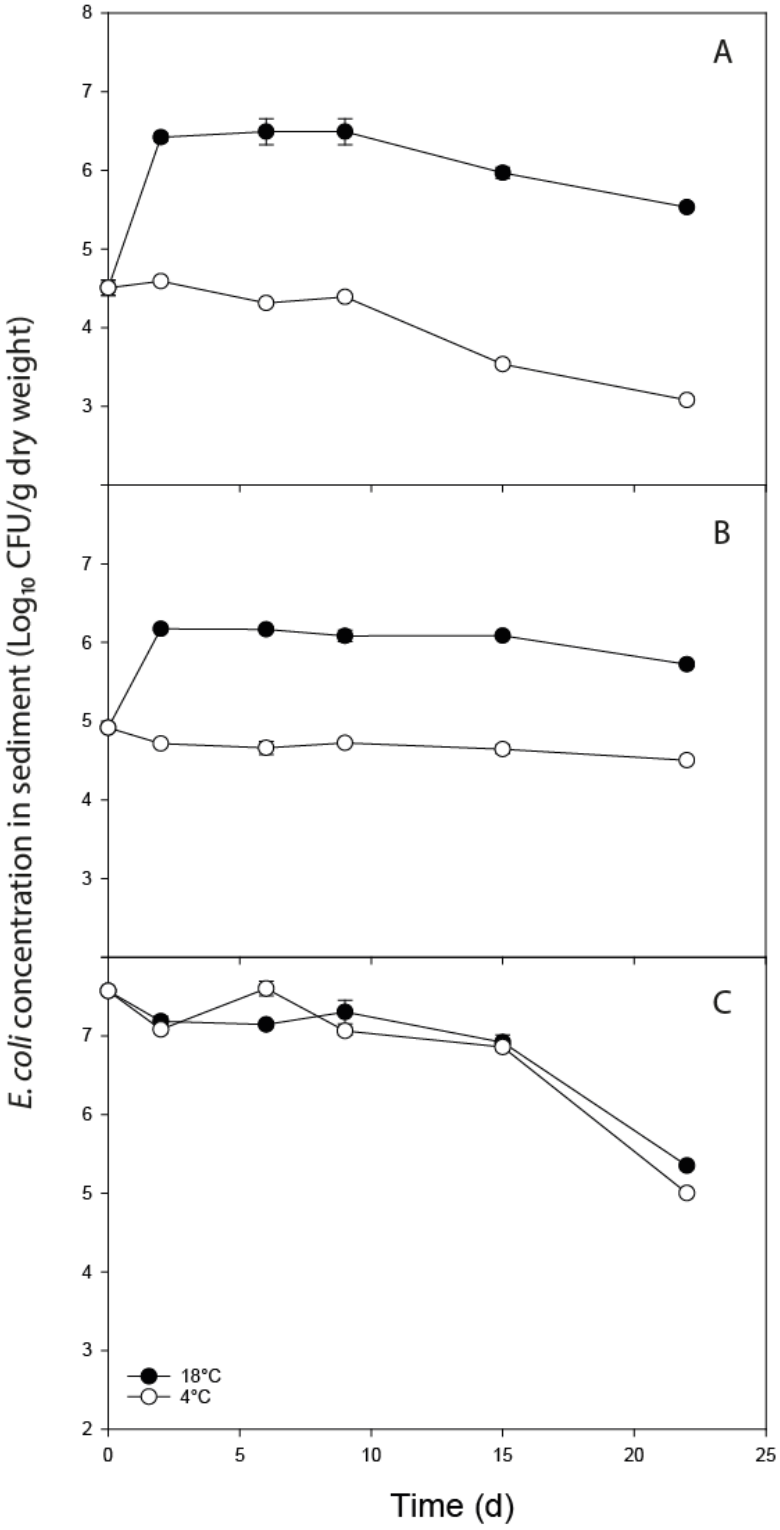

Figure 4.4: *Persistence profiles in streambed sediment of E. coli sourced from A) dairy cow B) red deer and C) greylag goose faeces. Data points are the mean of four replicates ± standard error.*

Linear regression was performed on the decline phase of the persistence profiles to model the die-off of *E. coli* across the different sediment treatments (Table 4.2). For those treatments that experienced regrowth, linear regression was initiated once the *E. coli* population began to decrease. Modelled decay constants were lowest for deer faeces and highest for goose faeces, with all three faecal types supporting significantly different rates of decline ($P<0.001$). Temperature did not significantly influence the modelled rates of decline ($P > 0.05$).

Table 4. 2. Linear model parameter values for E. coli decay in sediment contaminated with different faecal types.

	Die-off phase coefficients								
Treatment	**Dairy**			**Deer**			**Goose**		
	k (day^{-1})	R^2	*D*-value (days)	*k* (day^{-1})	R^2	*D*-value (days)	*k* (day^{-1})	R^2	*D*-value (days)
18°C	0.105	72.9	21.9	0.048	68.3	48.0	0.193	73.8	11.9
4°C	0.164	89.2	14.0	0.032	53.3	72.0	0.230	73.7	10.0

k = linear decay constant = 2.303 x Figure 2 modelled gradient of decline R^2 model fit *D*-value = time necessary for 90% inactivation = 2.303/*k.*

4.6.2 Complementary data to support experiment

The particle size composition of the streambed sediment used in this experiment is shown in Table 4.3. Silt dominated the sediment composition (70.6%), with clay and fine sand representing the other main constituents, albeit at much lower proportions (13.8% and 13.7%) respectively.

Table 4.3. Particle size composition of streambed sediment.

Particle class	Particle size fraction (mm)	Particle subclass (mm)	% Particle size
Clay	<0.002		13.80
Silt	0.002-0.06		70.54
Sand	0.06-2.0	fine 0.06-0.2	13.73
		medium 0.2-0.6	1.93
		Coarse 0.6-2.0	0.00

The faecal material from the three faecal sources was artificially mixed with this sediment. To understand better how faecal material would dissipate through the water column and accumulate in the streambed sediment an additional experiment was conducted to infer the rate of sedimentation of faecal material delivered to water. The changes in turbidity values of the faecally contaminated water (with associated standard error) over time are reported in table 4.4 and the resulting sedimentation rates as inferred from changes in turbidity, normalised to a percentage change over time, are shown in figure 4.5.

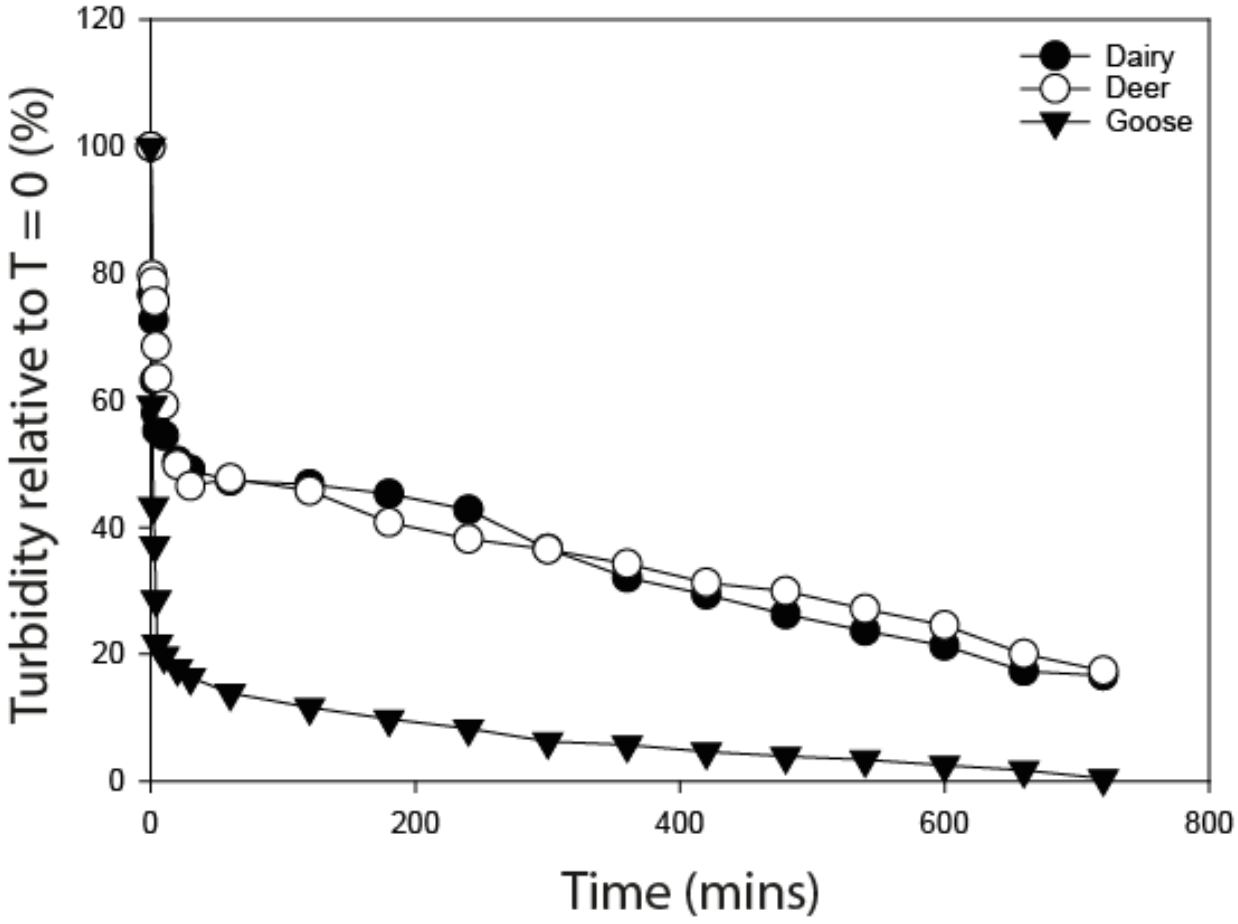

Figure 4.5: *Sedimentation rate as measured by percentage change in turbidity over time.*

Table 4.4. Turbidity (NTU) values over time for each of the faecal treatments.

	Turbidity (NTU)					
	Dairy faeces		***Deer faeces***		***Goose faeces***	
Time (mins)	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
0	9316.7	455.7	9926.7	27.3	6756.7	636.1
1	7146.7	81.7	7916.7	38.4	4006.7	327.5
2	6773.3	271.7	7810.0	268.5	2923.3	86.7
3	5886.7	128.4	7506.7	146.2	2510.0	90.7
4	5406.7	69.8	6803.3	236.9	1933.3	167.6
5	5146.7	33.3	6310.0	17.3	1460.0	35.1
10	5073.3	82.1	5886.7	349.2	1333.3	16.7
20	4700.0	65.1	4943.3	58.4	1200.0	37.9
30	4570.0	63.5	4613.3	172.3	1103.3	21.9
60	4406.7	17.6	4736.7	12.0	936.7	31.8
120	4356.7	82.5	4540.0	105.4	783.3	46.7
180	4216.7	18.6	4046.7	92.4	660.0	10.0
240	3983.3	101.7	3786.7	34.8	560.0	28.9
300	3410.0	258.9	3623.3	60.6	422.5	11.8
360	2986.7	173.7	3396.7	59.0	384.9	5.9
420	2736.7	49.8	3100.0	61.1	311.5	7.1
480	2446.7	80.1	2970.0	35.1	263.9	15.7
540	2203.3	103.7	2690.0	70.0	228.3	21.2
600	1986.7	145.3	2436.7	78.0	168.8	10.5
660	1613.3	240.4	1990.0	236.9	116.5	10.7
720	1546.7	147.2	1726.7	312.1	29.5	6.5

Table 4.4 highlights the lower starting turbidity associated with the goose faeces treatment. Normalising the turbidity data to percentage changes over time relative to the starting turbidity therefore provides a more meaningful visualisation of how patterns of sedimentation linked to the three faecal types differ (Fig 4.5). Patterns of sedimentation of the faecal constituents from dairy and deer faeces match very closely to each other. By contrast, goose faeces were observed to record much faster sedimentation times, reducing to ~ 20% of the original turbidity within 10 minutes, whereas a similar reduction in turbidity for dairy cow and deer faeces required over 600 and 660 mins, respectively. Despite the lower starting turbidity associated with goose faeces, the rate of change in the clarity of the water is evidenced by the more rapid changes in recorded NTU values too; the goose faeces treatment drops by a magnitude of 3833 NTU between 0 and 2 minutes, with dairy and deer faeces recording a drop of 2543 NTU and 2116 NTU, respectively, over the same time period.

4.6 Discussion

Legacy stores of faecal pollution in streambed sediments can result in further subsequent delayed impacts on environmental quality and human health if resuspended into the overlying water column (Holcomb and Stewart, 2020). Characterising how different sources of faecal pollution can contribute to the legacy store of FIOs is therefore important for improved targeting of management advice and mitigation. Farming and wastewater treatment can be key contributors to faecal pollution, but there is recognition that wildlife and wildfowl activity in catchments can link to elevated FIO concentrations, a proportion of which will settle and persist in sediment stores (Jeong et al., 2019). This chapter provides new evidence to improve understanding of the potential risks posed by sediments when contaminated with wildlife and wildfowl faeces. The persistence patterns of *E. coli* in streambed sediment were found to vary as a function of faecal source and temperature. Fresh goose faeces accommodated the highest concentrations of *E. coli*; however, this faecal source also experienced the largest drop in concentration over the experiment duration, which also reflected the most rapid die-off rate relative to deer and dairy cow faeces. Temperature did not significantly influence *E. coli* die-off rates, but it did play a role in shaping patterns of survival because the warmer treatment was associated with regrowth of *E. coli* in sediments contaminated with both deer and dairy cow faeces. This led to distinctly different concentrations of *E. coli* over time supported at 18 ^{o}C versus 4^{o}C for these faecal types, but the temperature-driven response was not mirrored in sediments contaminated with goose-derived *E. coli*. The goose faeces also differed from dairy cow and deer faeces with respect to the recorded speed of settling of faecal particles in a water column, suggesting a more efficient delivery of *E. coli* to streambed sediments would occur when faecal material from geese enters a waterbody.

Differences in *E. coli* concentration in fresh faeces excreted by livestock, wildlife and wildfowl are not unexpected and studies have reported variability in *E. coli* shedding across different sources (Jeamsripong et al., 2019). Differences in the initial concentration of *E. coli* likely reflect the diet associated with deer, dairy cows and geese and also reflect the digestive tract characteristics and likely the cross-contamination from exposure to other animals in their habitats (Biswas et al., 2016). The dairy cow and deer faeces used in our study were collected from a working dairy

farm and a deer park, respectively, where the animals were exposed to formulated feeds in addition to pasture. By contrast, the greylag goose population included a migratory and resident population that are much more free-roaming and largely unexposed to a managed diet. The high initial concentration of *E. coli* in goose faeces was consistent with previous studies (Meerburg et al., 2011; Moriarty et al., 2011; Moriarty et al., 2012). Experiments that use faeces as a natural carrier of indigenous FIOs to contaminate environmental matrices and then compare FIO survival responses across treatments provide an alternative to experiments that inoculate a known quantity of cells to a range of treatments. The former can make the assessment of the subsequent survival patterns more challenging because of uncertainties in how variation in starting concentrations of cell numbers may be propagated through the survival response, but such an approach is more reflective of real word scenarios and different experimental approaches offer different types of insight, provided strengths and limitations are recognised (Oliver et al., 2016). In our study, the percent survival plots help to reveal patterns in persistence after normalising for those initial differences in *E. coli* concentration.

The physical integrity of the different faecal matrices was lost through their combining with the streambed sediment. It is therefore difficult to suggest that differences in physical structure of the faeces played a role in determining the persistence patterns but the nature of the particles the faeces contain would differ and would persist when combined with sediment. As discussed, the starting concentrations did differ and perhaps this was responsible for the more rapid *E. coli* die-off in the sediments contaminated with goose faeces, which after 22 days reached a concentration equivalent to the starting concentration of the sediments contaminated with deer and dairy cow faeces. There is evidence to suggest that experiments that use higher starting concentrations of FIOs are likely to record more obvious die-off than those experiments that use lower starting concentrations (Kiefer et al., 2012; Hodgson et al., 2016). An alternative approach would have been to mix the sediment with different volumes of faeces to ensure the same FIO loading across all treatments; however, doing so would result in different ratios of in the faeces:sediment mix, which itself could influence the survival response of the FIOs because of differences in nutrient supply to the bacteria (Zheng et al., 2019).

The survival and growth of *E. coli* in the environment has been attributed to the influence of temperature (Petersen and Hubbart, 2020). In our study, the warmer (18°C) temperature treatment supported higher concentrations of *E. coli* over time relative to the lower temperature treatment (4°C). Temperature is recognised as an important factor that controls the survival of microorganisms in the environmental matrices, especially enteric bacteria excreted from warm-blooded animals' guts (Oliver & Page, 2016). While warmer temperatures supported regrowth of *E. coli* in the sediment contaminated with dairy and deer faeces, no significant regrowth was seen in *E. coli* held in sediment at 4°C for all faecal sources. The lack of regrowth of *E. coli* in goose faeces may be attributed to strain and genotype differences of *E. coli* in the faecal matter (Jang et al., 2017). The difference in *E. coli* response at different temperatures is clear when comparing each faecal treatment individually, e.g., the differences observed for temperature effects in dairy faeces and in deer faeces, because both temperature treatments for each respective faecal source started with the same *E. coli* concentration on day 0. Although the influence of temperature in itself is not a novel finding, the *E. coli* growth rate and magnitude of increase recorded for both faecal sources is still substantial and provides important evidence of how FIOs can increase in environmental matrices under varying conditions and provides information to support FIO fate and transfer modelling (Oliver et al., 2016). The lack of regrowth in all faecally-contaminated sediments at lower temperatures is probably due to a reduction in the metabolic process of *E. coli* in the environmental matrices (Guber *et al.*, 2015). Consequently, some cells may have experienced mechanical damage to their cell structure or entered a viable-but-non-culturable (VBNC) state (Wang et al., 2019), limiting opportunities for cell replication.

The inactivation rate of *E. coli* in the environment can be influenced by the sediment composition, which links to particle size and the organic matter content although the role of different sediment characteristics in FIO survival is not straightforward and interacts with other environmental factors (Kiefer et al., 2012; Pachepsky and Shelton 2011; Garzio-Hadzick et al.; 2010). In this chapter, only one type of sediment was used because the focus of the research was to determine the influence of different faecal types spanning livestock, wildlife and wildfowl sources combined with influences

of temperature. An investigation into whether the persistence patterns recorded for this sediment composition hold across other sediment types dominated by sand or clay fractions would be important to further support the evidence base of how different wildlife and wildfowl sources of FIOs persist in the environment. This would help to refine risk assessments of landscapes frequented by large wildlife and wildfowl populations by identifying the factors that combine to generate legacy FIO hotspots in stream and river networks.

Rates of FIO accrual in bottom sediments are governed by their attachment to particles (Wyness et al., 2019). Although some FIOs will enter a waterbody as freely suspended cells, a large proportion will be associated with mineral or organic particles. Those FIOs that have attached or remain associated with physical material will settle out into underlying bed sediment relatively faster than free floating FIOs (Wu et al., 2019). This is because the rate of settling of suspended particles depends on the mass of the particles (Auer and Niehaus, 1993). This chapter focused on quantifying persistence over time of FIOs in sediments contaminated with different faecal sources, but a secondary aim was to identify whether the different faecal sources would influence the rate of faecal material (and by association FIO) delivery to the streambed sediment. The constituent parts of goose faeces, when mixed with water, were found to settle at a more rapid rate than those associated with deer and dairy faeces. Diet again, as discussed earlier, probably influences the composition of the faecal material and will dictate to some extent how the faecal material disaggregates and settles through a water column (Kotz et al., 2021). All faecal types were fresh, but the goose faeces had marginally higher (< 0.5% difference) and higher (~ 6% difference) moisture content than the dairy cow and deer faeces, respectively. This would suggest that the drier the faecal matter, the more likely the faecal particles are to remain in suspension in the water column for a longer duration. However, the results of this chapter cannot conclude whether dry weight or faecal type is the factor driving the rate of settling. Further research is needed to investigate the settling rates of different faecal types across a spectrum of recorded dry weights, which would reflect different ages of faeces too.

4.6 Conclusion

Characterising how *E. coli* from different catchment sources survive in streambed sediment under varying temperature regimes can help catchment managers and environmental regulators understand the potential for faecal pollution following resuspension of legacy FIO stores. The concept of delayed impairment of water quality from legacy phosphorus is well recognised, but equally other pollutants, such as FIOs, can accumulate in catchment stores and cause rainfall-independent water quality impacts if disturbed, e.g., by recreational water users or livestock activity in water courses, and high flow impacts following sediment resuspension. This study did not use flowing water chambers such as those used by Kiefer et al (2012) and Smith et al (2019), instead the mesocosm design reflects a shallow water depth above the sediment; however, the lack of flow would have physical effects as well as impacts on oxygenation. There were some other limitations in that no sediment/faecal chemistry was undertaken and microcosms were capped, which would have some influence on aeration / anaerobicity. Despite these potential limitations, findings in this chapter underscore the importance of warmer temperatures in promoting higher concentrations of *E. coli* in sediments contaminated with deer and dairy cow faeces, which are then likely to result in hotspots of potential legacy pollution. The dynamic nature of FIO die-off means that these hotspots may have time-limited risk periods that respond to temperature influences on survival. Further laboratory research and field quantification on survival of wildlife and wildfowl derived *E. coli* in different sediment types and catchment settings through contrasting seasons will deliver further evidence to support our knowledge of other non-agricultural and non-human FIO pollution sources in catchments.

5. Stakeholder perceptions of wildlife-derived microbial pollution in rural catchments

Abstract

Deterioration of freshwater quality can be linked to agricultural and wastewater management practices. However, other sources of faecal pollution include remobilisation of legacy sources from streambed sediments and localised hotspots from poorly maintained and leaking septic tanks. Wildlife and wildfowl can also serve as an important source of microbial water pollution and contribute to the spread of antimicrobial resistance (AMR) genes in agricultural settings, but a lack of quantitative data on lesser known sources to confirm the relative differences between agriculture, wastewater and wildlife with regard to pollution loading can make it difficult to convince sceptical stakeholders about the sources of a faecal pollution incidents and the importance of managing the sources in the environment. Soliciting views of different catchment stakeholders can provide alternative insight into how sources of microbial pollution and the spread of AMR in agricultural catchment are perceived. The aim of this chapter was to use a survey-based approach to investigate how different stakeholders perceive the potential for wildlife to impact on microbial water quality and contribute towards spreading of AMR to the water environment. In general, stakeholders perceived that wildlife contribute to microbial watercourse pollution but to a lesser extent relative to other sources such as agricultural practices and wastewater discharge. Runoff from agricultural landscape and domestic waste effluent were described as major contributors to evolution and dissemination of AMR in the environment by stakeholders. Appreciating and integrating the perception of different stakeholders as expert data into the policy making process to protect water quality from microbial pollution can play an important role for improved management of surface water and help to reduce conflict among agricultural catchment stakeholders and facilitate co-construction of effective solutions.

5.1 Introduction

Microbial water quality can be compromised by a variety of catchment sources. Common contributors to faecal pollution include agricultural practices, e.g. grazing livestock and manure applications to land (Stoyanova and Harizanova, 2019; Kumar et al., 2021) and human wastewater effluent discharged from sewage treatment plants (Camara et al., 2019; Rebi et al., 2021). The importance of agricultural and human sources of faecal pollution varies depending on catchment characteristics such as land use and population density and environmental factors such as rainfall, which influences runoff and therefore base and high flow conditions in receiving waters (Karlsen et al., 2019).

However, other sources of faecal pollution include remobilisation of legacy sources from streambed sediments, localised hotspots from poorly maintained and leaking septic tanks and also contributions from wildlife and wildfowl. The importance (and resulting impacts on water quality) of these sources relative to agricultural or sewage treatment plant contributions is relatively unknown, although studies have reported on risks associated with all of these sources (Afolabi et al., 2020; Li et al., 2021; Nwugha et al., 2021). Environmental regulation of microbial water quality, inferred using faecal indicator organisms (FIOs) links strongly to the management of agricultural and wastewater sectors (Gilfillan et al., 2018), but increasingly there are questions over the risks from wildlife and wildfowl, and in the absence of quantitative data to confirm the relative differences between agriculture, wastewater and wildlife it can be difficult to convince sceptical stakeholders about the origins of a faecal pollution incidents and the importance of managing particular sources in the environment.

There is growing interesting in AMR in the environment (e.g., Avery et al., 2022) and there remains much uncertainty in terms of relative risk of different sources promoting the dissemination of AMR in the environment (Polianciuc et al., 2020). AMR is pertinent to faecal pollution because it is a potential key source with the environment providing a ‘melting pot’ for AMR proliferation and persistence. The perception of the importance of wildlife and wildfowl in contributing FIOs and AMR genes to receiving waters is likely to vary across different stakeholder communities, but little research has explored how these views may differ with respect to the impacts on waterbodies. Previous chapters in this thesis have so far contributed new evidence to support understanding of how wildlife and wildfowl can contribute to the first three components

of the source-mobilisation-delivery-impact (SDMI) transfer continuum with respect to FIO pollution. This final data chapter now considers the final stage in this continuum by soliciting the views of different stakeholders about the perceived 'impact' of wildlife on microbial water quality in rural catchments.

The views and perspectives of different catchment stakeholders are likely to vary because of differences in levels of experience and knowledge that they have gained through their respective roles. It is well recognised that different stakeholder 'typologies' are likely to accommodate different world views and accrue different types of knowledge depending on their role and remit (e.g., Stosch et al., 2019). In seeking to determine stakeholder perceptions of the role of wildlife in contribution to potential water quality issues it is important to recognise a spectrum of expertise that can be consulted. For example, research scientists and academics who work in the area of soil and water science and environmental pollution, and who have specifically investigated microbial pollution, will likely have an understanding of the published evidence-base on this topic, however limited it may be. Those with a responsibility for regulating the environment are more likely to have anecdotal evidence of on-the-ground observations from their catchment investigations. Farmers are also a key stakeholder group and need to be valued as a source of expert data; farmers and land managers are expert observers of landscape processes and provide diverse information on land use practices and underpinning attitudes towards risk (Oliver et al., 2010). Likewise, the farm advisor community and water industry provide an additional source of knowledge concerning land and water quality challenges faced in agricultural settings.

Soliciting the views of different stakeholder groups can be achieved using a variety of different methods. In-depth qualitative research approaches often deploy interviews to obtain a rich transcript of perspectives from key individuals, e.g.(Haan et al., 2017; Ongena and Dijkstra, 2021). Such approaches can be time intensive and focus on depth of detail from fewer participants. Alternative approaches include the use of focus groups, whereby a panel of different stakeholders can engage in a facilitated discussion and debate around particular questions of interest. This approach enables the possibility of further insight drawn out through conversation across multiple

stakeholders, but the method can be limited if a particular member of the focus group panel tends to dominate the conversation. A more common approach to solicit views and perspectives is through the deployment of a questionnaire or survey. Survey-based approaches allow for larger sample sizes to be obtained and are often regarded as a more rapid and efficient approach to data collection (Ball, 2019; Sturgis and Luff, 2021). In contrast to an interview approach, the data is less rich and detailed, but the advantage is that a greater volume of perspectives and views can be sought through a wider programme of survey dissemination.

Therefore, the overarching aim of this chapter was to use a survey-based approach to investigate how different stakeholders perceive the potential for wildlife to impact on microbial water quality and contribute towards spreading of antimicrobial resistance to the water environment. The specific objectives were to (i) quantify differences in stakeholder (e.g., farmer, farm advisor, regulator, environmental scientists) perceptions of wildlife threats to microbial water quality in agricultural catchments; and (ii) understand stakeholder perceptions of wider issues of microbial pollution and AMR-related challenges to water quality in catchments in general.

5.2 Materials and Methods

5.2.1 Overview

An UK-wide online survey was developed to elicit perceptions from across four key stakeholder groups with experience of water quality issues in rural catchments. The four stakeholder groups were: (i) farmers; (ii) farm advisors; (iii) environmental regulators; and (iv) researchers in land and water management (academics). The online questionnaire was designed using the JISC ‘Online Surveys’ software (https://www.jisc.ac.uk). The questionnaire was developed in two formats; one for the advisors, regulators and academics (stakeholder questionnaire) and another specifically targeted at the farmer community (farmer questionnaire). The ‘stakeholder’ questionnaires comprised 12 questions and the ‘farmer’ questionnaire 14 questions. Many sections of the two questionnaires were the same, but the farmer questionnaire sought specific information relating to the farm environment managed by the farmer. Stakeholders were invited to participate in the web-based survey, which was designed to collect information on their own perceptions of the risk posed to surface waters by

faecal pollution from deer, geese and gulls. The survey asked questions about their own experience and background, their views on different sources of faecal pollution in the environment and their opinions on factors that might influence the spread of AMR to the water environment. A range of different question styles were used in the survey, including five-point Likert scale questions, multiple-choice questions, open-ended questions and questions seeking views of how stakeholders apportioned their levels of importance to particular issues (in percentage terms). Where relevant, the option of 'prefer not to say' was included as a response. The Likert scale was used for its simplicity and ability to measure a series of attitude-related propositions and to use non-parametric tests such as Chi-Square (cross-tabulation) for statistical analyses (Chyung et al. 2017). Please refer to the appendix for the full list of questions used

5.2.2 Recruitment

Stakeholders who were 18 years old or above and resided in the UK were invited to participate in the research. A link to the questionnaire was shared through direct email solicitation and via distribution on social media as well as via interest groups and stakeholder organisations. Invitations to participate in a short survey were sent to targeted individuals who were known in the relevant catchment microbial dynamics community as researchers, regulators or farm advisors. In addition, requests were sent to key organisations asking for the survey link to be distributed in member newsletters or via social media. Groups contacted included the British Cattle Veterinary Association (BCVA), Sheep Veterinary Society, British farming forum, National Farming Union, West Cumbria Rivers Trust, Lune River Trust and Ribble River Trust.

The questionnaire was available for completion from 10th of December 2020 to 10th of May 2021 Ethical approval for the survey was obtained via the University of Stirling General University Ethics Panel (GUEP).

The survey was distributed electronically rather than face-to-face in order to maximise survey return rates and due to remove geographical, financial and time-related barriers to participation in the study. Furthermore, some COVID-19 regulations were ongoing in Scotland, making face-to-face survey techniques logistically challenging.

5.2.3 Analysis

The responses from all participants were arranged into the four stakeholder groups (academics, environmental regulators, farmers, and farm advisors) prior to further analysis. Many questions required calculation of a percentage response rate for the different response options. Where appropriate, descriptive data analysis was performed on some of the responses to determine the mean and standard error (e.g., when stakeholders were asked to apportion values to particular pollution sources). Pearson's Chi-Squared Test of Association was used to analyse the association between the stakeholders' responses to different questions. Chi- square significance thresholds were set at $P < 0.05$. Following on from Chi-square tests, Cramer's V analysis (represented by φ_c) was performed for any statistically significant associations to determine association strength. Cramer's V thresholds for association strength were classified as: Very Strong >0.25, Strong >0.15, Moderate >0.10, Weak >0.05, Very weak >0 (Akoglu, H. 2018). Minitab 18.0 software, Minitab Inc.; State College, PA, USA was used to determine the association between the factors while SigmaPlot 13.0 was used to produced bar plot charts.

5.3 Results

It was not possible to report a response rate given the approach used for survey dissemination. The total number of responses received from the online survey was 60. Background information about the composition of the stakeholder responses is provided in Tables 5.1 and 5.2. Academics represented the largest stakeholder type (Table 5.1). In terms of geographic location of respondents, academics, regulators and advisors were mostly based in England whereas the farmer response rate was highest for Scotland (Table 5.2).

Table 5.1. Summary of stakeholder group composition.

Participants	**Number of participants**	**% of participants**
Academic	23	38.3
Regulator	17	28.3
Advisor	10	16.7
Farmer	10	16.7

Table 5.2. Geographic location of respondents by stakeholder type.

	Country in UK where participants reside		
	England (%)	**Scotland (%)**	**Wales (%)**
Academic	56.5	39.1	4.4
Regulator	58.8	35.3	5.9
Advisor	90	10	0
Farmer	20	70	10

All survey participants had self-reported levels of awareness of environmental issues that were considered average, or above (Fig 5.1). The majority of academics, regulators, and advisors indicated that they had ‘high’ levels of awareness of environmental issues (i.e., 60.9%, 64.7% and 60%, respectively), with the remainder of these groups (~35-40%) having above average awareness of the environment. Responses from the farming community were less confident in self-reported levels of environmental awareness, with the most common response being ‘above average’ as opposed to ‘high’ levels. Thus, 60% of the farmers claimed above average levels of awareness of environmental issue, 20% suggested ‘high’ levels and 20% had average understanding of the environment (Fig. 5.1).

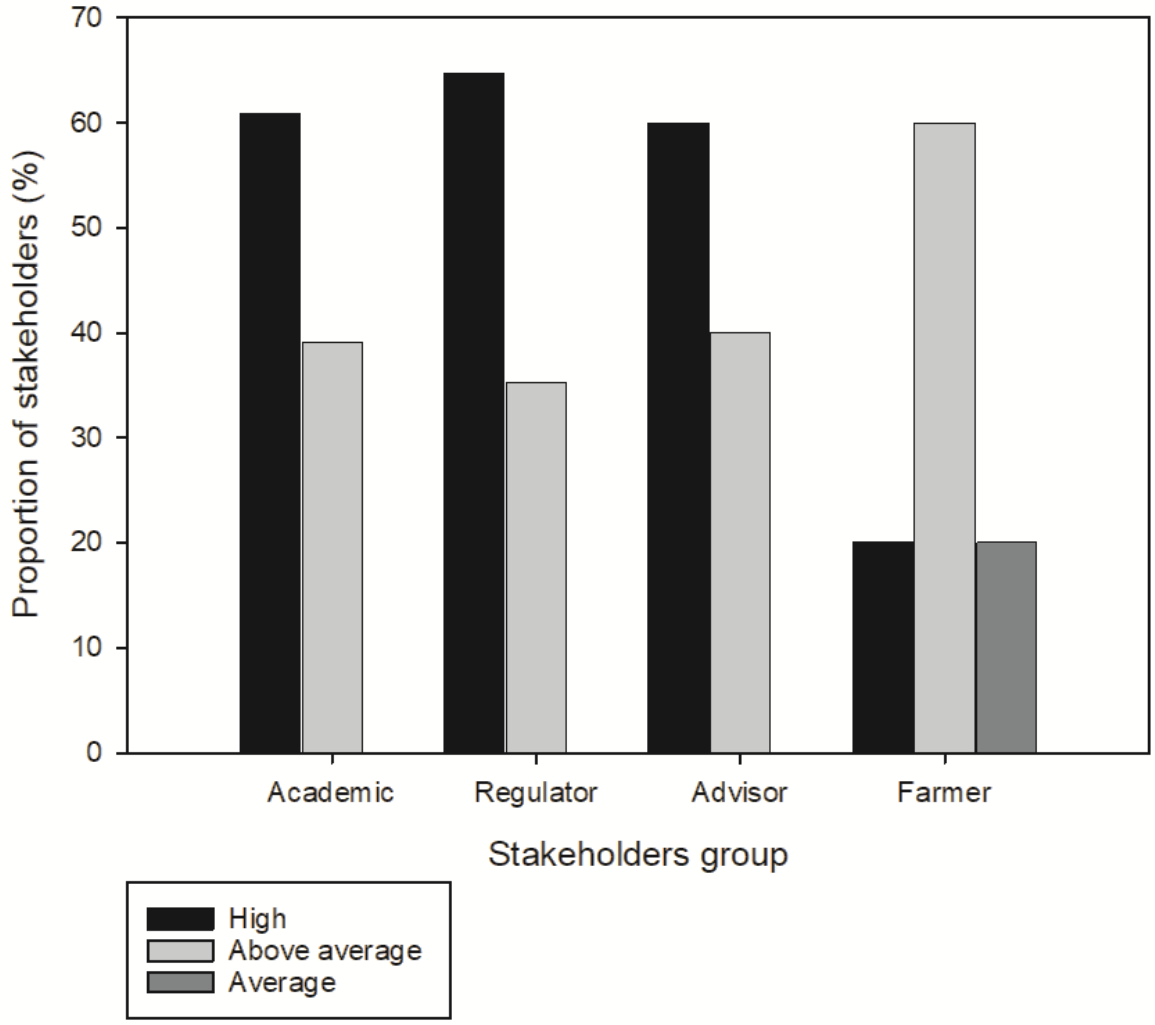

Figure 5.1: *Stakeholders' self-reported level of awareness of environmental issues.*

The academic, advisor and regulator stakeholders were asked for their perception on the importance of wildlife for contributing to microbial pollution in rural catchments relative to livestock and agricultural sources (Fig 5.2) (the farmer questionnaire did not address this question directly to avoid bias in later questions in the farmer-specific questionnaire). There was significant association between stakeholder type and perceived importance of wildlife contributions to microbial pollution ($P < 0.05$, $\varphi_c = 0.126$). Those stakeholders linked to the 'academic group (i.e. researchers, scientists) were more likely to view wildlife as an important contributor rather than neutral/not

important, which differed relative to the advisor and regulator responses. Indeed, 70% of academics registered a response of 'important' to this question, compared with only 45% and 40% of regulators and advisors, respectively (Fig. 5.2). There was no significant association determined between self-reported level of environmental awareness and perceived importance of wildlife to microbial pollution relative to agricultural sources (P = 0.08).

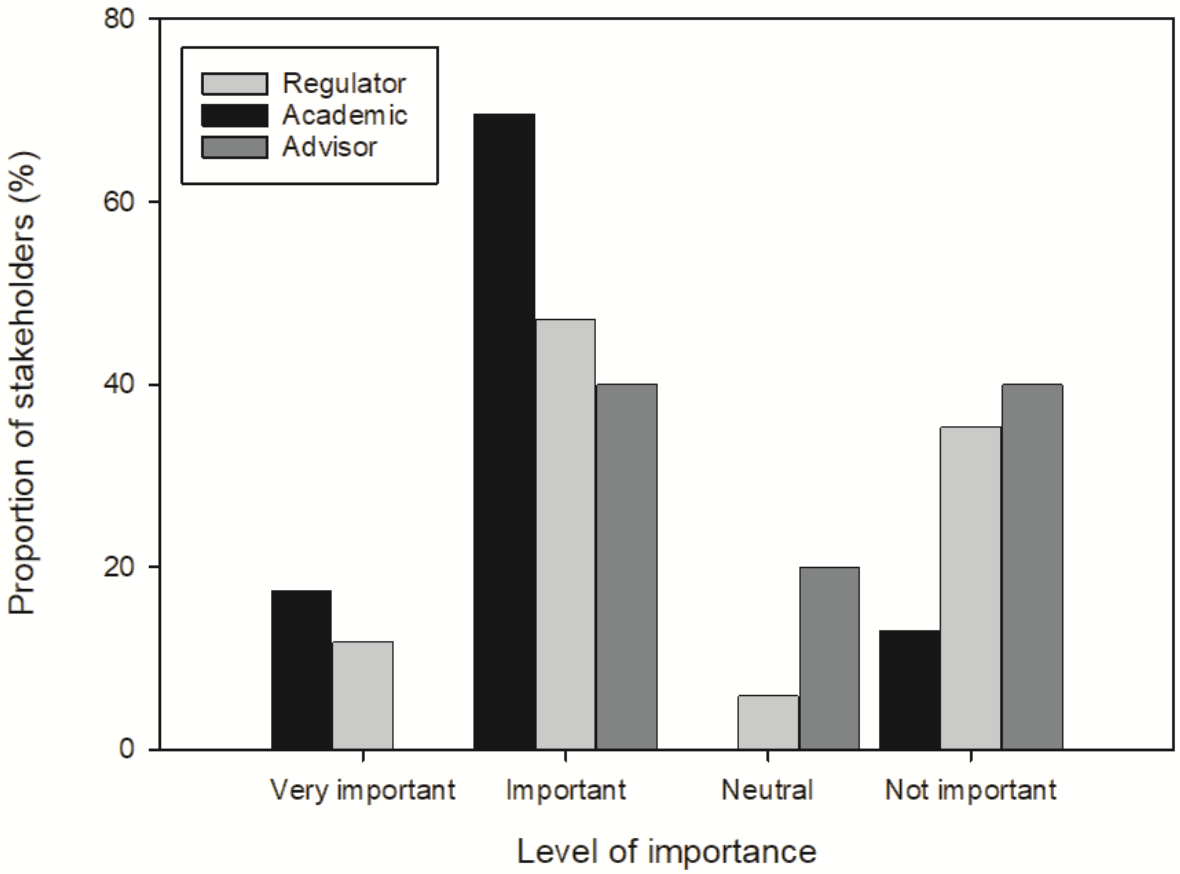

Figure 5.2: *Stakeholders' perception on the importance of wildlife for contributing to microbial pollution of water in rural catchments.*

Views were sought on the relative contribution of different catchment sources to microbial pollution in catchments, specifically contributions from: arable land, livestock & grazed pasture, wildlife, urban wastewater and other sources. The two dominant contributors were 'livestock and grazed pasture' and 'urban wastewater' (Figure 5.3). Farm advisors rated 'livestock and grazed pasture' the highest of all stakeholders, with

a 45% contribution. Regulators attributed a value of 36% of microbial pollution to ‘livestock and grazed pastures’, but overall considered urban wastewater to be a slightly larger contributor at 38%. All stakeholders considered contributions from arable land to be much lower and the wildlife contribution in general was slightly less than what the arable land in a typical catchment was perceived to contribute. Consistent with earlier questions about the relative contribution of wildlife to microbial pollution of surface waters, academics recorded a slightly higher contribution for wildlife (12.3%), compared to 9.9%, 11.6% and 9.3% contributions registered by the regulators, advisors and farmers, respectively. Responses from farmers included over a 16% contribution to microbial pollution from ‘other’ sources, although the nature of those other sources was not specified.

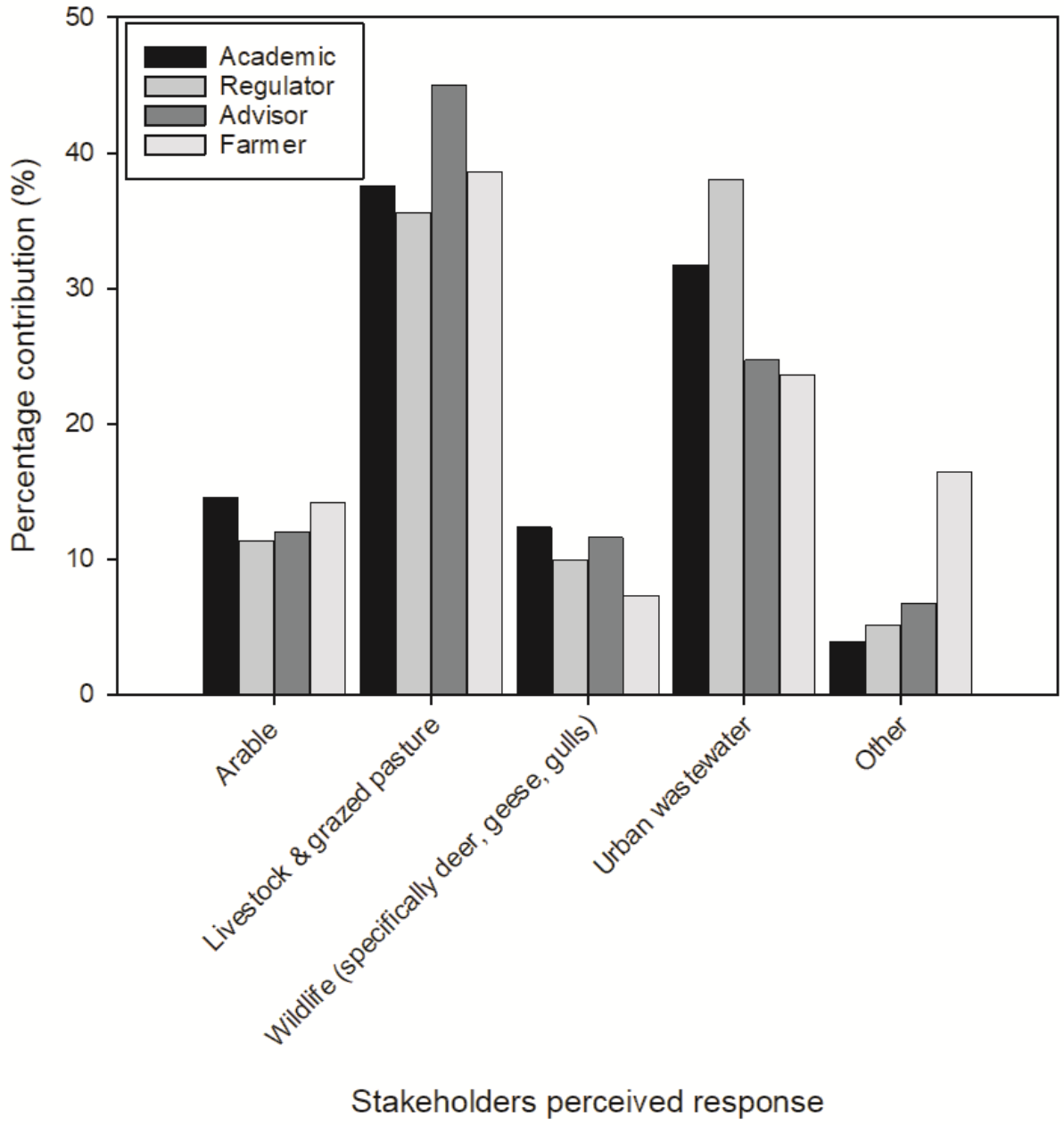

Figure 5.3. *Stakeholders' perception of relative contribution of different catchment sources to microbial water pollution.*

The importance of different environmental matrices (soil, water, air, faeces) in facilitating the dissemination of AMR in the environment was considered by the different stakeholders, with results from all stakeholders combined shown in Figure 5.4. Water and faeces emerged as the two environmental matrices that recorded higher responses as being 'very important' (44% and 59% for water and faeces, respectively) or 'important' (47% and 30% for water and faeces, respectively) for AMR dissemination. Responses on perceived importance of soil were more mixed, whereas the role of air for facilitating AMR dissemination was perceives as being 'not important' by most respondents.

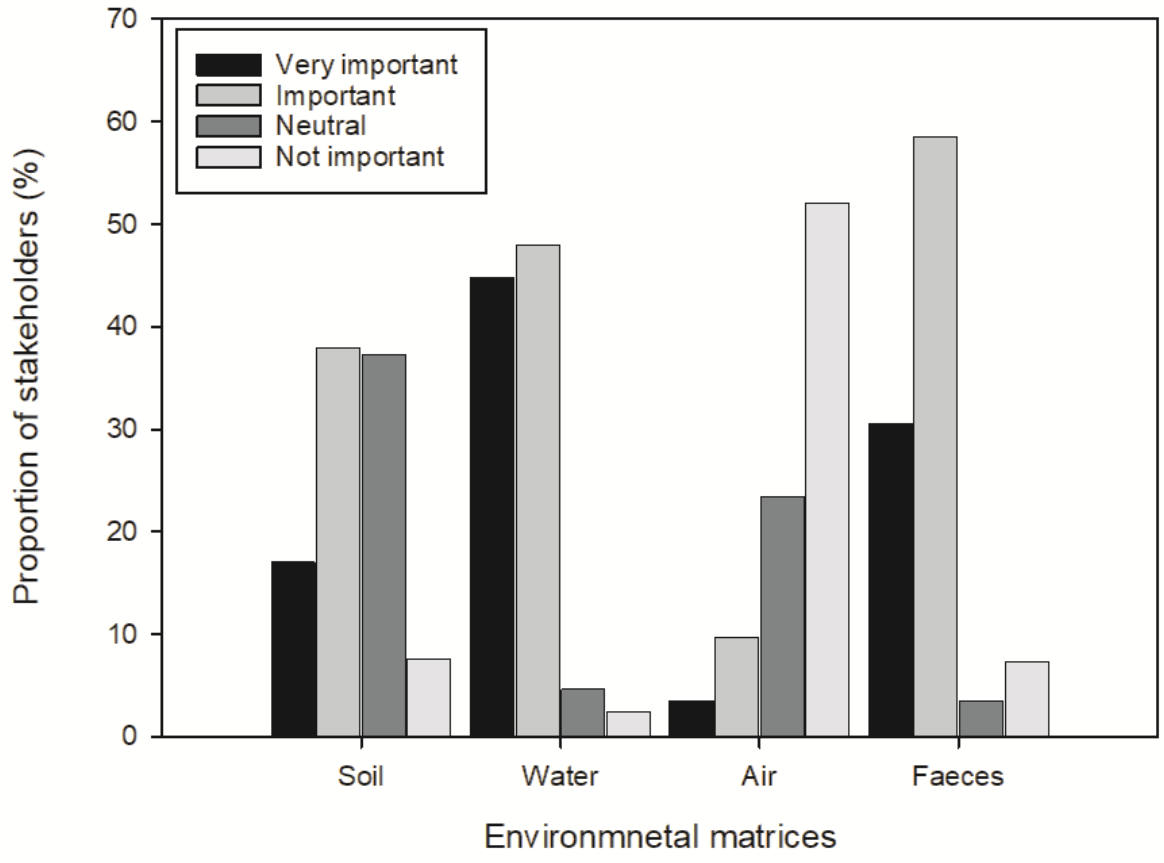

***Figure 5.4**: Perceived importance of different environmental matrices in facilitating AMR dissemination (all stakeholders combined).*

The perceived importance of each environmental matrix in facilitating AMR dissemination according to different stakeholders was also investigated. No significant associations between stakeholder type and perceived importance of different matrices were determined ($P > 0.05$). The breakdown of stakeholder perceptions on the importance of soil for AMR dissemination highlighted the majority of views across all stakeholders were associated either with an important or neutral response, rather than a very important or not important response (Fig. 5.5A). Some clear differences were apparent, for example a divergence of opinion between regulators and advisors on the ability of soil to disseminate AMR to the wider environment. While 52% of regulators considered soil as an important contributor of AMR to the surface water, 60% of advisors were neutral in their opinion (Fig. 5.5A). The breakdown of stakeholder responses to perceived importance of water, air and faeces in facilitating AMR are also provided in figure 5.5 B, C and D, respectively.

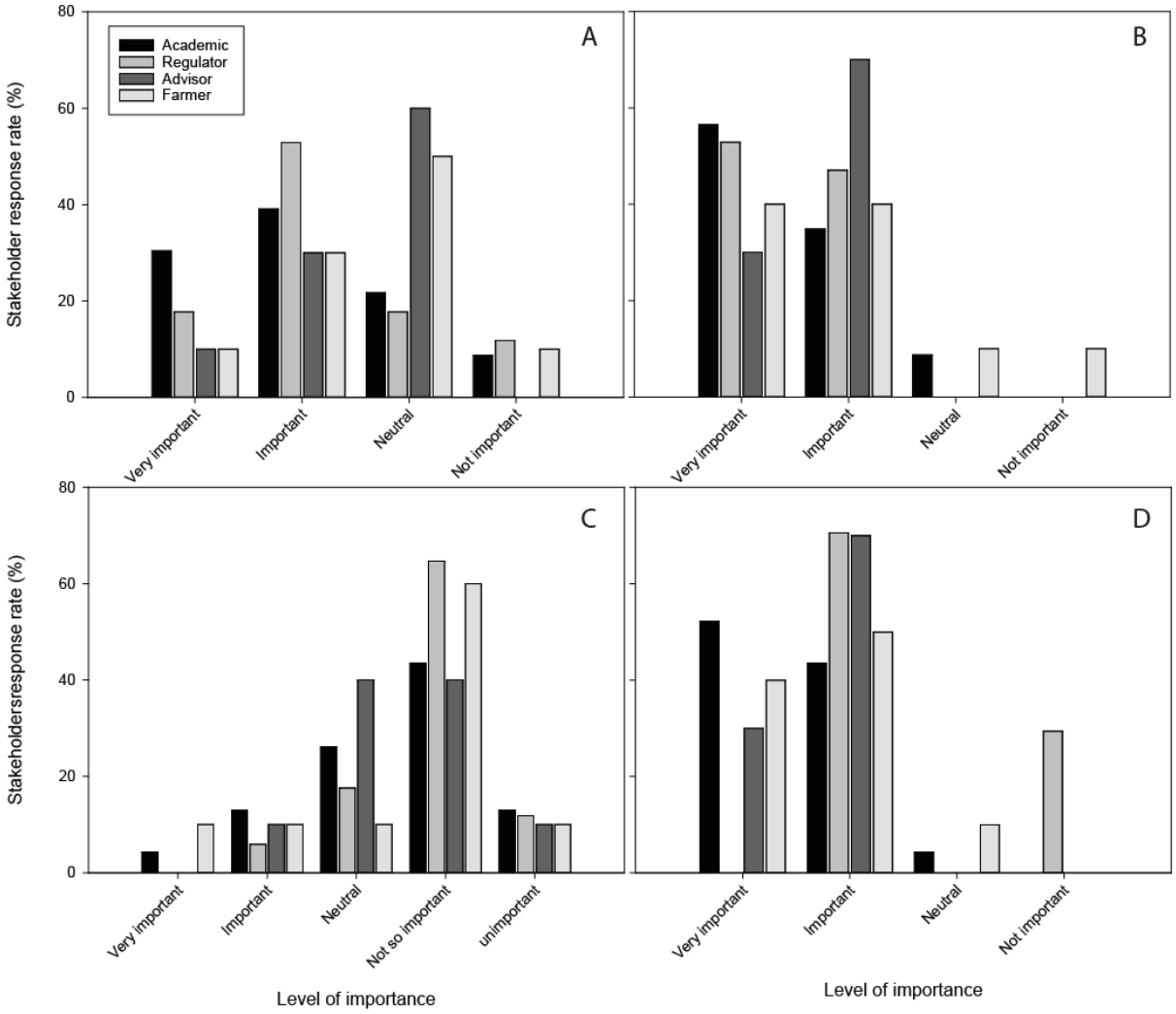

Figure 5.5: *Perceived importance of A) soil B) water C) air D) faeces as a facilitator of AMR dissemination according to different stakeholder groups.*

No significant association between stakeholder group and perceived importance of the agricultural sector in disseminating AMR to the environment was found ($P > 0.05$; Fig 5.6). In total, 70% of advisors considered the agricultural sector to play an important role in disseminating AMR, compared to 41% of regulators and 39% of academics (Fig. 5.6). The dominant view of academics and regulators was that the agricultural sector plays a very important role in disseminating AMR to the wider environment (47% and 52%, respectively).

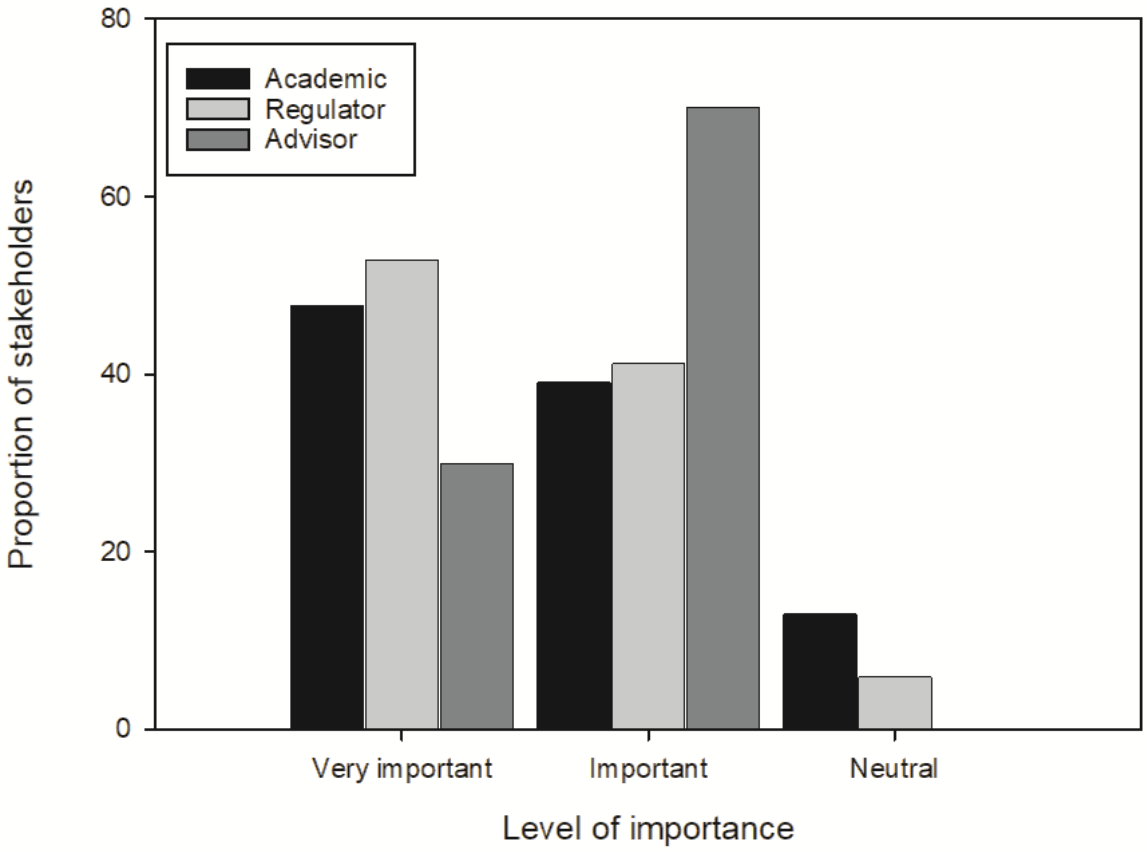

***Figure 5.6**: Perceived importance of the agricultural sector in the dissemination of AMR to the environment.*

Stakeholders were asked to explain their answer when considering the importance of the agricultural sector in disseminating AMR. Table 5.3 shows some of the common responses to this question.

Table 5.3. Selected quotes from respondents explaining perceived importance of agricultural sector for disseminating AMR to the wider environment.

Perceived importance of agricultural sector as a potential contributor for disseminating AMR to the wider environment	**Example responses to explain level of perceived importance**
Very Important	*"Administration of veterinary medicines and antibiotics to livestock: potential for wider dispersal into the environment from livestock wastes deposited/applied to land"*
	"You said "potential" - I don't think we know enough to say it isn't so it's "important" until we do"
	"The use of antibiotics in livestock rearing especially coupled with slurry spreading to land is one of the major sources of AMR in the water environment"
	Antimicrobial use, heavy metal 'supplements' in feed, manure application, feeding of antibiotic-laden milk to calves, defecation directly on-land leading to land runoff to water courses
	"Antibiotics in medical use is largely responsive but in livestock agriculture it is used in larger doses and in a preventative mode. Human waste is for the most part digested/composted at WWTW but less treated in the agric sector"
	"There is no clearly defined boundary between agricultural systems and the wider environment, this issue and the significant land coverage that agriculture represents make it crucially important in controlling the dissemination of AMR to the wider environment"
Important	*"Antimicrobial compounds, antimicrobial resistant microorganisms, and antimicrobial resistance genes have all been found in sewage sludge and farmyard manures and slurries that are applied to agricultural land and this pathway for transmission of AMR in the environment is widely recognised in the scientific literature"*
	"The use of antibiotics within agriculture has increased dramatically in the last 20 years, diffuse, or point sources of pollution from agriculture contain a wide variety of pollutants including antibiotic residues. Given the scale and intensification of farming in the UK, it is difficult to consider the agricultural sector as being anything but an important contributor to the dissemination of AMR. Clearly there are other contributors, which should be addressed"
	"Overuse of antibiotics in animal welfare and spreading of slurry on land containing bacteria which then get into the environment and food chain"

Again, there was no significant association between stakeholder group and perceived level of importance of AMR as a global environmental issue. The majority of all stakeholder groups identified AMR to be either a very important or important global issue. (Fig. 5.7).

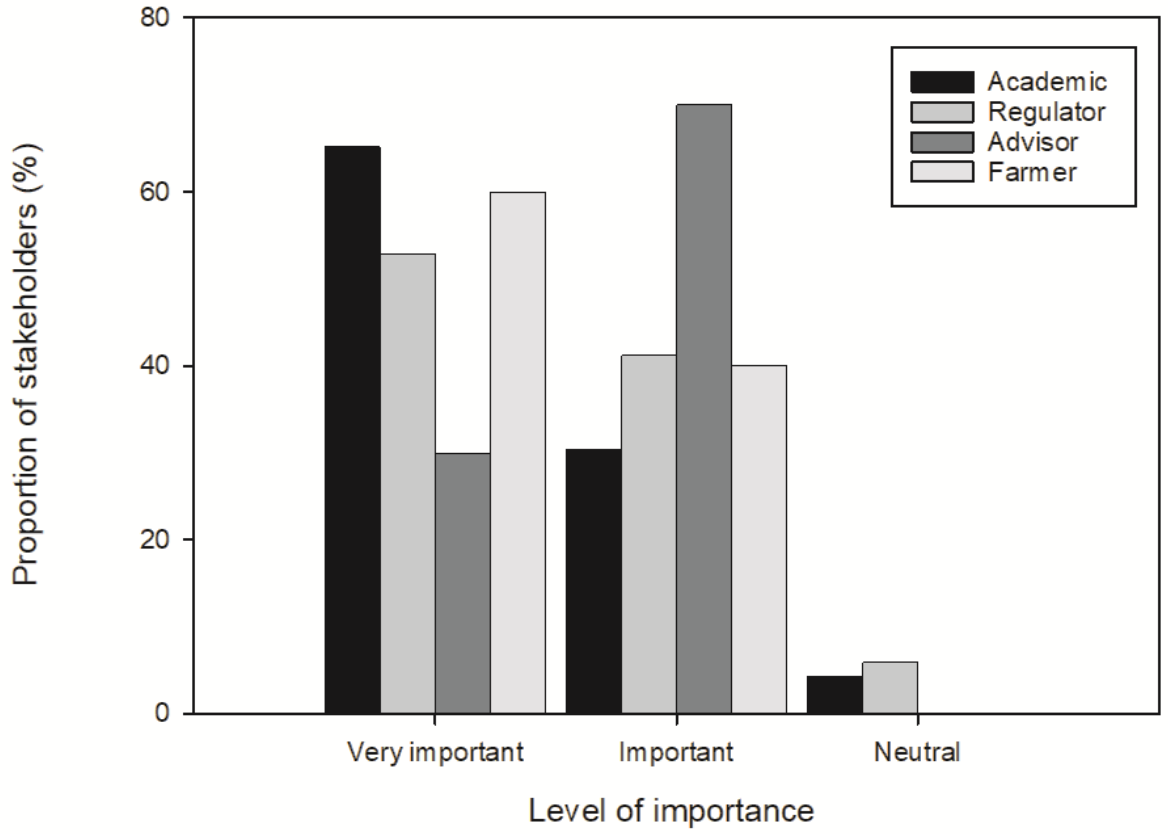

***Figure 5.7**: Perceived importance of AMR resistance challenges in the environment on a global scale.*

Stakeholders were asked to consider the relative importance of a range of different sources (domestic wastewater effluent, industrial (including hospital) wastewater effluent, runoff from agricultural land (including veterinary sources), aquaculture and other) in contributing to the evolution and dissemination of AMR within UK surface water. In general, runoff from agricultural land (including veterinary sources) and domestic wastewater effluent were considered the most important sources, followed by industrial (including hospital) wastewater effluent (Fig. 5.8). Aquaculture and other sources were considered to contribute less to dissemination of AMR in the environment.

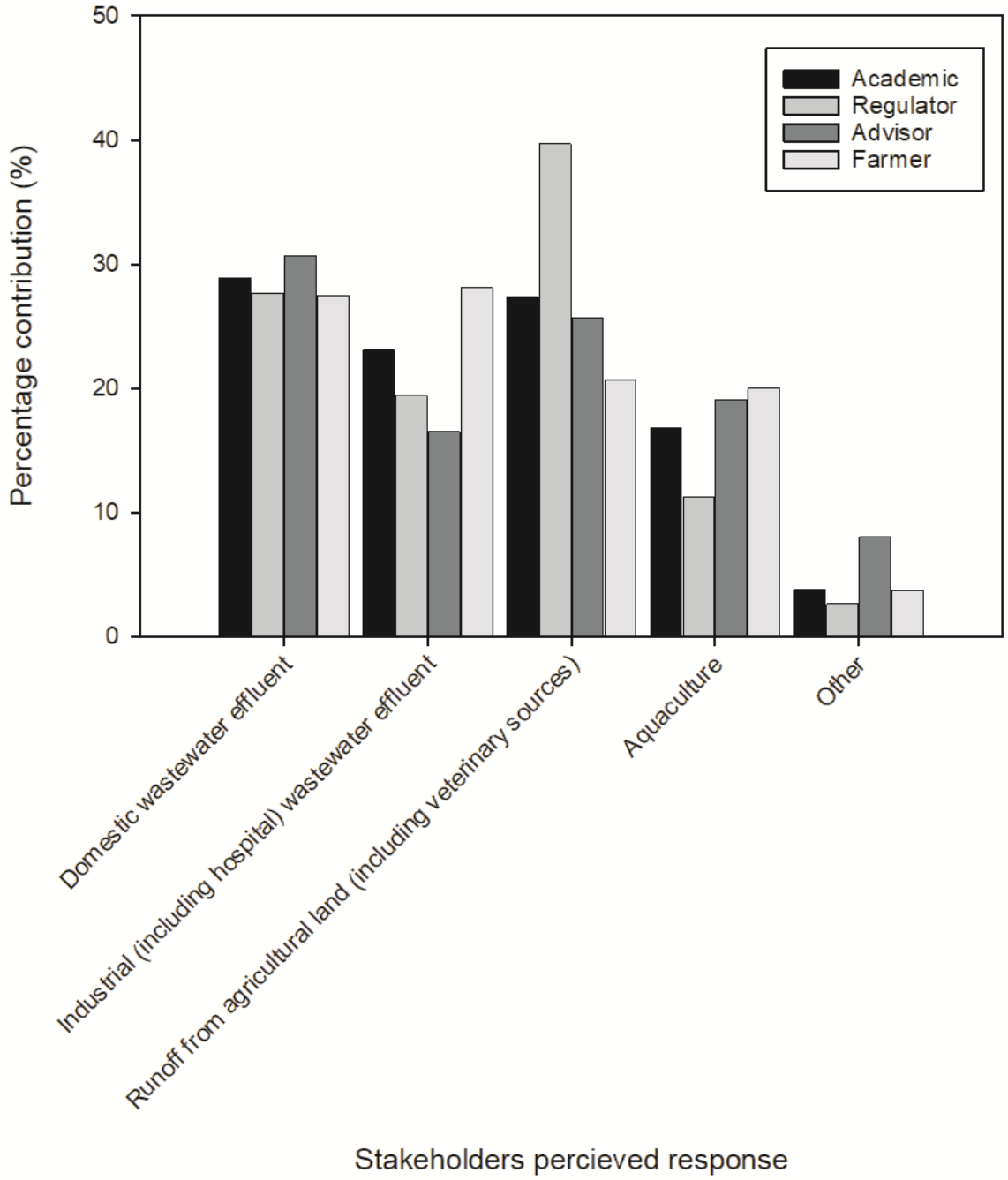

Figure 5.8: *Contributions of different sources to the evolution and dissemination of AMR within UK surface water according to different stakeholder groups.*

Stakeholder perceptions on which wildfowl/life type (deer, gull or goose) was most likely to impact on faecal pollution of surface water in UK is shown in figure 5.9. Geese and gull were considered by stakeholders as more of a threat to water quality than

deer, although farmers were more critical of the contribution from deer relative to other stakeholders (the same was true of their perception of geese too relative to other stakeholders). Similar proportions of academics and regulators considered gulls to be the biggest threat to faecal pollution of water from the wildlife/fowl being evaluated, with 65% and 64% response rates, respectively. No significant associations between stakeholder type and wildlife type were determined ($P > 0.05$).

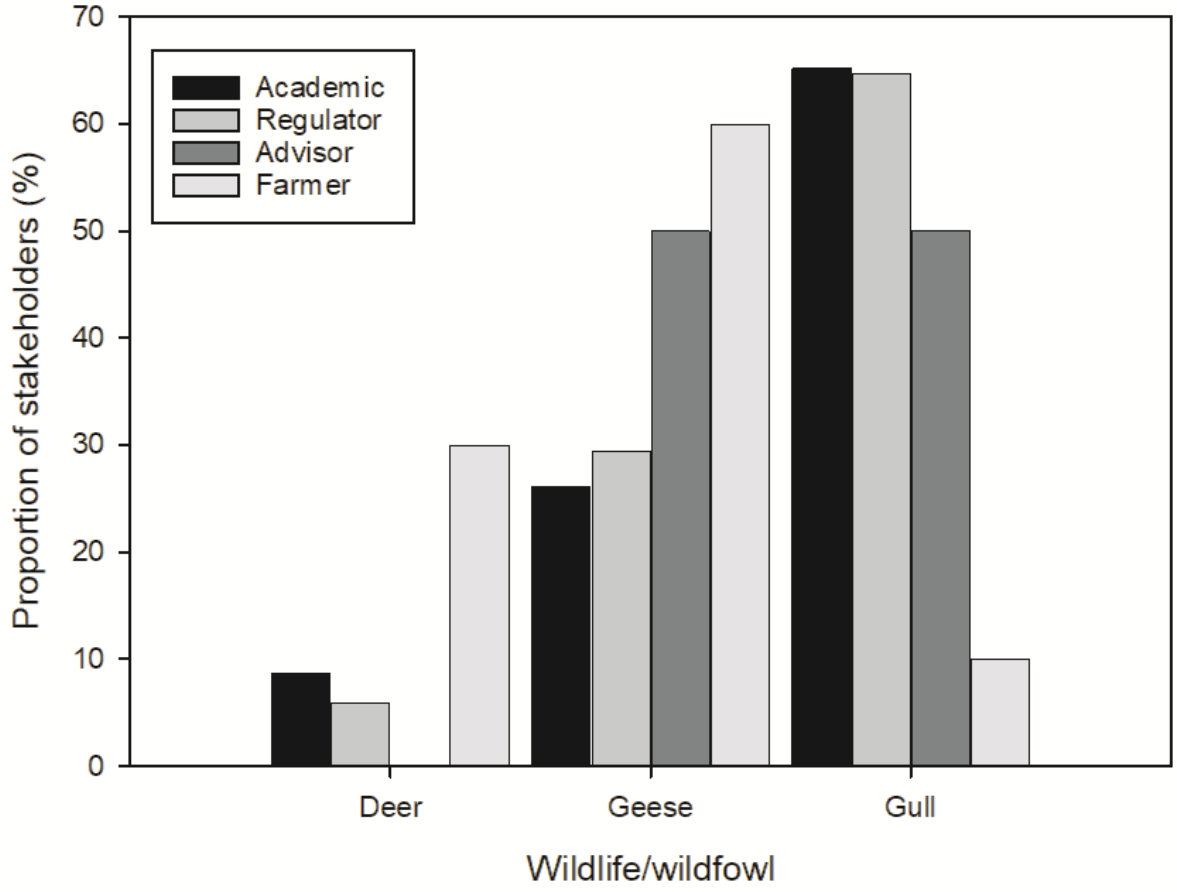

Figure 5.9: *Which wildlife/fowl type do different stakeholders perceive as having the greatest impact on faecal pollution of surface waters?*

Again, geese and gulls were considered by stakeholders as the wildlife type with greatest possibility for disseminating AMR to surface water in the UK (Fig 5.10). While the responses of stakeholders on the potential of wildlife type to disseminate AMR were mixed for geese and gull, their opinion were broadly similar for deer. Academics considered gull as the wildlife type with the highest potential to disseminate AMR in

UK surface waters, with a 65% response rate among this stakeholder group whereas 58% of regulators attributed gull to be the most important of these animal types to disseminate AMR. This compared to a 40% and 30% response rate for farm advisors and famers on the importance of gulls. Farmers believed that geese had most potential to disseminate AMR with 50% responses attributed compared to 17%, 23% and 30% responses from academics, regulators, and farm advisors, respectively. No significant associations between stakeholder type and wildlife type were determined (P > 0.05).

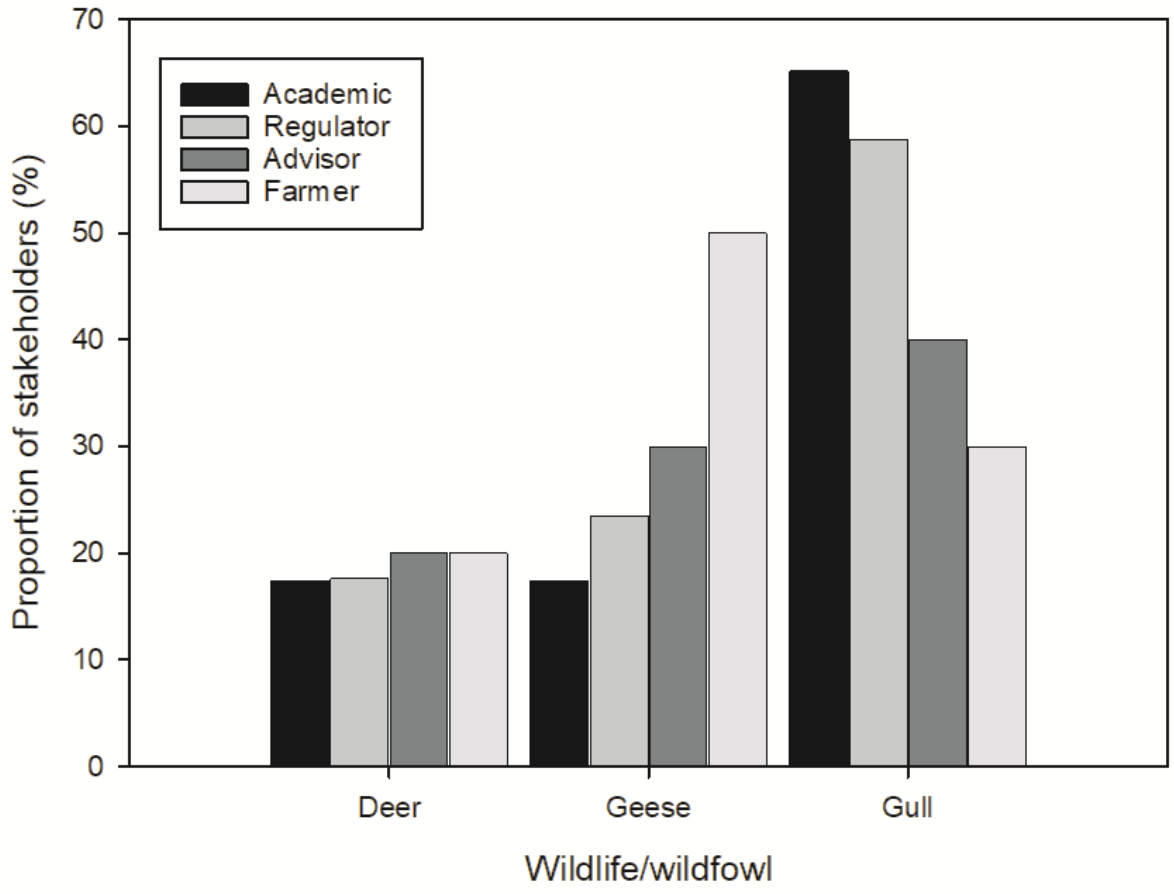

***Figure 5.10**: Which wildlife/fowl type do different stakeholders perceive as having the greatest potential for disseminating AMR to surface water?*

Stakeholders were asked to consider which sectors could play an important role in reducing AMR in the environment. The free-text responses were combined to generate a word cloud in order to visualise most common responses (indicated by the size of the word; Figure 5.11)

Figure 5.11*: Visualisations of most common stakeholder responses to 'which sector can play an important role in reducing AMR dissemination in the environment.*

A final question asked stakeholders to consider whether more needs to be done to reduce the potential water quality impacts of wildlife such as deer, geese and gulls? This open-ended question yielded a range of responses. Some example quotes from respondents are grouped below into several themes.

The first common theme identified was one of general uncertainties:

> *"Yes - but we currently lack good quality understanding in terms of a quantitative evidence base of data".*

"Research to identify the relative contributions and importance of these sources and to explore the efficacy and cost-effectiveness of interventions"

"Not sure, we can. But we certainly would need to know what the contribution of wildlife is, so that we have a better picture for source apportionment and see where/which methods would work best"

"Yes, but from a policy perspective who owns the issues is the barrier. Many diseases in wildlife pose the difficulty of implementing controls on animals whose ownership and interaction with habitat is unclear. AMR is even more difficult to ascribe ownership to than traditional diseases".

A second common theme linked to gull management for bathing water protection:

"Educate people using recreational waters not to leave food lying about and to discard waste in a way that prevents gulls feeding on it. This will reduce the number of gulls in an area and hence the faecal loading".

"There are effective measures to reduce the number of gulls in coastal towns, which have helped reduced their impact on water quality. However, for effective longer-term solutions to reduce potential water quality impacts, measures such as Sustainable Urban Drainage, and improvements in treating wastewater and surface water would be more effective".

A third theme recognised the need to balance wildlife conservation alongside water quality:

"Where there are demonstrable issues backed up by accurate and robust data and evidence the impacts should be assessed on a site-by-site basis. The impacts of wild animals are highly marginal when compared to other impacts in catchments. Wild animals should not be persecuted in the name of solving a problem which is primarily caused by wastewater and agriculture".

"Wildlife are important and should be protected but i am supportive of controlling populations that can expand exponentially without being kept in check".

"*Yes - not sure what though as difficult conflict with conservation etc"*

A fourth theme was that little/nothing more needs to be done:

"*No I think there are more important issues"*

"No it is the humans that need to clean up their act!"

*"*A little but I think there are other actions that take priority".

"Its difficult to control wild animals and as they all require access to water I'm not sure what action can be taken that firstly reduces the impact on water quality and secondly is practicable and cost effective."

5.4 Discussion

This chapter reports on the perceptions and viewpoints of different stakeholders who have an interest in microbial pollution of surface waters in catchments. Specifically, academics, regulators, advisors and farmers were surveyed with the aim of understanding how these different communities perceived the importance of wildlife relative to other catchment sources, e.g., agriculture and wastewater, with respect to their role of contributing to microbial pollution of waterbodies and the wider dissemination of AMR to the environment. Understanding the varying perspectives of different stakeholders linked to landscape management for water quality benefits is important, particularly when investigating aspects of soil and water science that are relatively under-researched and lacking extensive published evidence. Regarding the impacts of wildlife on faecal pollution and microbial contamination of watercourses, evidence is largely anecdotal and access to good quality information is challenging and often only discussed anecdotally in informal settings such as workshops. In earlier chapters, this thesis has provided empirical evidence on: (i) the survival of FIOs in sources of wildlife faeces, (ii) their mobilisation and (iii) FIO dynamics in streambed sediments once delivered to a receiving water As a result of this final data chapter there is now: (iv) insight into the perceived potential impacts of wildlife derived FIOs

on water quality relative to other catchment sources of pollution following solicitation of views from 60 stakeholders with knowledge of catchment microbial dynamics.

The interpretation of data from this survey needs to be treated with caution to avoid overstating any findings given the limited sample size of 60 respondents. The study provides initial insight into how different stakeholder groups view wildlife contributions to microbial pollution of surface waters, and their wider views of AMR in the environment and the approach represents a template that could be used in future research as part of a larger programme of elicitation of expert knowledge to further understand lesser-known sources of faecal pollution and associated environmental issues. For example, targeted distribution of online surveys across relevant government departments and environmental bodies, e.g., the Environment Agency, Scottish Environment Protection Agency, Natural England, DEFRA, Scottish Government, Natural Resources Wales and other organisations, would likely generate a more robust dataset for the general views of regulators and advisors on this topic area. Likewise, greater involvement of farmer networks, e.g., via the NFU, would help to deliver a much larger sample of farmers perspectives. Farmers are often considered a hard-to-reach community (White et al., 2021) and the distribution of a survey via a weblink is not necessarily the best approach for capturing a large response rate from the farming community; however, the COVID-19 pandemic prevented in-person surveys due to public health guidance and restrictions at the time of this study. Limited engagement by the farming community may also link to their day-to-day requirements of managing their land, with less priority given to completing surveys (Lamarque et al., 2011). Indeed, physical surveying of farmers at livestock auction markets, for example, would probably have enabled an increased return rate for farmer surveys because of the removal of barriers associated with the need to entice respondents to participate via clicking a weblink and due to the congregation of this stakeholder type at a particular event. Others have used smaller sample sizes (n = 43) to investigate how stakeholder understanding varies, e.g., when considering ecosystem service trade-offs, and have compared views of academics, regulators, water industry professionals and farm advisors using participatory engagement methods (Stosch et al., 2019; 2022). While the methodology used by Stosch et al (2019; 2022) differs from that of an online survey, differences in stakeholder views were identified even when using stakeholder cohorts of ~10 participants per stakeholder type, thus highlighting the

potential for generally representative views from relatively small cohorts of stakeholders linked to a particular domain/remit, though of course there will always be variability within stakeholder organisations/typologies with respect to personal views and perceptions (Kujala et al., 2022). Irrespective of these caveats, the data provides some initial findings and a blueprint for conducting a larger and broader survey, but the statistical power of the analysis in this chapter is limited. What is provided is a general overview and an informative commentary on how different stakeholders view the role of wildlife as a source of microbial pollution.

One of the key findings from this chapter was that a significant association was found between stakeholder type and perceived importance of wildlife contributions to microbial pollution. A divergence in opinion was observed, with academics more likely to consider wildlife as a somewhat important contributor to water quality deterioration. By contrast, farm advisors and regulators appeared less convinced, with responses more frequently associated with a neutral or not important view. Perceptions are shaped by the experiences, situations, knowledge and day-to-day environments that people are exposed to (Siegrist and Arvai, 2020). It is likely that all stakeholder types who participated in the survey appreciate that there are different faecal sources in a catchment, of which wildlife/fowl are one; however, academics who work in the field of land and water management, with microbial pollution as one of their interests may have been exposed to more recent literature of published studies that have reported on wildlife issues as part of their ongoing research. Or perhaps this group is more open to being curious about a more diverse range of pollution sources and the need to fill a research gap. This is not to say either perception is incorrect, but the perception of academics is likely to be more heavily influenced by published academic material, and their having a more direct aspect of research in this field may lead to greater awareness of a growing body of material concerning this topic. Those perceptions could also be linked to their interest in pushing forward a research agenda, and by suggesting the importance of a particular issue there could be a degree of self-promotion of a particular topic of interest. It is interesting to consider the responses of the regulators and advisors too. The regulators did acknowledge the importance of wildlife but their day-to-day exposure to regulating agricultural and wastewater sources perhaps constrains their view of how important wildlife are relative to these two major sources of pollution that they typically deal with.

Overall, all stakeholders did view wildlife as the smallest contributor to microbial watercourse pollution in terms of percentage contribution when considered alongside arable land, grazing pasture and urban wastewater. This does not mean that wildlife are an irrelevant source of pollution, but when considered against some of the larger sources there was a consistent view that some bigger priorities exist in terms of where effort should be focused. This was reinforced by some of the free-text responses in the survey whereby a number of participants highlighted that other more important issues should be addressed ahead of wildlife contributions. This probably needs to be considered at different scales, because at some very local levels the role of wildlife could be extremely important for influencing microbial water quality, as suggested by some responses to the free-text questions, e.g., highlighting the need for site-by-site assessments and that risks from wildlife are highly marginal. Certainly, gull management on beaches has received research attention (Converse et al., 2012) because large numbers of birds do have the potential to congregate and defecate near to bathing water/recreational water sampling locations, which can jeopardise regulatory standards. The reputational damage and lost revenue from such occurrences can be severe for local economies that rely on tourism and so wildlife impacts at specific catchment locations can perhaps be magnified depending on the end-point receptor (Koskey et al., 2014). In Scotland during the summer of 2022, 'Keep Scotland Beautiful' ran a campaign at a number of designated bathing water beaches using posters and social media to warn the public to not feed seagulls because their faeces contribute to water pollution (BBC, 2022). However, this generated conflicting views and led to a response from other groups such as conservation charities who challenged the message that such awareness-raising was generating. Conservation groups argued that seabird droppings provide key sources of nutrients for marine life and their faecal contributions are minimal relative to sewage discharges from wastewater treatment plants. Clearly there are divergent views within different expert communities.

The survey also provided an opportunity to seek views on wider issues of AMR in the environment and the general view from all stakeholders was the importance of both faeces and water in helping to facilitate AMR dissemination, and that these environmental matrices were more important than air and soil. Academics, in

particular, associated both water and faeces as important environmental matrices though overall there was no significant association between stakeholder type and perceived level of importance of any single environmental matrix for AMR dissemination. The dataset does provide a useful overview of general views across different communities, which in turn can help to focus awareness raising campaigns. A variety of quotes were provided by respondents with regard to the importance of the agricultural sector as a potential contributor to AMR in the environment, with the majority linking to the administering of veterinary medicines and antibiotics to livestock. However, few if any made the direct link to possible exposure of wildlife to land and water that had received land applications of faecal material from agriculture that would be a source of AMR. Agricultural sources and domestic wastewater were identified as the two largest contributors to the evolution and dissemination of AMR within UK surface waters and this maps well to the response to the question about which sector can play an important role in reducing AMR dissemination in the environment, with both agriculture and the water industry identified as the prominent suggestions.

How stakeholders perceived geese, gulls and deer with regard to their importance for contributing to (i) faecal pollution of surface waters and (ii) AMR dissemination via water revealed no statistically significant associations, but some patterns were observed. Farmers clearly identified geese as being a threat for both faecal pollution and for AMR dissemination. Farmer perceptions of geese are often negative and there are a number of farmer concerns over geese reported in the literature concerning their conservation value versus damage to farmland when large flocks of geese descend on agricultural areas (Redpath et al., 2015; Simonsen et al., 2017, Rakotonarivo et al., 2021; Tombre et al., 2013). The environmental damage and cost to farmer's livelihoods can be substantial from geese (McKenzie & Shaw, 2017) and this potential negative perception may be responsible for how farmers perceived geese in general for other environmental issues such as water pollution and AMR risks. The importance of gulls likely links to perceived issues around bathing water quality and gull populations, and there is evidence to suggest that large populations of gulls in some coastal resorts can be detrimental for recreational water quality (Alm et al., 2018; Thorstensen et al., 2021), though as discussed earlier there are clearly conflicting views on the importance of gulls. Their scavenging behaviour at landfill sites may also

contribute to perceptions of the role of gulls for AMR dissemination (Langley et al., 2021).

A wide-ranging set of views were returned in response to the question of whether more could be done to reduce the potential impacts on water quality from wildlife, which could include possible risks associated with AMR spread too. No formal qualitative analysis was done as this was beyond the scope of the chapter, but it was possible to identify some broad themes around issues of: (i) uncertainty; (ii) gull management; (iii) wildlife conversation/water quality trade-offs and (iv) a need to focus on other priorities. The theme concerning uncertainty reinforces the rationale for the body of research reported in this thesis with a number of respondents highlighting that the research community lack good quality quantitative information to support our understanding of the relative contributions and importance of wildlife/fowl and how this might vary spatially and temporally across different landscape types. Without a more substantial evidence-base it becomes challenging to identify and implement interventions because uncertainty is propagated into levels of (mis)understanding of likely efficacy and cost-effectiveness of those interventions, as reported previously when highlighting challenges in empirical data to support catchment modelling (e.g. Oliver et al., 2016). Furthermore, a key issue that was identified was the challenge of managing wildlife movements and how control could ever be imposed, or indeed who if anyone is considered to 'own' this particular problem. Gull management linked to bathing water quality was raised by several respondents as possible options to take forward, linked mainly to efforts to raise awareness, though others balanced this with suggestions that mitigation effort further up catchment targeted at point and diffuse sources would be more effective. Several responses recognised the need to carefully manage any efforts of reducing impacts of wildlife/fowl given the water quality issues are driven largely, in their opinion, by wastewater and agriculture and so ideas of potential conflicts with conservation were listed by several respondents. This complemented a series of responses that made clear that little if anything should be done because there were more important issues to address in catchments (although arguably, the evidence to support this remains limited and this provides tension between views put forward by other respondents who indicated a lack of evidence to be sure how important wildlife/fowl sources really are).

5.5 Conclusion

Management of diffuse microbial pollution in surface water from agricultural catchment requires understanding of various sources of faecal pollution. Uncertainties and gaps in our understanding of the relative contributions of different FIO sources can undermine messages to land managers when they query how important other potential contributors may be relative to their own industry, e.g. farmers querying the importance of wildlife and wildfowl for impacting on microbial water quality. This chapter provided an overview of how different types of stakeholders perceive wildlife and wildfowl with regard to their role in facilitating FIO and AMR dissemination in the environment relative to other key ctchment sources such as agriculture and wastewater treatment. Livestock and grazing pasture are recognised as important contributors to the deterioration of water quality, but the views of respondents did also highlight situations whereby wildlife/wildfowl can impact on microbial pollution of surface water at more local levels, especially the contributions of gull faeces around recreational/ bathing water which can cause potential economic consequences for local authorities through lost revenue from tourism and bad publicity (although in such cases the bathing water quality is probably impacted by a mix of agricultural, human and wildlife sources). The data from this chapter also suggest that runoff from agricultural landscape and domestic waste effluent are perceived as major contributors to the evolution and dissemination of AMR in the environment but importantly the study also provides insight into how different types of stakeholders perceive wildlife and wildfowl with regard to AMR dissemination too. A larger (inter)national scale survey of stakeholder perceptions of wildlife/fowl risks to water quality with a much larger response rates across the different stakeholder types is recommended to identify a more detailed and nuanced overview of how perceptions and challenges vary in both space and time.

6. Synthesis and Conclusions

6.1 Introduction

The findings from this thesis deliver new quantitative evidence to support our understanding of the likelihood for wildlife and wildfowl derived FIOs to persist in and be mobilised from faecal sources. While it has been recognised for some time that agricultural and human sources are not the only contributor to microbial water quality impairment, we have lacked good quality data, particularly in a UK context, that highlights levels of FIO survivability and mobilisation from diverse catchment sources, e.g., wildlife and wildfowl faeces. This thesis provides insight into some key aspects of understanding the risk of wildlife and wildfowl impacting microbial water quality in rural catchments, but to fully appreciate the 'riskiness' of these less common catchment sources, we need to couple the likelihood of associated FIO survival or mobilisation in the environment with estimates of the consequence or impact (i.e., the environmental impact of FIO pollution contributed from the wildfowl and wildlife communities in catchments).

6.2 Towards Assessing the Risk of Wildlife/Fowl FIO Pollution

Quantifying FIO survival and mobilisation dynamics enables an appreciation of some of the environmental drivers that influence the likelihood of wildlife-derived FIOs being transferred across the landscape and delivered into receiving waters (Huang et al., 2022). This thesis has reported on FIO persistence patterns under various conditions (Chapter 2, 3, 4). Wildlife and wildfowl are common in rural catchments and therefore the presence of wildlife/fowl faeces occurs throughout the landscape and is subjected to a variety of environmental conditions. The pathway of source-mobilisation-delivery-impact of faecal matter from livestock and wildlife is shown in figure 6.1 New data reported in this thesis has identified that FIOs can persist in the faecal matrix under harsh environmental conditions associated with sub-freezing temperatures (Chapter 2), faecal material is detached during simulated rainfall activity and results in the mobilisation of FIOs when conditions allow (Chapter 3) and those FIOs that are successfully delivered to receiving waters can be stored in bank or bed streambank sediments and persist as a legacy store (Chapter 4). For wider considerations of risk (e.g. likelihood x consequence), an assessment of the magnitude of wildlife-derived

FIO impacts is now needed to further advance the evidence provided by this thesis. For example, how big a contribution to the overall catchment FIO burden to land is associated with wildlife/fowl excretion? Furthermore, 'risks' of wildlife/fowl faeces will differ depending on stakeholder interests; there are risks to water quality and associated standards and there are potential risks to human health. The latter requires another level of investigation and would need to consider specific pathogens of concern contributed by wildlife/fowl but was beyond the remit of this thesis. This thesis has also made some inroads into considering the consequences of FIO pollution from wildlife and wildfowl by using an online survey of key stakeholders involved in catchment management (Chapter 5). Integration of new scientific datasets with the involvement of relevant stakeholders to provide expert input and steer is recognised as an approach to improve water quality (Merrett et al., 2020). However, to fully assess 'risk' there is a need for larger scale surveys and for on-the-ground empirical research to monitor and determine consequences and environmental impacts from wildlife, probably using multi-scaled approaches (e.g. plot studies, hillslope experiments, paired (sub)catchment studies etc.). With continued research across scales, a more robust assessment of the risk posed from wildlife and wildfowl to microbial water quality will be possible.

6.3 Reflecting on Common Questions Concerning Wildlife/Fowl Contributions to FIO Pollution

In Chapter 1, this thesis identified common questions concerning wildlife and wildfowl contributions to FIO pollution in rural catchments. Four key objectives were also identified. Here those questions and the thesis objectives are revisited, and the research undertaken in this thesis is mapped to each question/objective to identify how new insight delivered in the data chapters can help to underpin a response to these questions.

1) *How important are common UK wildlife, e.g. deer, geese, in terms of their contribution to FIO loading of catchments?*

To quantify overall FIO loading requires an understanding of wildlife numbers and this data can be difficult to obtain. For context, obtaining good quality data on livestock numbers is fraught with spatial data challenges and caveats, uncertainties and confidentiality issues (Oliver et al., 2009) and yet livestock data from agricultural audit records represents information that is far more organised in terms of its collection. However, Chapter 2 (*Obj1, characterise FIO die-off*) has provided clear evidence that deer faeces can persist under sub-optimal temperature conditions and that legacy stores of FIOs are essentially protected to some extent by the faecal matrix when exposed to sub-freezing and freeze-thaw conditions. In addition, Chapter 5 (*Obj 4, investigate stakeholder perceptions*) provides information on stakeholder perspectives with respect to wildlife and wildfowl contributions and has highlighted both similarities and differences across and within different stakeholder types with regard to viewpoints on relative risk of these faecal sources. However, to fully address this question requires a larger programme of research and detailed analysis of typical FIO values excreted by a wider suite of wildlife/fowl types. Characterising FIO contributions (magnitudes, variability) and combining with typical values for faecal excretion loads per wildlife/fowl type would enable an extrapolation to estimate the relative contribution of wildlife/fowl to the overall catchment burden but would require some knowledge of wildlife/fowl numbers in a given area to guide the upscaling. A GIS mapping and modelling approach could be developed to provide spatial risk maps of FIO burden from wildlife/fowl sources, similar to what has been done for livestock (e.g. Oliver et al., 2018), but the associated uncertainties are likely to be much higher given the more limited availability of data on wildlife/fowl numbers in rural catchments.

2) *Does the magnitude of FIOs sourced from common wildlife/fowl types vary in space (e.g. in different catchments) and time (e.g. across seasons)?*

This is difficult to answer from the thesis alone, but previous studies have reported variability of FIOs concentration from different wildlife/wildfowl (Table 1.3). The COVID-19 pandemic prevented any field-based research and instead a stakeholder survey was deployed to generate data of a different nature. Spatial and temporal assessments of wildlife derived FIOs were therefore not possible. If undertaken, this

would have provided an opportunity to consider how differences in catchment characteristics and seasonal influences can impact FIO survival and mobilisation under field-relevant conditions, but clearly this was not possible. Chapter 2 (*Obj1, characterise FIO die-off*) investigated freeze-thaw influences vs constant temperature conditions and this therefore allowed some consideration of changes in temporal conditions that may be experienced in colder seasons, but the data were generated under controlled conditions that enabled a manipulation of temperature regimes.

3) *To what extent does FIO die-off vary within different wildlife faeces under specific environmental conditions?*

Chapters 2, 3 and 4 (*Obj1, characterise FIO die-off; Obj2, determine FIO mobilisation; Obj3 quantify FIO persistence in streambed sediments, respectively*) all provide new information relating to wildlife/wildfowl derived FIO survival in environmental matrices and a core component of this thesis is a contribution to understanding conditions that influence FIO die-off curves. If we consider the source-mobilisation-delivery-impact conceptual framework (Haygarth et al., 2005), the area where most knowledge exists tends to be around 'sources' of pollution. This is true not only for FIOs (whether considering livestock, human or wildlife/fowl sources) but for other pollutants too, e.g nutrients (Vero and Doody, 2021), heavy metals (Alam et al., 2021), suspended sediments (Bloodworth et al., 2015) and emerging contaminants such as microplastics (Park and Park, 2021). However, for FIOs it is clear that our understanding of source dynamics related to wildlife and wildfowl faeces lags behind the extensive understanding we have of FIO sources contributed from agricultural systems. For decades, survival curves of FIOs have been reported under different experimental and field-relevant conditions and the field has advanced significantly resulting in a robust characterisation of FIO persistence profiles for cattle and sheep faeces in particular. The lack of comparable data for wildlife/fowl is likely a result of challenges associated with securing fresh faeces in sufficient quantities from these sources. Practical constraints can therefore limit levels of understanding. This thesis has, however, revealed differences in persistence associated with varying faecal type (and associated characteristics) and different temperature conditions.

4) *What factors control the efficiency of FIO mobilisation from wildlife faeces?*

Chapter 3 (*Obj2, determine FIO mobilisation*) dealt specifically with the topic of FIO mobilisation, which is under-reported in the literature. The limited data of FIO mobilisation from wildlife/fowl sources is probably due to the same reasons reported for limited FIO survival in faeces from these sources. There is much that can be done with this topic though, as alluded to earlier in the thesis, and in particular there are opportunities for investigating different types of mobilisation processes (e.g., raindrop impact versus sloughing from faeces) and applying this to scenarios of desiccation of faecal material and mobilisation following freeze-thaw (and therefore added value of coupling concepts from different chapters together to further develop the evidence base in FIO fate and transfer dynamics).

6.4 Scientific Implications of the Thesis

The results from the three laboratory experiments (chapter 2, 3 and 4) of this study demonstrate that both livestock and wildlife are important sources of diffuse microbial pollution in the environment. While livestock in agricultural catchment (either housed or grazed) have restricted movement, wildlife roam freely in woodland and agricultural landscapes covering a long-range distance without restriction and often across national boundaries (Arnold et al., 2016). Although, dairy faeces present high risk of FIO pollution in terms of longer survival in harsh conditions and high mobilisation rates, both faecal sources (dairy and wildlife) contribute to deterioration of water quality through diffuse and point sources in agricultural and woodland landscapes. The data collected revealed that FIOs from both dairy cow and wildlife can survive subfreezing conditions and serve as a legacy store for FIOs in the environment. The range of FIO concentration that survive freeze-thaw cycles reported in this thesis (1.7 $\log_{10}$-4.5 $\log_{10}$ for dairy, and 2.5 $\log_{10}$-3.8 $\log_{10}$ for deer) improves our knowledge of possible concentrations of FIOs that may be transferred during rainfall event following detachment from faecal matter. More environment-tolerant cells and viable-but-non-culturable (VBNC) population are likely to resuscitate following warmer weather and re-mobilise in run-off during rainfall events (Afolabi et al., 2020). The concentration of FIO that would be delivered into watercourse depends on the concentration of FIO detached during rainfall events and the concentration of FIO trapped in the soil after

detachment (Sepehrnia et al., 2018; Kim et al., 2016). If more FIOs survive the unfavourable weather and persist in the environment (including in streambed sediments) in high concentration, a continuous loading of FIO into the watercourse during rainfall would become a greater challenge for water managers. Hence there may be a possible outbreak of public health diseases if the recreational waters are not properly monitored or managed.

Therefore, classification of hotspots of FIO legacy stores within landscapes that are considered to harbour large wildlife populations may have less intensive agriculture still requires attention in order to facilitate effective management of microbial pollution in and around recreational waters. The use of modern equipment and deployment of technology such as monitoring cameras and sensors rather than traditional counting of wildlife would deliver a better output in monitoring the frequency of wildlife visits around recreational waters, or feeder streams that eventually drain to waters used by members of the public (Prosekov et al., 2020). This is important in identifying microbial risk prone areas and developing measures to mitigate their impact on human health.

6.5 Policy recommendations

Wildlife can potentially contribute to faecal pollution, although the magnitude of that impact was not the remit of this thesis. However, their faecal sources can provide mobilised cells to surface waters (Oliver et al., 2012; Afolabi et al., 2020). Some pathogens of public health risk have been associated with faecally polluted water including *Cryptosporidium* spp and *Campylobacter* spp (Chan et al., 2021). In addition, some other pathogens associated with waterborne illnesses are reported in Table 1.1. Around the world, the impact of unsafe water on humas and can be severe ranging from the public health diseases to economic loss due to hospital treatment. For example, approximately 829,000 people die yearly from diarrhoea due to unsafe drinking water, sanitation, and hand hygiene with close to 300,000 under five years children representing 5.3% of all deaths recorded in this age group (Lin et al., 2022). Furthermore, about $7.3 million is spent globally in the health sector for the treatment of waterborne diseases, apart from economic losses due to citizen's inability to work because of health-related issues from waterborne diseases which results in loss of income and productivity in the work environments (Mumbi and Watanabe, 2022). This

section therefore outlines proposed actions which can be considered by environmental and water managers to further reduce the risk of microbial pollution of waters from wildlife. Some science, policy and knowledge exchange considerations are outlined below.

We know that rainfall mobilises FIOs. Understanding where localised hotspots of wildfowl/wildlife congregation occur can help guide advice about possible risks of water contamination. While popular bathing waters are protected by the bathing water directive (Quilliam., 2019), including some inland freshwater sites, many rural water bodies do not get visitor numbers that warrant regulation. However, the growth in popularity of wild swimming means that those who use rural waterbodies for open water swims may be more exposed to wildlife derived FIOs. Awareness raising about these possible risks is vital to curtail potential outbreaks of ill-health associated with microbial water pollution.

Environmental departments of government promote agri-environment schemes and land stewardship practices that often encourage practices that protect and enhance the natural environment, which may include taking land out of production, encouraging hedgerow growth and development of riparian corridors; however, this may also attract wildlife and unintentionally increase contributions of faecal pollution from these less well recognised sources. Of course, this would probably only be problematic in areas where such changes would lead to excessive congregations of wildfowl/life around sensitive waterbodies (and therefore likely be rare), but considering potential trade-offs of land management decisions, such as those described above, is important.

Clearly wildlife can contribute to faecal pollution and when pollution incidents at bathing beaches are identified and regulators attempt to use catchment forensics to pinpoint particular sources, we know that wildlife can potentially represent a contributing FIO load. In catchments where wildlife and wildfowl are common, these sources should be considered as possible risk factors alongside traditional sources such as livestock and human wastewater and a wider variety of wildlife-specific markers should be developed to help ascertain catchment contributions.

Finally, there are opportunities for awareness raising campaigns among stakeholder groups such as regulators, policy makers and farmers to highlight that there is some evidence of survival and mobilisation of FIOs in wildlife faeces and that this evidence helps contextualise the relative contributions from wildlife, human and livestock sources in catchments. Wildlife and wildfowl are not completely overlooked, but data on their contributions is less documented and more challenging to obtain. More needs to be done to fully understand the relative impact of these different FIO sources in catchments of varying land use.

6.6 Reflections of the Experimental Work Undertaken

While the experimental work undertaken adds value to the empirical evidence base, it is recognised that there were some limitations. For example, including a wider range of environmental variables within the laboratory studies would add breadth to the datasets. This could have included more temperature conditions (Chapters 2, 3 and 4); variable DESPRAL rotation speeds to mimic rainfall events of different sizes (Chapter 3); a range of sediment types to explore their influence of FIO survival (Chapter 4). Time and resource constraints clearly limit what is possible, but the above highlights some possibilities for future developments that build on the research reported in this thesis.

All experimental work focused on laboratory-scale experiments and a field-based study would have complemented the more controlled, mechanistic experimental work to deliver data considered to be more ‘field-relevant’. A field-study would have allowed a degree of scaling-up to have featured in the thesis. This would likely have taken the form of field scale persistence and mobilisation studies that accounted for interacting and varying environmental factors, such as fluctuating temperature and rainfall conditions.

Finally, the online survey used a questionnaire-based approach, and this did enable capture of 60 responses to a range of different question styles seeking views on FIO contributions and risks from wildlife and wildfowl. An alternative approach could have been to complement this with some more detailed interviews with key representatives

from different stakeholder groups. Such an approach would have provided richer knowledge from the stakeholder community but like the laboratory versus field-based discussion, different methodologies deliver different types of benefits and future research could use more detailed interviews, but the sample size would be much reduced given the larger demands on time that is associated with interview methods.

6.7 Recommendations for Future Research

Potential avenues for continued research into the fate and transfer of FIOs from wildlife and wildfowl have been identified during production of this thesis:

- The range of wildlife considered in this PhD was restricted to red deer and graylag geese; however, there are a variety of deer and goose species and indeed a wider range of wildlife and wildfowl that need to be considered. These include wildlife such as rabbits, voles, beavers, swans and ducks, a number of which have very close associations with the water environment. Beavers may be a particularly interesting wildlife source given UK programmes for their reintroduction into the wild because of their potential to act as ecological engineers (Auster et al., 2020) and deliver benefits such as reduced flood risk and improved water quality. What is less understood is their potential to contribute faecal material and the role that beaver ponds may play in storing a legacy store of FIOs, both from the beavers themselves, but also in terms of the changes in hydrological dynamics of water moving through such pond systems.

- Developing further understanding of a) the behaviours of different *E. coli* and enterococci strains and b) which animal faeces harbour those strains. This could be further enhanced by combining FIO and MST data gathering as part of field campaigns.

- Exploring the impact of a wider range of freeze-thaw and wetting-drying cycles on wildlife/fowl derived faeces and in turn FIO survival and mobilisation would provide further data to aid parameterisation of models and enhance fundamental understanding of FIOs in the environment. Investigating different

mobilisation processes (e.g., raindrop impact versus sloughing from faeces) is also an area that can be exploited further to further our understanding of FIO risks in the environment and how mobilisation potential may interact with varying scenarios of desiccation of faecal material.

- Continued integration of scale is critically important. Studies focused on a single scale do not allow for an appreciation of wider understanding of FIO fate, mobilisation, transfer in the environment and studies that move from controlled laboratory environments to more complex catchment systems will be inherently more uncertain, but more reflective of field-relevant conditions. Multi-scale studies linking field and laboratory investigation should be encouraged.

- Finally, advances in modelling efforts of wildlife/fowl contributions to faecal burden in catchments would be well received by researchers in the catchment microbial dynamics community. To aid modelling of wildlife/fowl faecal loading there is a requirement for improved underpinning data on wildlife/fowl numbers, distribution ranges, seasonal movements etc. Through a combined GIS mapping and modelling approach deliver spatial maps of FIO burden from wildlife/fowl sources & stop there to reduce repetition.

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Appendix 1

FARMER SURVEY ON WILDLIFE CONTRIBUTION TO MICROBIAL
WATER QUALITY IN AGRICULTURAL CATCHMENTS

Page 1: WILDLIFE CONTRIBUTION TO MICROBIAL WATER QUALITY IN AGRICULTURAL CATCHMENTS

Opening statement

This survey is part of a wider PhD research project on catchment management.

The survey will ask questions about your own experience and background, your views on different sources of faecal pollution in the environment and your opinions on factors that might influence the dissemination of antimicrobial resistance to surface waters. The survey should take approximately 10 minutes to complete.

Participant Information and Consent Sheet

Do I have to take part?

No. Your participation in this survey is voluntary. You may refuse to take part in the research or exit the survey at any time without penalty by closing the browser. If you want to leave a response blank please just enter 'n/a'

Are there any potential risks in taking part?

There are no foreseeable risks involved in participating in this survey and the answers that you provide will be anonymous

Are there any benefits in taking part?

There will be no direct benefit to you from taking part in this research and there will be no payment for taking part in this project.

What happens to the data I provide?

Your answers will be completely anonymous. Your data will be stored in a password protected file and may be used in academic publications. Your IP address will not be stored.

Will the research be published?

The University of Stirling is committed to making the outputs of research publicly accessible and supports this commitment through our online open access repository STORRE. Unless funder/publisher requirements prevent us, this research will be publicly disseminated through our open access repository.

Who is organising and funding the research?

The Nigerian government via the Petroleum Technology Development Fund is sponsoring/funding this research

Who has reviewed this research project?

The ethical approaches of this project have been approved via The University of Stirling [General University Ethics Panel].

Your rights

You have the right to withdraw from this survey at any time without giving reasons and without consequences to you.

Whom do I contact if I have concerns about this study or I wish to complain?

If you would like to discuss the research with someone please contact the researcher via e.o.afolabi@stir.ac.uk or supervisor via david.oliver@stir.ac.uk. You can also contact the Head of Division at: a.s.jump@stir.ac.uk

You have the right to lodge a complaint against the University regarding data protection issues with the Information Commissioner's Office (https://ico.org.uk/concerns/). The University's Data Protection Officer is Joanna Morrow, Deputy Secretary. If you have any questions relating to data protection these can be addressed to data.protection@stir.ac.uk in the first instance.

Thank you for your participation.

1. Electronic Consent Form * Required

Please don't select more than 1 answer(s) per row.

Please select at least 2 answer(s).

	Please tick the box to state that you agree:
I agree to take part in this study	☐
I am 18 years old or over	☐

Page 2: Background: Section 1

2. Within which county of the UK are you located (e.g. Stirlingshire, Lancashire, etc)?
Required

3. What type of farm do you run (e.g. dairy, beef, mixed etc.)? Please state Required

3.a. Please give an approximate value for the:

Required

Livestock head on your farm

Size of your farm in hectares

4. Is your farm organic or conventional? (Please select one) Required

- Organic
- Conventional

Page 3: Background: Section 2

5. For how many years have you been farming your land? Required

Please enter a whole number (integer).

5.a. How would you describe your level of experience of environmental management? Required

- Low
- Below average
- Average
- Above average
- High

5.b. In general, how would you describe your level of awareness of environmental issues? Required

- Low
- Below average
- Average
- Above average
- High

Page 4: Background: Section 3

6. What is the dominant land use on your farm? (Please select one). Required

Page 5: Background: Section 4

7. Which of the following is most commonly seen on your farm (Please select only one): Required

7.a. For the animal selected, is their presence common year-round or seasonal? Please comment. Required

7.b. At peak times, what typical (approximate) numbers would you see of deer/geese/gulls on your farm? (Please answer only for the most common wildlife type identified) Required

Please enter a whole number (integer).

7.c. Can you describe the impact (on land, water, or in general) that their presence has on your farm, if any? Required

Page 6: Part 2: Your thoughts on challenges to water quality

8. In your opinion, how do you apportion the relative contribution of faecal pollution to surface water in the UK (Apportion a value to each of the following categories to give a total of 100).

	Optional
Arable land	
Livestock & grazed pasture	
Wildlife (specifically deer, geese, gulls)	
Urban wastewater	
Others	

Page 7:

Section 2: Antimicrobial Resistant Genes & Dispersal

Please read the following passage of text before answering the questions below

Antimicrobial resistance (AMR) in bacteria is a response to the use of antibiotic medicines used to prevent and treat infections in humans and animals. The resistant bacteria may cause infections that is more difficult to treat than those caused by nonresistant bacteria, resulting in higher medical costs, prolonged hospital stays and higher mortality.

9. In your opinion, how important are the following environmental matrices for facilitating the dissemination of AMR?: ✱ Required

Please don't select more than 1 answer(s) per row.

Please select at least 4 answer(s).

Please don't select more than 4 answer(s) in any single column.

	Very important	Important	Neutral	Not so important	Unimportant
Soil	☐	☐	☐	☐	☐
Water	☐	☐	☐	☐	☐
Air	☐	☐	☐	☐	☐
Faeces	☐	☐	☐	☐	☐

10. In terms of global environmental challenges, how important do you consider the

Required

- Very important
- Important
- Neutral
- Not so important

issue of antimicrobial resistance in the environment?

Unimportant

11. In your opinion, how do you apportion the relative contributions of the following to the evolution and dissemination of AMR within UK surface water? (Apportion a value to each of the following categories to give a total of 100).

	Optional
Domestic wastewater effluent	
Industrial (including hospital) wastewater effluent	
Aquaculture	
Runoff from agricultural land (including veterinary sources)	
Other	

Page 8: Section 3

12. Which sector(s) do you think can play an important role in reducing antimicrobial resistance in the environment? Required

12.a. Please explain your answer. Required

13. Which of the following do you think has the greatest potential to impact

Required

- Deer
- Geese
- Gull

on faecal pollution of surface water in the UK? (Please select one).

13.a. Which of the following do you think has the greatest potential for

Required

- Deer
- Geese
- Gull

13.b. Please explain your answers to both of these questions. Required

disseminating AMR to surface water in the UK? (Please select one).

14. Does more need to be done to reduce the potential water quality impacts of wildlife

Required

such as deer, geese and gulls? If yes, please explain your answer.

Page 9: END OF SURVEY

Key for selection options

6 - What is the dominant land use on your farm? (Please select one).
- Improved grassland
- Rough grazing
- Arable
- Forestry

7 - Which of the following is most commonly seen on your farm (Please select only one):
- Deer
- Geese
- Gulls

Appendix 2

STAKEHOLDER SURVEY ON WILDLIFE CONTRIBUTION TO MICROBIAL WATER QUALITY IN AGRICULTURAL CATCHMENTS

Page 1: WILDLIFE CONTRIBUTION TO MICROBIAL WATER QUALITY IN AGRICULTURAL CATCHMENTS

Opening statement

This survey is part of a wider PhD research project on catchment management.

The survey will ask questions about your own experience and background, your views on different sources of faecal pollution in the environment and your opinions on factors that might influence the dissemination of antimicrobial resistance to surface waters. The survey should take approximately 10 minutes to complete.

Participant Information and Consent Sheet

Do I have to take part?

No. Your participation in this survey is voluntary. You may refuse to take part in the research or exit the survey at any time without penalty by closing the browser. If you want to leave a response blank please just enter 'n/a'

Are there any potential risks in taking part?

There are no foreseeable risks involved in participating in this survey and the answers that you provide will be anonymous

Are there any benefits in taking part?

There will be no direct benefit to you from taking part in this research and there will be no payment for taking part in this project.

What happens to the data I provide?

Your answers will be completely anonymous. Your data will be stored in a password-protected file and may be used in academic publications. Your IP address will not be stored.

Will the research be published?

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Who is organising and funding the research?

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Who has reviewed this research project?

The ethical approaches of this project have been approved via The University of Stirling [General University Ethics Panel].

Your rights

You have the right to withdraw from this survey at any time without giving reasons and without consequences to you.

Whom do I contact if I have concerns about this study or I wish to complain?

If you would like to discuss the research with someone please contact the researcher via e.o.afolabi@stir.ac.uk or supervisor via david.oliver@stir.ac.uk. You can also contact the Head of Division at: a.s.jump@stir.ac.uk

You have the right to lodge a complaint against the University regarding data protection issues with the Information Commissioner's Office (https://ico.org.uk/concerns/). The University's Data Protection Officer is Joanna Morrow, Deputy Secretary. If you have any questions relating to data protection these can be addressed to data.protection@stir.ac.uk in the first instance.

Thank you for your participation.

1. Electric Consent Form ✱ Required

Please don't select more than 1 answer(s) per row.

Please select at least 2 answer(s).

	Please tick the box to state that you agree
I agree to take part in this study	☐
I am 18 years old or over	☐

Page 2: Background: Section 1

2. Within which county of the UK are you located (e.g. Stirlingshire, Lancashire etc)? Required

3. What is your profession? Required

4. How would you describe your level of experience in your current profession? (5

Required

1.
2.
3.
4.
5.

representing long-term role, 1 representing new to role)

5. In general, how would you describe your level of awareness of environmental issues? Required

1. Low
2. Below average
3. Average
4. Above average
5. High

Page 3: Part 2: Your thoughts on challenges to water quality

6. Relative to the contribution from livestock and agricultural sources, how important do you think wildlife (e.g. deer, geese and gulls) might be for contributing to microbial

watercourse pollution? Required

- Very important
- Important
- Neutral
- Not so important
- Unimportant

7. In your opinion, how do you apportion the relative contribution of faecal pollution to surface water in the UK? (Apportion a value to each of the following categories to give a total of 100).

Arable land	
Livestock & grazed pasture	
Wildlife (specifically deer, geese, gulls)	
Urban wastewater	
Other	

Page 4: Section 2

Please read the following passage of text before answering the questions below

Antimicrobial resistance (AMR) in bacteria is a response to the use of antibiotic medicines used to prevent and treat infections in humans and animals. The resistant

bacteria may cause infections that are more difficult to treat than those caused by nonresistant bacteria, resulting in higher medical costs, prolonged hospital stays and higher mortality.

8. In your opinion, how important are the following environmental matrices for facilitating the dissemination of AMR?: * Required

Please don't select more than 1 answer(s) per row.

Please select exactly 4 answer(s).

Please don't select more than 4 answer(s) in any single column.

	Very important	Important	Neutral	Not so important	Unimportant
Soil	☐	☐	☐	☐	☐
Water	☐	☐	☐	☐	☐
Air	☐	☐	☐	☐	☐
Faeces	☐	☐	☐	☐	☐

9. How important do you consider the agricultural sector as a potential contributor to

Required

- Very important
- Important
- Neutral
- Not so important
- Unimportant

the dissemination of AMR in the environment?

9.a. Please explain your answer. Required

9.b. Does more need to be done to reduce the risk of AMR dissemination to surface water from agriculture? If yes, please explain your answer. Required

Page 5: Section 3

10. In terms of global environmental challenges, how important do you consider the

Required

- Very important
- Important
- Neutral
- Not so important
- Unimportant

issue of antimicrobial resistance in the environment?

11. In your opinion, how would you apportion the relative contributions of the following to the evolution and dissemination of AMR within UK surface water? (Apportion a value to each of the following categories to give a total of 100).

Domestic wastewater effluent	
Industrial (including hospital) wastewater effluent	
Runoff from agricultural land (including veterinary sources)	
Aquaculture	
Other	

12. Which sector(s) do you think can play an important role in reducing antimicrobial resistance in the environment? Required

12.a. Please explain your answer. Required

12.b. In your opinion, what are the barriers to reducing AMR dissemination to surface water? Required

13. Which of the following do you think has the greatest potential to impact Required

- Deer
- Geese
- Gull

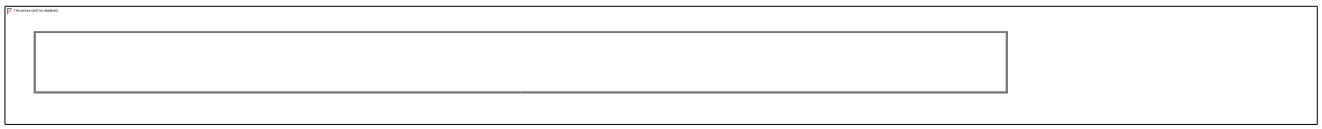
on faecal pollution of surface water in the UK (Please select one)? 13.a. Which of the following do you think has the greatest potential for Required

- Deer
- Geese
- Gull

13.b. Please explain your answers to both of these questions. Required

disseminating AMR to surface water in the UK (Please select one)?

14. Does more need to be done to reduce the potential water quality impacts of wildlife such as deer, geese and gulls? If yes, explain your answer. ✱ Required



Page 6: END OF SURVEY