Environmental reservoirs of bovine tuberculosis in the European badger (*Meles meles)*

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List of Abbreviations

AF	Acid fast
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APHA	Animal and Plant Health Agency
ATP	Adenosine triphosphate
BCG	Bacillus Calmette-Guérin
BEV	Badger edge vaccination
BSA	Bovine serum albumin
bTB	Bovine tuberculosis
CFU	Colony forming unit
CI	Confidence interval
Defra	The Department for Environment Farming and Rural Affairs
di-H_2O	Deionised water
DNA	Deoxyribose nucleic acid
EA	Edge area
EMA	Ethidium monoazide
GAM	Generalised additive model
GB	Great Britain
GE	Genome equivalent
GI	Gastrointestinal
HRA	High risk area
IMC	Immunomagnetic capture
IFN-γ	Interferon gamma
LED	Light emitting diode
LOD	Limit of detection
LRA	Low risk area
mAB	Monoclonal antibody
MIA	Minimum infective area
mRNA	Messenger ribonucleic acid
MTC	Mycobacterium tuberculosis complex
NPV	Negative predictive value

NVL	No visible lesions
OADC	Oleic acid, bovine serum albumin, dextrose, catalase
OD	Optical density
OTF	Officially TB free
OTF-W	Officially TB free withdrawn
pAB	Polyclonal antibodies
PANTA	Polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin
PA-PCR	Presence-absence polymerase chain reaction
PBS-T	PBS + 0.05% Tween 20
PCR	Polymerase chain reaction
PI	Propidium iodide
PMA	Propidium monoazide
PPD	Purified protein derivative
PPS	Protein precipitation solution
PPV	Positive predictive value
qPCR	Quantitative polymerase chain reaction
RBCT	Randomised badger culling trial
RD	Regions of difference
RMSE	Root mean-square error
rRNA	Ribosomal ribonucleic acid
RTA	Road traffic accident
SD	Standard deviation
SE	Standard error
SICCT	Single intradermal comparative cervical tuberculin
SNP	Single nucleotide polymorphism
STW	Sterile water
TB	Tuberculosis
UK	United Kingdom
VBNC	Viable but not culturable (under standard conditions)
VL	Visible lesions
v-qPCR	Viability quantitative polymerase chain reaction
WGS	Whole genome sequencing

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree.

Summary

Four years after the implementation of the current bTB strategy (2014), bTB was responsible for the premature slaughter of 44,656 cattle and estimated to cost the taxpayers and dairy industry a combined £120 million. Badger culling is a key component of the strategy, with at least 32,601 badgers culled in England in 2019. Despite the decrease in the number of herds classified as OTF-W across England (-4 %), this is not necessarily reflective of an improvement in circumstances, with incidents increasing by 13% in the EA and 37% in LRA in the year to May 2019. This increase is thought to be partly attributable to the breakdown of badger social group territories and the subsequent movement of infected badgers; however, the disease dynamics within badger populations typical of cattle farms are poorly understood and much is based on speculation. Furthermore, despite the knowledge that *M. bovis* is capable of long-term extra-host survival, there have been no persistence studies under UK conditions using methods with higher sensitives' than of culture, or studies conducted in naturally infected badger faeces -a hypothesised badger-to-cattle transmission mechanism. These knowledge gaps could result in errors in farm-level biosecurity design and have consequences for national disease control strategies.

For this project, the seasonal stability of *M. bovis* excretion levels by badger social groups was demonstrated on a chronically infected and un-culled dairy farm in the HRA of England. This information was supplemented by an assessment of environmental persistence and identified a biphasic decay rate within badger faeces. Furthermore, it determined that soil temperature is the best predictor of *M. bovis* persistence and that UV is an unreliable method of faecal decontamination. Finally, additional methods were optimised for the rapid differentiation of hypothetically viable *M. bovis* in environmental matrices by v-qPCR and immunomagnetic capture. Proposed future work will use these methods to establish the state in which *M. bovis* bacilli persist within the environment, with important implications for the detection of *M. bovis* and subsequent methods of decontamination.

Chapter 1:

A general introduction to bovine tuberculosis in the United Kingdom

1.1 History of *Mycobacterium bovis* in the UK

Mycobacterium bovis (M. bovis) is the aetiological agent of bovine tuberculosis (bTB), a notifiable disease which has been the focus of an eradication programme in Great Britain since 1950. While it is classed as a zoonotic disease, in recent decades it accounts for just 0.5-1.5% of the UK's clinical TB infections (Drobniewski et al., 2003, de la Rua-Domenech, 2006). However, *M. bovis* remains a significant threat in developing countries where pasteurisation of milk is not standard practice, and a relatively high HIV prevalence compromises the immune system of many individuals (Cosivi et al., 1998, Muller et al., 2013). While *M. bovis* is no longer considered a direct threat to the health of the UK's human population, its control remains a significant economic burden, estimated to cost taxpayers circa £70 million per annum and farmers an additional £50 million (Godfrey, 2018). Furthermore, there is a significant impact on the social and psychological well-being of farmers associated with breakdowns and chronic bTB infection, the impact of which has only been addressed in recent years (FCN, 2009, FarmingUK, 2019).

Areas of England have been categorised according to their risk of infection, with different approaches applied in a trade-off between disease risk and the benefits of the chosen strategy. In the High Risk Area (HRA) and Edge Area (EA) of England, current control strategies include the licensed culling of the main environmental reservoir, the European badger (*Meles meles*), across 31 zones and the annual or 6-monthly testing of cattle by the single intradermal comparative cervical tuberculin test (SICCT) (Donnelly and Nouvellet, 2013, Downs et al., 2013, Godfrey, 2018). In the Low Risk Area (LRA), there is only a four-yearly testing regime unless trading from higher risk areas has occurred. The latest figures for herds that are not officially TB free (OTF-W) counts 2945 herds under movement restriction across England due to an unresolved positive test; this is a 6% year-on-year decrease (Anon., 2019a). Despite the decrease in the number of herds with unresolved outbreaks, the geographic range of the disease has continued to increase. While there has been a 10% decrease in OTF-W herds in the HRA of South-West England, there has been an increase of 15% in the EA and 14% in the LRA (Anon., 2019a). A map of point locations (Figure 1.1) demonstrates the distribution and increasing spread of farms registered as OTF-W in the years 1986 and 2009 (Anon., 2011). Figure 1.2

demonstrates the situation as of 2017 though using herd level incidence which smooths the 100 closest herds to each herd location (Anon., 2019a).

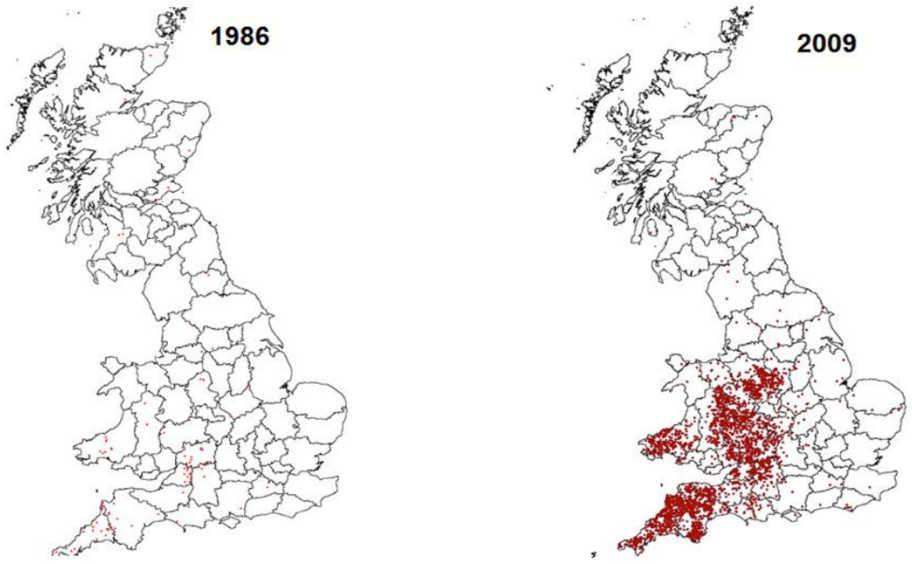

Figure 1.1: Point locations of bTB breakdowns where herds were registered as OTF-W in 1986 and 2009 (Anon., 2011).

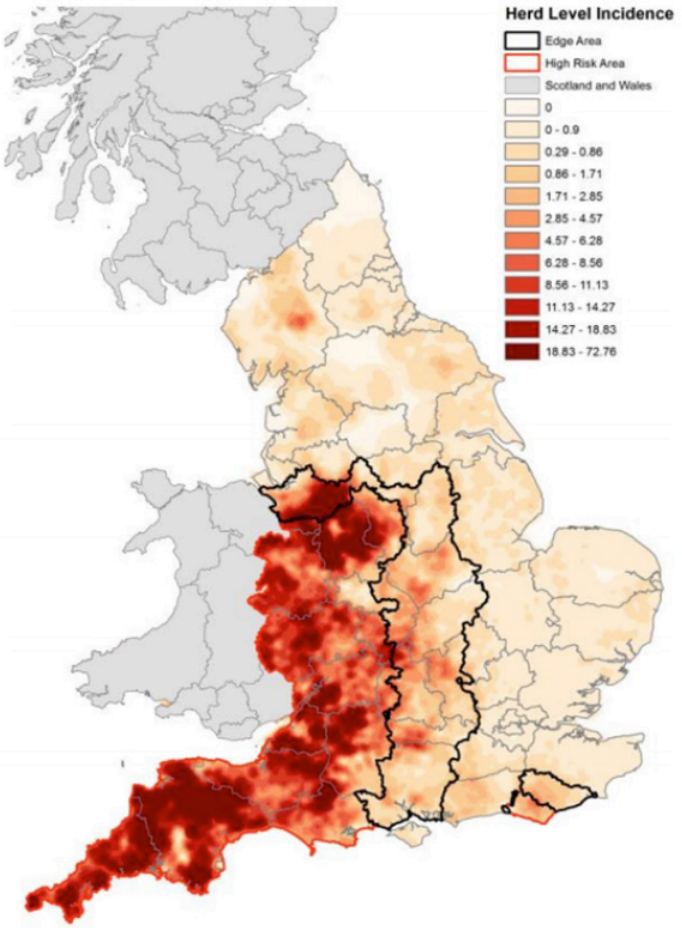

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Figure 1.2: Herd level incidence (the average incidence in the 100 closest herds to each herd location) of bTB breakdowns in England, 2017. Edge area boundaries (outlined in black) were altered in 2018 (Godfrey, 2018).

1.2 *Mycobacterium tuberculosis* complex

The genus *Mycobacterium* is a member of the *Actinobacteria* phylum and contains in excess of 170 members (Tortoli, 2014, Witney et al., 2016). Mycobacteria possess a notoriously slow growth rate yet are separated into two categories based on this criterion; the (relatively) fast growing and the slow growing species. Fast growing mycobacteria are generally saprophytic and include *Mycobacterium smegmatis* and *Mycobacterium fortuitum*. These bacteria exhibit a doubling time of 2-6 hours with colonies appearing on solid media within 7 days (Wayne, 1986, Stephan et al., 2005). In contrast, slow-growing mycobacteria, such as *Mycobacterium tuberculosis* (*Mtb*) and *M. bovis*, are more frequently pathogenic and display doubling times of greater than 16 hours, taking weeks for colonies to develop on media (Wayne, 1986, Beste et al., 2009). A library of transposon mutants identified 84 genes that are responsible for slow growth, and 256 genes that permit switching from slow to fast growth in *M. tuberculosis* and *M. bovis* (Beste et al., 2009).

The mycobacterium tuberculosis complex (MTC) consists of nine highly genetically related pathogens. Despite sharing greater than 99.9% identical genomes and identical 16S rRNA sequences they vary greatly in levels of pathogenicity and host tropisms (Brosch et al., 2002). Sections of the genome, termed regions of difference (RDs), of which there are at least 20 across the MTC, are responsible for the alterations to their characteristics, (Ru et al., 2017). For example, RD1 encodes type VII secretion system associated with virulence and is present in *M. tuberculosis* and *M. bovis* yet absent in the attenuated vaccine strains of *M. bovis* BCG. These RDs facilitate the differentiation of species by PCR, eliminating the requirement for laborious biochemical testing (Figure 1.3) (Brosch et al., 1998). Of relevance to this research is RD4, a 12.6 kb fragment that encodes for 11 genes linked to the biosynthesis of trehalose glycolipids. This region is absent in 'classical' *M. bovis* strains and the scar region is targeted by PCR primers to specifically identify this species from other *Mycobacteria spp.* as part of a validated qPCR assay (Travis et al., 2011, King et al., 2015b). PCR based assays are perceived to have greater sensitivity and therefore ability to quantify the target organism due to eliminating the requirement for harsh decontamination methods.

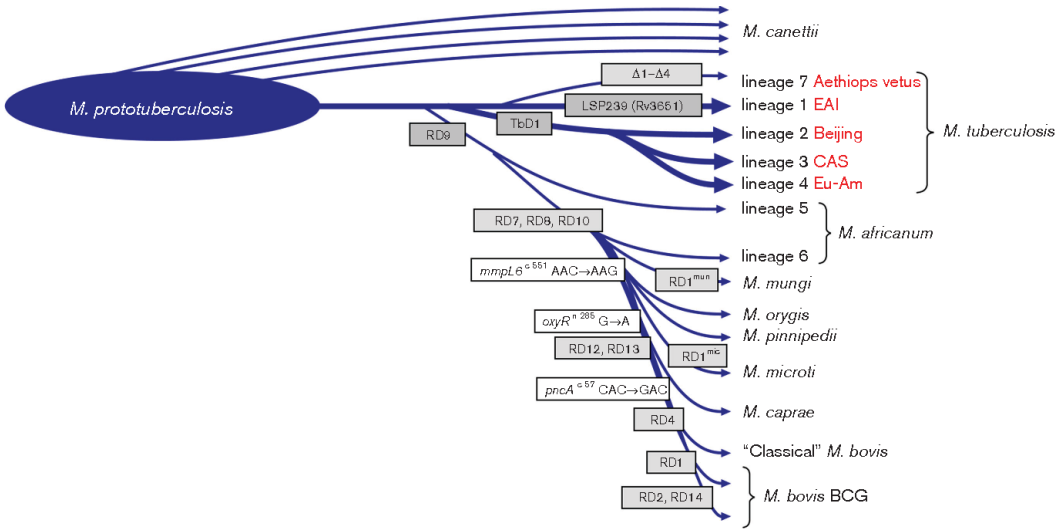

Figure 1.3: Mycobacterial classification by presence or absence of RDs (regions of difference) given specific numbers in the grey boxes and SNPs (single nucleotide polymorphisms) within the white boxes (Nebenzahl-Guimaraes et al., 2016).

1.3 The European badger

The European badger (*Meles meles*) is a member of the *Mustelidae* family and the largest remaining carnivore in the UK with a population of approximately 485,000 (Judge et al., 2017). They are predominantly nocturnal and live in social groups of variable sizes, sexes and ages in subterranean setts that are usually located in or near woodland and range in size and usage (Rogers et al., 1998). Sett classes vary and when surveying for badgers the different classes must be recorded as either: main setts of continual occupancy; annex setts of frequent occupancy and in close range to the main sett (<150 m) with well-worn paths; outliers which are infrequently occupied (and predominantly during the summer months) and often used by other animal species (Roper, 1992). Badger social groups inhabit territories which they will actively and aggressively defend when they encounter badgers which are not of their social group (Macdonald et al., 2004). The edges of group territories, and areas in close proximity to main setts, will be demarked with a series of latrines containing faeces, urine and glandular secretions to communicate their presence via olfactory signals (Kilshaw, 2009, Roper et al., 1993). Social group size is thought to be dictated by the availability of food and space. Groups typically consist of 3-8

individuals (mean = 6.74), but larger numbers have been recorded in South-West England (personal witness to a main sett in Oxfordshire containing at least 12 individuals) (Judge et al., 2017). Despite the territorial nature of badgers, there are movements between social groups, extra-group mating events and the use of communal latrines on territory boundaries (Rogers et al., 1998). These factors influence the rates of contact and are likely to contribute to the transmission of disease between neighbouring social groups.

1.31 Badgers and bovine TB

The first British case of a badger infected with bovine TB was identified in 1971, and thus the species was implicated as a reservoir of infection (Murhead and Burns, 1974). There are no current estimates for disease prevalence in badger populations in England, but in the Krebs trial, 20-25% of badgers removed tested positive for *M. bovis* infection (Krebs et al., 1997). Meanwhile, during a latter survey of RTA badgers found that within the HRA, 15% of badgers tested positive (CI 0.13-0.19%) for *M.* bovis (Anon., 2005). In 2019, carcasses from culled badgers in Cumbria demonstrated a total disease prevalence of 11.1%; ranging from 1.7% in the outer buffer to 21% in the minimum infective area. This was higher than that found in the 'found-dead' badger collection in which only 4.9% of badgers tested positive, though this was in the 'potential hotspot' area in an LRA rather than in a designated HRA. In Gloucestershire, 17.7% of badgers collected from the culls tested positive for *M. bovis* by *post mortem* (Anon., 2019e). These data suggest that the prevalence of disease in badgers has remained relatively consistent over the last 20 years of sporadic testing.

Circumstantial evidence for the role of badgers in the transmission and maintenance of bTB in cattle populations was provided by areas of highest badger population density correlating strongly with high herd breakdown numbers (Barrow and Gallagher, 1981, White, 1995). More recently, spoligotyping of bacterial isolates demonstrated common strain types in neighbouring cattle and badger populations while whole genome sequencing (WGS) identified that there was a maximum of four single nucleotide polymorphisms (SNPs) between strains isolated from sympatric cattle and badgers (Biek et al., 2012, Barron et al., 2018). Though a larger SNP

database is required, the WGS study on 5 herds (26 isolates) and four roadkill badgers (5 isolates) indicated that genetic lineages persisted locally though could not infer directionality (Biek et al., 2012). Alongside local badger culling influencing the number of new breakdowns and total number of herds infected with bTB, this evidence provides a strong case for the involvement of badgers in localised disease maintenance and transmission (Donnelly et al., 2003).

1.4 The pathology and pathogenesis of tuberculosis in badgers

Badgers are highly susceptible to *M. bovis* and infection is generally transmitted by aerosolisation and subsequent inhalation of bacilli (Gallagher et al., 1976, Corner et al., 2007). *Post mortem* examination has detected gross lesions in circa 13% of culled badgers and bacteriological analysis of pooled tissue samples detected *M. bovis* within 36.3% of culled badgers (O'Boyle, 2003, Murphy et al., 2010).

Differences in disease development are thought to be linked to the milder innate immune response exhibited by badgers towards *M. bovis*. This was demonstrated experimentally by badger monocytes failing to produce nitric oxide in response to being challenged with *M. bovis* (Bilham et al., 2017). Initial infection is subclinical with no visible lesions or symptoms (NVL), progressing to a mild disease state with at least one visible lesion circa 1-2 mm in diameter. Visible lesions are most frequently located in the tracheobronchial and broncho-mediastinal lymph nodes as well as the lungs (Murphy et al., 2010). Unlike cattle, at *post mortem* a significant number of badgers will yield positive cultures of *M. bovis* from lymph nodes yet display no visible lesions and thus have been in a potential state of latency (Gallagher et al., 1976, Murphy et al., 2010). There is also a high frequency of secondary spread, with disease frequently being detected within the kidney; this is hypothesised to be in part responsible for the high bacterial counts in urine (Gallagher et al., 1998). This is suggestive of dissemination during early infection, prior to the development of an adaptive immune response (Corner et al., 2012).

In badgers which have been infected via the aerosol route the disease will generally remain latent, however a secondary transmission route via biting behaviour contributes significantly to *M. bovis* transmission (Gallagher et al., 1998, Murphy et

al., 2010, Corner et al., 2012). Infection via bite wounds manifests in more significant disease progression with the formation of subcutaneous granulomas, abscesses and large areas of ulceration as well as increased dissemination (Gallagher et al., 1998, Corner et al., 2011).

1.5 The pathology and pathogenesis of tuberculosis in cattle

Inhalation remains the most common route of infection and results in lesions of the nasopharynx, tonsils, dorso-caudal apex of the lung and the lymph nodes associated with these regions (Neill et al., 2001, Van Rhijn et al., 2008). Most other potential routes for direct cattle-to-cattle transmission have little significance due to *M. bovis* usually being contained within the lungs (McIlroy et al., 1986, Domingo et al., 2014). That said, faeces may also pose a transmission risk due to the swallowing of bacilli from the sputum or tracheal mucus of the bovine (Williams and Hoy, 1927, Stenhouse Williams and Hoy, 1930, Neill et al., 1988).

Lesions develop due to the immune system response through the activation of macrophages by proinflammatory cytokines; similarities in structure and distribution have been observed between human and cattle lesions (Palmer et al., 2007, Alvarez et al., 2009, Metcalfe et al., 2010, Waters et al., 2010). The body will form a mass of epithelioid histiocytes enclosed by a further layer of lymphocytes in order to prevent widespread dissemination. Once contained within this, an outer layer of macrophages and neutrophils will form which is then further bound by a layer of fibroblasts (Figure 1.4) (Flynn et al., 2011, Guirado and Schlesinger, 2013). As the disease progresses and overcomes the immune response, the distribution of the lesions becomes miliary and will amalgamate to form large areas of necrotic tissue and caseation and disseminate to other organs (Gallagher et al., 1976, Fagan, 1993, Murphy et al., 2010). However, in countries such as the UK with ongoing control programmes the majority of disease is detected in the early stages pre-dissemination, with lesions located within the lymph nodes associated with the respiratory tract and with a lower frequency found in the lung parenchyma (McIlroy et al., 1986, Domingo et al., 2014).

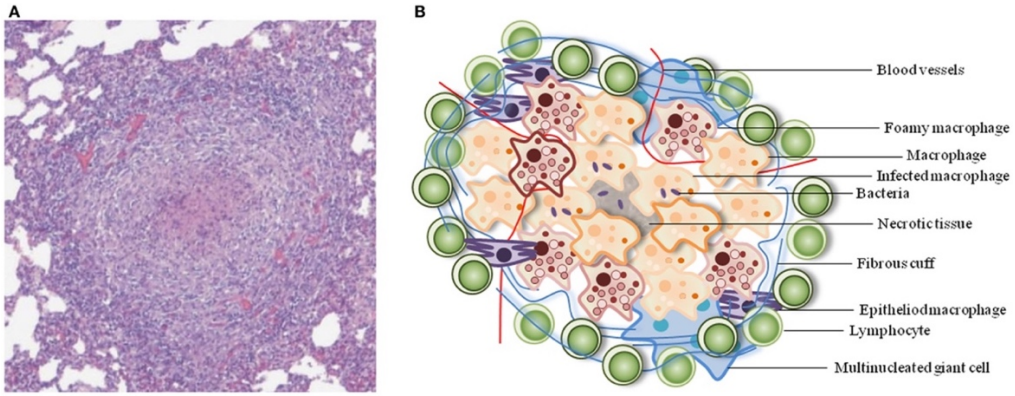

Figure 1.4: Infection of cattle with M. bovis has been suggested to be a potential model for TB in humans (Waters et al., 2010) . Typical structure of a TB granuloma, as occurs in humans (Guirado and Schlesinger, 2013).

The length of the latency period in cattle and the factors triggering the progression from latency to active disease is poorly understood. It has been shown to be affected by the age, nutritional state, level of demand being placed on the cow and the level of coinfection. Within the literature, the hypothesised or detected latency period varies between 87 and 226 days in calves or from 6 months up to 7 years in adult cattle (Stoneham, 1986, Neill, 1989, Barlow et al., 1997).

Investigating the presence and prevalence of latent infection is not possible under current slaughter regulations however circumstantial evidence is suggestive of its existence. In a herd which contains a reactor, and assuming that all the animals have been exposed to the reactor, a maximum of 30% of the animals will display any clinical signs of infection suggesting either clearance, or latency (Phillips et al., 2003). Furthermore, animals testing positive for *M. bovis* have been found many years after the completion of a test-and-slaughter regime despite no herd introductions (Cousins, 1998). Clinical evidence is based largely on assumptions however, in humans the development of latent infection is associated with caseation; while it has not been definitively proved that there are dormant bacteria in cattle, caseous tubercles that progress towards cavitation have been found at *post mortem* (Alvarez-Herrera and Flores-Valdez, 2014).

Infected animals may also be capable of clearing the infection, supported by evidence of bovines being SICCT test or IFN- γ positive but displaying no clinical signs, NVL at slaughter or are culture negative (Pollock et al., 2000). To summarise, there is no set-progression-route for the disease, and research suggests that it could be cleared, remain latent, progress slowly or be fatal within months (Phillips et al., 2003). Our understanding of the factors that determine the route which infection takes is limited, but housing conditions and sanitary standards are thought to be key determinants (Francis, 1947, Phillips et al., 2003).

1.6 Detection in cattle - the SICCT

The SICCT (single intradermal comparative cervical tuberculin) is the licensed method of testing cattle exposure to *M. bovis* and has been compulsory in the UK since 1950 (Macrae, 1961). This test is performed by veterinarians or licensed lay testers who inject 0.1 mL of purified protein derivative (PPD) from *M. bovis* into the dermal skin layer in the neck of the bovine, as well as a control injection to reduce the risk of false positives associated with cross reactivity with *Mycobacterium avium paratuberculosis* (*map*) (Buxton, 1928, Goodchild et al., 2015). If the animal has been exposed to *M. bovis* then a swelling will form within 72 hours which is comparatively larger than the swelling at the *map* injection site. Variations in the classification of animals' reactivity status exist depends on whether the standard or severe interpretation is applied. Furthermore, while the standard interpretation is the same across England, Scotland and Wales, when the severe interpretation is applied there are stricter rules enforced in Wales than in England (Summary Table 1.1) (APHA, 2019a). Differences in the severity of interpretation are applied depending on the relative risk in the area; if the area is of high risk then the severe test interpretation is applied which increases the sensitivity of the assay but increases the likelihood of detecting false positives. In areas of high risk, the possibility of a false negative is considered more costly in the longer term than that of the removal of false positives, therefore the trade-off is weighted in favour of increased sensitivity.

Table 1.1: Summary of interpretations of the SICCT across England, Scotland and Wales.

	Standard (England/Scotland/Wales)	**Severe (England/Scotland)**	**Severe (Wales)**
Pass	1. Negative bovine reaction and positive or negative avian reaction. 2. Positive bovine reaction ≤ avian reaction.	1. Negative bovine and avian reactions. 2. Positive bovine reaction ≤ positive avian reaction.	1. Negative bovine reaction. 2. Positive bovine and avian reactions where avian reaction is > 2mm larger than bovine.
Inconclusive (retest)	Positive bovine reaction ≤ 4mm larger than positive or negative avian reaction.	Positive bovine reaction ≤ 2mm greater than positive avian reaction.	Positive bovine and avian reaction; bovine reaction is within 2mm of avian reaction reading.
Fail (remove reactor)	Positive bovine reaction > 4mm greater than negative or positive avian reaction.	1. Positive bovine reaction and negative avian reaction. 2. Positive bovine reaction > 2mm greater than positive avian reaction.	1. Positive bovine reaction and negative avian reaction. 2. Positive bovine reaction > 2mm greater than positive avian reaction.

In the UK, the SICCT is considered to be the most appropriate method for detecting *M. bovis* infection in live cattle (with a compromise reached between the number of false positives and false negatives), however the test and its operators are not infallible. At best, the test is estimated to be 70-89% sensitive when determining the infection status of an individual; therefore in a herd containing at least one positive animal, there is a 11-30% probability that the animal will not be detected (Karolemeas et al., 2012). The sensitivity of the test can be compromised by immunosuppression or anergia as well as pregnancy, early lactation or contemporaneous disease (Monaghan et al., 1994). The test can also be compromised by the veterinarian's operating procedure, or by the batch of protein antigen - although steps are being made in order to improve control systems for the production of purified protein derivative (PPD) (Downs et al., 2013). It is also noteworthy that the specificity of the SICCT decreases as the herd size increases and is vital in our assessment of the test under current farming practices as the average herd size has increased from 75 adult cows per farm (2004) to 143 adult cows in 2016 (AHDB, 2017). There are also concerns regarding the classification of cattle with swellings between 1 and 4 mm as 'intermediate reactors' (or IRs) and any animals presenting as such should be re-tested within 42 days (Anon., 1964, Green, 2005, Karolemeas et al., 2012).

For nearly thirty years following the introduction of routine cattle testing there was a decrease in the number of cattle being classed as reactors by the SICCT. In 1979, the lowest prevalence of bTB in GB was reported, with 0.49% of herds containing a reactor and 0.018% of cattle testing positive (Anon., 2013). However, since the 1980s, there has been a steady increase in numbers and expansion radiating from the South-West of England; this was particularly noticeable after the foot and mouth disease outbreak in 2001 (Figure 1.5). In the 12 months to the end of March 2019, 6.2% of herds in England tested positive for bTB, with an average of 11.7% in the South-West of England (Anon., 2019b). The disease is considered to be endemic in West and South-west England, as well as parts of Wales (Brunton et al., 2015). Compulsory TB testing schedules are specific to the area of the UK in which the farm is based. In the low-risk area (LRA) it is a 4 yearly testing regime, and annual in the high-risk area (HRA). Edge areas have now been divided with either annual or six-monthly testing depending on whether a lesion/culture positive TB herd is

within 3 km radius of the farm. All keepers on 6 monthly or annual testing intervals also must comply with pre-movement testing regulations (APHA, 2017a). In systems designed to test more frequently in areas believed to have a higher prevalence, and less so in areas of perceived lower prevalence, an epidemic can be hidden by bias (Medley, 2003). Similarly, increasing the frequency of testing can result in the appearance of an epidemic due to increased detection. This presents a significant challenge for data analyses and means that interpretation of levels of 'success' attributable to any one method must be delivered with caution (Green, 2005).

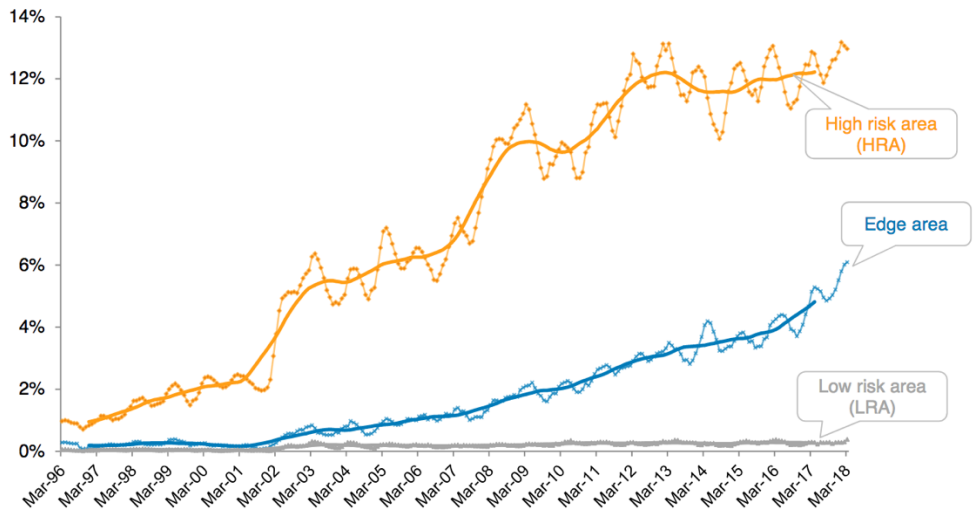

Figure 1.5: Number of herds under disease restriction as a percentage of registered and active herds across the HRA, EA and LRA. Bold lines indicate the 23 month centred rolling averages (Anon., 2018c).

1.7 Wildlife control: the badger cull

Badger culling became an integral (though unmonitored) part of the government's control strategy in 1973 and continued until the RBCT was established in 1998 to determine the effect of badger removal on local and neighbouring bTB prevalence in cattle. The trial found that bTB incidence decreased by 23.2 % (95% CI: 12.4 – 32.7%) within the cull zone but was offset by a 24.5% (95% CI: -0.6% - 56% higher) increase on the land up to 2 km beyond the cull zone, dubbed the perturbation effect (Bourne, 2007). In order to see an overall beneficial effect, the RBCT recommended cull zones of at least 200 km^2. When the current badger culling strategy was launched in Gloucestershire in 2013 it included smaller target areas at just 150 km^2, with the aim of removing 2900 of the estimated 3644 badgers (79.5%). However, just 708 badgers were removed within this first year totalling 19.4% though this was later revised to 30% when original population estimates were adjusted to 2350 badgers (IEP, 2014). Over the following years, the cull has been expanded to more counties in the HR and edge areas and more zones included and in 2018, 32,601 badgers were culled across 30 zones (Anon., 2017, Anon., 2018e).

Current approaches for determining the success of the cull are rooted in the target outcome – a reduction in the prevalence of herd breakdowns within the cull zones (APHA, 2018). There has been little success from any attempt the UK Government has made to establish badger infection levels either prior to or after a cull due to unsuitable sample storage methods. Results from Ireland indicate that, in line with the reduction of bTB in cattle, culling reduced infection in local badger populations from 26 to 11% in the years spanning 2007 to 2011 (Byrne et al., 2015). However, large-scale culls are considered to be unsustainable and it is unclear what the maintenance level of infection would be in areas capable of supporting large badger populations even if the disease were eradicated from cattle. The current badger culls are costly and as of yet do not yield results of any significance (APHA, 2018), however it is likely to take at least 4 years of continuous culls to yield meaningful results with the strongest effect hypothesised to be detectable post the cessation of culling (APHA, 2018, Jenkins et al., 2008).

1.8 Detection of badger infection

There are two states to consider when assessing whether an animal poses a risk: whether the animal is infected, and whether the animal is infectious. To firstly address the question of whether an animal is infected, there are two lines of testing to be considered. Firstly, there is trap side sampling for *in vivo* testing by licensees, as badgers are a protected species under European regulations and the 1981 Countryside Act. Secondly, is by examination at *post mortem* for the presence of lesions and/or culture of routine laboratory samples (Murphy et al., 2010). Unlike cattle, there is no enforced or routine testing method.

1.81 Invasive testing

Immunoassays are currently the recommended method of trap side diagnosis and are either based on detecting IFN-γ from lymphocytes that have previously been exposed to *M. bovis* or previously by the use of the Brock Stat-Pak assay (now discontinued) based on a variety of antigens. The methods have sensitivities of 57-85% and 49-59% respectively which are influenced by the stage of the disease, age and immune state of the individual tested e.g. whether the badger has become anergic (Greenwald et al., 2003, Dalley et al., 2008, Chambers et al., 2009). Also when assessing the prevalence of disease in a population there is bias introduced by the likelihood of catching an individual badger which varies by trapping method, age and season (Tuyttens et al., 2001, Byrne et al., 2012). Cage trapping has highly variable levels of success and for seven out of ten areas there were rates between 71 and 85% of the hypothesised total population. The remaining areas achieved capture rates of between 35 and 46%, with success thought to have been limited by adverse weather conditions or trap interference (Smith and Cheeseman, 2007). These values are corroborated by recent results on the success of cage trapping during cull operations in which a range of 40-70% is cited (Jones, 2018). It is therefore likely that a substantial percentage of the population will not be surveyed by the use of trap side testing alone.

1.82 Non-invasive testing

The presence of infection, does not necessarily mean that the animal is excreting the organism. While there is verbal and photographic evidence of badgers and cattle interacting, studies indicate that the two species infrequently come into contact but do utilise the same areas of space; this suggests that indirect transmission as a likely source of infection in herds living near infected badgers (Benham and Broom, 1989, Woodroffe et al., 2016). Assuming little direct interaction, this thesis focusses on the risk of environmental transmission via detection in environmental samples.

1.83 Culture

Faecal culture in combination with that of other clinical samples will exhibit a sensitivity of less than 8.0% (Drewe et al., 2010). Currently there is no true consensus method for the isolation of *Mycobacterium spp.* from complex sample types, with many being listed in the literature (Allen, 1991, Morris et al., 1993, Livanainen, 1995, Radomski et al., 2010, Chambers et al., 2017). All methods use strong chemicals at variable concentrations and contact times to remove less resilient non-target organisms. The method used for the above study was only briefly outlined in the paper but included the decontamination of the sample with 10% oxalic acid ($C_2O_6H_6$), centrifugation and inoculation of the pellet on to triplicate modified Middlebrook 7H11 agar slopes. The low sensitivity of the method is attributed to loss of cells by sensitisation due to harsh chemical treatment, clumping of mycobacteria and presence of viable-but-non-culturable cells (Loebel et al., 1933, Allen, 1991, Sweeney et al., 2006).

1.84 Quantitative PCR

This method has low sensitivity if incorrectly applied as a diagnostic method rather than as a method to establish the infectiousness of a group, as it is assumed that only animals exhibiting more advanced disease will be actively excreting bacilli (Gallagher et al., 1998, Corner et al., 2011). Quantitative PCR is a method used to enumerate target organisms by detecting specific sequences within their DNA and comparing the amplification reaction to a standard curve including known numbers of genome equivalents. Using the detection of specific targeted sequences as a proxy

for the presence of *M. bovis* in environmental samples negates the requirement for long and laborious culture methods which also greatly increase the number of viable organisms and risk within the category 3 facilities. While qPCR is limited by its inability to distinguish between viable and non-viable organisms, it is far more sensitive than traditional culture-based techniques (Figure 1.6) and provides a 'worst-case-scenario' if it is assumed that all detected genomes are from viable cells (Nocker et al., 2007).

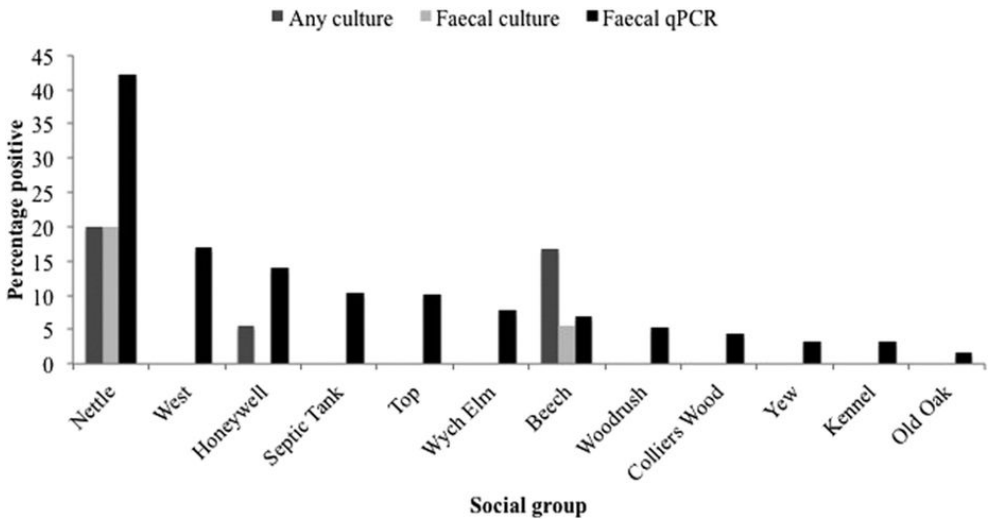

Figure 1.6: Detection of M. bovis by the culture of both tracheal and faecal samples, by the culture of faecal samples or by the faecal qPCR on samples (King et al., 2015a)

1.9 Cattle infection

The most probable transmission route is unknown, other than the principal method proposed being via aerosolisation and subsequent inhalation (Phillips et al., 2003). It is likely that a multitude of factors contribute towards transmission and persistence of bovine tuberculosis in natural, sympatric populations. This section will address a number of the most likely factors, as well as the hypothesised involvement of badgers within this transmission cycle.

1.91 Transmission to cattle

Aerosol transmission has been proposed as the most likely route, firstly because the majority of tuberculin reactor cattle will produce positive cultures from the broncho-mediastinal and lymph nodes associated with the respiratory tract. Secondly, because the minimum infective dose via aerosol is potentially as low as one bacillus, whereas by the oral route several million are required to establish an infection (Chausse, 1913). However, the possibility of aerosolisation of *M. bovis* from infected material during feeding or sniffing cannot be ruled out; nor that the development of respiratory lesions may simply happen more rapidly than the mesenteric, but the disease development is cut short due to current testing regimes.

Badgers have been implicated in both the introduction and maintenance of local disease (Godfrey, 2018, Anon., 2019e). The overall contribution of badgers to the likelihood of an individual bovine testing positive is approximately 50%, with 5.7% due to direct transmission and the remainder resulting from onwards cattle-to-cattle spread (Donnelly and Nouvellet, 2013). Environmental contamination is the biggest contributor to the infection of an individual and hypothesised to be responsible for 15% of new breakdowns (Brooks-Pollock et al., 2014). However, the single largest factor responsible for 84% of new herd breakdowns is the movement of cattle or the consequences of having moved these cattle (Brooks-Pollock et al., 2014). The roles of both cattle and badger in the transmission of disease will be discussed in the following paragraphs.

1.92 Direct badger-to-cattle transmission

Studies suggest high levels of badger infection in areas of high cattle infection, but the current evidence demonstrating direct transmission from badgers to cattle is under highly artificial situations. The only known study contained three calves and eight infected badgers housed in one pen, and nine calves and thirteen infected badgers housed in another. After six months, all the cattle were found to be tuberculin reactors and displayed lesions in the retropharyngeal, pulmonary and bronchial lymph nodes; this distribution is suggestive of aerosolised droplet infection rather than ingestion (Little et al., 1982b). However, this prolonged proximity and duration of contact is entirely artificial and merely demonstrates the possibility, not the plausibility of direct transmission. If this were to be applied to a natural situation, transmission will be inhibited by cattle actively avoiding badgers, preferring to remain at distance of 15-20 m, while the hypothesised distance required for aerosol transmission is 1.5 m (Benham and Broom, 1989, Sauter and Morris, 1995). Under natural conditions, there is only circumstantial evidence that badgers are capable of transmitting the disease to cattle. For example, the risk of a breakdown is increased by having an infected badger sett within one km of the herd but the relationship was not strong and would only explain 9-19% of breakdowns (Martin et al., 1997).

1.93 Indirect badger-to-cattle transmission

The role of indirect transmission is less clear; while many hypothesise that this is the most likely route of transmission, once again the evidence is circumstantial. Badgers shed *M. bovis* intermittently in their urine, faeces, sputum and pus and it is likely to only be individuals in more advanced stages of the disease (Clifton-Hadley et al., 1993, Allen et al., 2011). Sputum and pus are less likely to be encountered by cattle and thus most research focuses on the impact of more frequent excreta.

It is thought that due to the slow progression of the disease in badgers, lesions will contain high numbers of bacilli relative to that which are seen in animals with stronger immune responses (Gallagher et al., 1976, Bilham et al., 2017). These high counts can be produced in lung discharges or swallowed by the infected animal resulting in contamination of the faeces and subsequent environmental

contamination (Little et al., 1982c). Furthermore, significant numbers of lesions are discovered in the kidneys of infected badgers providing another potential route for excretion into the environment (Gallagher and Clifton-Hadley, 2000). An average urine sample from an infected individual contains approximately 3 x 10^6 bacterial mL^{-1}, and an average faecal sample circa 7.5 x 10^4 – 4 x 10^5 bacteria g^{-1} (Anon., 1979, King et al., 2015a). While badger faeces are usually concentrated into latrines urination patterns are less predictable, with badgers capable of producing trails greater than 0.5 meters in length (Brown, 1993). However, cattle actively avoid grazing land contaminated with badger urine and faeces when there is suitable herbage available elsewhere (Smith et al., 2008, Benham and Broom, 1991). Occasionally, despite non-contaminated land being available, some cattle are willing to graze close to both types of excreta and are likely to be animals that are lower ranking with less access to more preferential feeding grounds (Hutchings and Harris, 1997). High density stockings reduce the availability of suitable land and forces less-dominant animals onto the less preferential ground, with levels of avoidance decreasing as time since excretion increases (Hutchings and Harris, 1997, Benham and Broom, 1991). This may be an increasingly important factor with current housing densities and increasing herd sizes (AHDB, 2017). It must be noted that more dominant cattle may also demonstrate greater amounts of sniffing and interest in the potentially infectious material and increase their transmission risk by potential aerosolisation from infective material (Sauter, 1995).

1.94 Direct cattle-to-cattle transmission

Current testing regimes are likely to result in most infected cattle being removed in the early disease stages prior to development of a highly infectious state (Phillips et al., 2003). However due to imperfections with the current test and its application there is a chance that infected animals will be undetected (de la Rua-Domenech et al., 2006). It has been suggested that the maximum immune response develops between 8 and 65 days post-infection though this will be influenced by a multitude of confounding factors (Kleeberg, 1960). However, animals kept under good husbandry conditions are unlikely to excrete significant numbers of bacilli until between 4 and 9 months post infection (O'Reilly and Daborn, 1995). The current regime includes risk-based testing depending on the location of the farm, the

purchase of high-risk cattle and whether the farm had a reactor within the last compulsory testing cycle. If a reactor or intermediate reactor is discovered the farm enters a cycle of three-monthly testing until the farm is clear of reactors. This includes either resolution of IR cattle as either being non-reactors on the re-test or a second IR reading resulting in the removal of the bovine (APHA, 2019a). Due to the risk-based intervals, there is a reasonable level of confidence of detecting infectious and excreting cattle prior to them posing a significant risk to their cohort unless they have entered an immunosuppressive state in which case the reaction to the SICCT is reduced (Lepper et al., 1977, Houlihan et al., 2008). In recent decades there has been a greater tendency to house cattle, despite the knowledge that turning cattle out on to pasture results in a significant decrease in the likelihood of within-herd transmission (Garner, 1946, Johnston et al., 2005). That said, animal nutrition and health and hygiene standards have also improved and adapted since this publication in order to support this lifestyle, and cattle testing was not as frequent as it is today and therefore animals would likely have progressed to a more infectious state. Regardless, the area provided to cattle in housing is smaller than that which would routinely be provided out on pasture, as well as intrinsically being less ventilated.

Sharing a common boundary with an infected herd significantly increases the likelihood of a breakdown occurring. In two studies, 23% and 25% of breakdowns were attributable to lateral disease spread, though this cannot be attributed to cattle-to-cattle or with badgers or other environmental factors acting as a potential intermediate in the transmission cycle (Griffin, 1995, Munroe, 1999). The combination of lateral between herd spread, the prevalence of short cattle movements to neighbouring farms, and the overlapping of badger territories over multiple farms are all likely to contribute to the radial spread demonstrated in the South-West of the UK.

1.95 Indirect cattle-to-cattle transmission

Cattle have been shown to acquire *M. bovis* infection when placed on pasture previously grazed by infected cattle (Schellner, 1956). Cattle excrete small numbers of bacteria in their faeces, though the burden is not suspected to be heavy both due to the rapid innate immune response and the volume of faeces diluting the number

per gram to below the detection limit of the tests. In 1955 prior to the current testing regimen, the proportion of cattle that excreted *M. bovis* in their faeces was circa 10%, though could be as high as 80% (Reuss, 1955). However, it is broadly assumed that, as in badgers, only bovines that are demonstrating advanced clinical disease will excrete *M. bovis* which under current testing regimes are likely to be removed prior to this stage of disease (Phillips et al., 2003).

A key aspect in the likelihood of transmission is the likelihood of the cattle coming into contact with infective sources. Similar to the avoidance observed with badger faeces, cattle will initially avoid grazing in close proximity to cattle faeces however this is dependent on the availability of alternative herbage (Broom, 1975). The amount of pasture that will be covered in cattle excreta depends on the density of cattle and frequency of rotation practices. The average herd size has been on an upwards trend (Figure 1.7) and current estimates of faecal coverage are not available (AHDB, 2017). However, when studies were conducted, faecal coverage was estimated to reach circa 2-4% of total available pasture towards the end of the grazing season (Arnold, 1958, Greenhalgh, 1968).

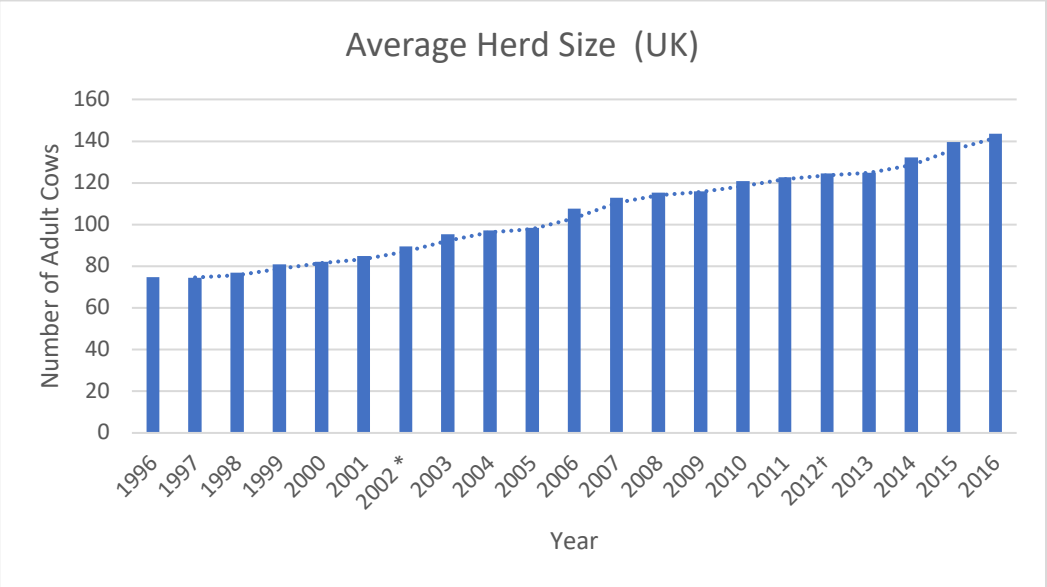

Figure 1.7: Average herd size in the UK (1996-2016) with rolling average indicated by the dotted line (AHDB, 2017). Asterisk marks the year of foot and mouth disease during which routine bTB testing was suspended for 10 months (Moustakas et al., 2018).

Sources other than contaminated pasture are plausible; *M. bovis* has been detected by qPCR detection methods in natural spring water on pasture in France that is grazed by cattle and frequented by wildlife (Barbier et al., 2016). Drinking water also regularly harbours NTM which can cause infection in humans (Falkinham, 2011). *M. bovis* can persist in water, as it does in a variety of other matrices, and has been shown to do so for up to 58 days (Fine et al., 2011b). However, this study used artificially inoculated samples with high levels of mycobacteria and used 'time until last positive' so this may not be realistic under natural conditions and bacterial loads. Contaminating cells in water are likely to be highly dilute and starved of bioavailable compounds unless concentrated at the bottom of the trough in organic matter (Chausse, 1913, Whittington et al., 2004). However, cattle splashing during drinking could provide a route of entry into the respiratory pathways of the cow as mycobacteria are highly hydrophobic, and this property lends itself to transferring from water to air (Blanchard and Syzdek, 1970). If it is possible for cattle to aerosolise the bacteria while drinking it would hugely reduce the hypothesised infectious dose and increase the viability of this transmission pathway.

1.96 How advances in WGS could be applied

Whole genome sequencing (WGS) has identified clusters of human TB with better resolution than that of historical methods that focus on defined regions of the mycobacterial genome (e.g. mycobacterial interspersed repetitive unit variable number tandem repeats) (CDC, 2015, Alaridah et al., 2019). Steps have been made towards the WGS of samples collected from infected badgers in Gloucestershire (Anon., 2019e). Of the 23 isolates from culled badgers, five genotypes were identified which were also represented within the local cattle populations, though one isolate was found beyond the 'home range' of that genotype. Three additional badger isolates had sequences that were identical (or very closely related) but were not found within cattle, indicating possible badger-to-badger transmission. However, the reduced number of cattle isolates due to increased surveillance decreasing the presence of visible lesions and ease of culture is likely to have reduced the potential for identifying the genotype in sympatric cattle.

If a WGS database were to be established and related to the proposed database for cattle movements this could provide greater understanding of transmission dynamics within cattle populations (Godfrey, 2018, van der Werf and Ködmön, 2019). The collection of sequences and their associated metadata over a period of time could provide an epidemiological clock sufficient to ascertain the direction of transmission, as has been done in clinical studies. Introducing data from sympatric badger populations (either those culled or the result of RTAs) or other wildlife from which *M.* b*ovis* sequences are obtained will provide additional clarity regarding within-species evolution and transmission.

This method is currently being developed and applied within studies conducted by the APHA on culled badgers. Data from the Cumbrian cull zone demonstrated 22 unique clades within the culling area and, though not yet complete, is establishing this epidemiological clock (Anon., 2019e). Clade A is the most likely the common ancestor of the other 21 clades and was found in cattle and badger populations (along with two other cross-species clades). A further fifteen clades were identified in badgers only, and four in cattle (though less sequences were available from cattle due to the aforementioned reasons). While it is extremely likely that there is interspecies transmission occurring (as well as within species transmission), it is not yet possible to infer directionality from the above data. Increased surveillance of badgers and the development and implementation of more sensitive methods for the sequencing of *M. bovis* DNA from cattle will be required in order to appropriately infer directionality by the removal of missing links when analysing SNPs in the bacteria WGS data.

1.10 Study Aims

Large gaps remain in our knowledge of *M. bovis* transmission, and the role of the environment in transmission and maintenance of local infection. While studies have examined the spatial relationship of infection in badgers, this has been at a distinct time point (i.e. during a cull), and thus the stability of infection, and the effectivity of a non-invasive sampling methods for detecting infection has not yet been trialled. Furthermore, the time for which the sample contains detectable levels of *M. bovis* has been studied under variable conditions and the interaction between the naturally infected samples and complex meteorological factors is poorly understood. The aims of this project were as follows:

1. To adapt a viability qPCR method for the purpose of environmental detection while eliminating the discussion surrounding the viability of cells within the sample and increasing the sensitivity of detection from current culture-based methods.
2. To collect fresh badger faecal samples from a chronically infected dairy farm within the HRA to explore the distribution and stability of environmental *M. bovis* within and between badger social groups.
3. To determine the decay of *M. bovis* in naturally infected badger samples under natural environment conditions across the seasons.
4. To adapt the immunomagnetic capture method for the isolation and concentration of *M. bovis* cells from badger faeces.

1.11 Statement

Over the last four years I have been exposed to a range of farms and situations which have given me first-hand experience of the heartbreak and suffering of farmers as a result of the current bTB crisis. As a result of this, my dissatisfaction with current policy and legislation has grown, including both the range of approaches taken but also the lack of incorporation of fundamental mycobacterial biology into the information provided to farmers. This thesis is the culmination of work founded on my desire to examine the disease at the farm-level, and to create research which can be directly applied to the people who the science is funded by and meant to support.

Chapter 2:

Investigation of approaches for the quantification of viable *M. bovis* in naturally infected badger faeces.

2.1 Abstract

Distinguishing between viable and non-viable bacteria presents a major challenge when attempting to understand complex disease dynamics and epidemiology. In the case of *M. bovis*, the majority of studies focus on the use of standard laboratory culture as the gold-standard for definitive proof of the presence of viable bacteria within a sample. However, the sensitivity of culture, even from relatively clean samples that require little or no decontamination, is low due to aggregation of hydrophobic bacilli and the presence of viable but not culturable bacteria. Furthermore, culture is extremely time consuming in processing stages and can take months for colonies to form on standard agar. The aim of this study was to optimise a viability-qPCR assay for use in environmental samples by the assessment of two membrane impermeable dyes that are capable of intercalating with DNA (PMA and PMAxx™). It was found that for the application of these methods to environmental samples that there needed to be a large decrease in sample turbidity as well as increased final concentrations of the dye. Due to the increased expense associated with the final method, opposed to that recommended by the manufacturer's for application to culture, this method was not suitable for application for later elements of the project.

2.2 Introduction

Our current understanding of *M. bovis* transmission within and between species is limited. While it is possible to take measures to prevent the active mixing of known-to-be infected individuals and susceptible individuals, it is harder to prevent indirect mechanisms of transfer, particularly when they have not yet been fully elucidated. Badger faeces has been widely implicated as a reservoir of *M. bovis* and a potential indirect transmission mechanism between badgers and cattle (Clifton-Hadley et al., 1993, Allen et al., 2011, Pontiroli et al., 2011, King et al., 2015a). However, the two current methods of faecal mycobacterial quantification produce widely varied results; viable count by culture is likely to underestimate the number of viable bacilli, whereas qPCR is likely to produce an overestimation (Adams et al., 2013, Carini et al., 2016, MacDougall et al., 2018).Therefore, it is important that novel methods of environmental bacterial quantification are considered while seeking to assess levels of environmental contamination for the purposes of biosecurity implementation strategies or assessing the effect of badger control methods (i.e. culling and vaccination) on levels of environmental *M. bovis*.

2.21 Why not culture?

The discovery of the 'viable but not culturable' (VBNC) state was a significant turning point in our understanding of the microbial world (Xu et al., 1982). Having been shown across many bacterial genera, it is now widely accepted that cells exposed to environmental stresses, can remain metabolically active but lack the ability to divide under standard laboratory conditions without resuscitation (Shleeva et al., 2002, Mukamolova et al., 2010, Ramamurthy et al., 2014). This state has been well studied in the context of mycobacteria and many models have been established for the routine, *in vitro* production of such cells (Mukamolova et al., 2010, Alnimr, 2015). Two models, and their derivatives, dominate the literature; the Wayne model focuses on the depletion of oxygen in a nutrient rich environment, while the Loebel model (or later adapted Corper and Cohn experiment) uses nutrient deprivation in an oxygen rich environment (Loebel et al., 1933, Wayne, 1986). These models permit the investigation of the persistent state of mycobacteria which is key to their

long-term *in vivo* survival, as well as their decreased susceptibility to first-line antibiotics within this state (Sarathy et al., 2013).

The existence of persistent *M. tuberculosis* cells is accounted for in both research and clinical treatment. However, little work has concentrated on how VBNC cells could influence the persistence and transmission of disease in the environment. *M. bovis* transmission in livestock is hypothesised to be partly due to indirect mechanisms (Brooks-Pollock et al., 2014, Woodroffe et al., 2016). The application of methods for investigating biocontamination must account for the highly flexible strategies mycobacteria can employ to survive harsh environments, and how they impact on the sensitivity of detection methods. The following paragraphs will discuss why the gold standard method (culture) is unsuitable for the application to environmental *M. bovis* detection.

Achieving a reliable viable count relies on the existence of a population of cells that are active, replicating, and non-clumped in state. However, many of the stresses to which badger-excreted cells are exposed to can greatly limit the sensitivity of the assay, with detection levels proposed to be circa 8% (Drewe et al., 2010). Firstly, the badger GI tract would hypothetically present as a suitably hostile environment so as to activate the suite of persistence genes in *M. bovis* (Oliver, 2010). The bacilli would be subjected to large and rapid fluctuations in pH as they passed through the badgers' GI tracts, being exposed to pH levels of 2 for several hours before rapidly being returned to alkaline conditions, pH 8 (Williams et al., 2019). While recent literature yielded little information regarding the effect of acidic conditions on mycobacterial culture, it was discussed during the establishment of the Loebel model in which high concentrations of hydrogen ions induced mycobacterial dormancy (Loebel et al., 1933). Extrapolation of the effect on the acidic phagosomes (as low as pH 3.8 recorded) of activated macrophages on *M. tuberculosis*, also leads to the conclusion that the decrease in pH experienced within the GI tract would be sufficient to decrease the population of cells detectable by traditional culture methods (Vandal et al., 2009).

Furthermore, within the GI tract, bacilli will have to compete with the normal microbiome of the badger. While the gut bacteria of badgers have not been studied

in great detail, a recent paper examining the effect of Lactobacillus faecal isolates from badgers showed that all 40 of the isolated strains were capable of secreting antibiotics that exhibited bacteriostatic or bactericidal effects on cultures of *M. smegmatis* and *M. bovis* BCG (Stedman et al., 2018).

Assuming the cells had remained both viable and culturable during their passage through the GI tract, the bacteria would then be subjected to nutrient deprivation in a rapidly altered habitat. While faeces are nutrient rich environments, information regarding the bioavailability of nutrients to microorganisms is limited, and conclusions are therefore being drawn from the extrapolation of data from sediment and soil. Morita discussed the existence of a starvation-survival state and drew upon evidence that in sediment, the amount of available carbon is approximately 0.5 - 1.0% of the total organic carbon (Morita, 1990). While hypothetically in a utilisable state, the amount which is biologically available is likely to be lower; for example, alanine in nearshore sediment was demonstrated to exist in a ratio of 1:80 for bioavailable to non-bioavailable (Bakken and Olsen, 1987, Morita, 1990). It is hypothesised that in soil, the amount of available energy would limit microbial biomass to 0.4 - 5.5 generations per annum (Morita, 1990). Cell maintenance requires approximately 2% of the cell's carbon biomass in organic carbon per year; therefore microbes must rely on exogenous catabolism for long-term persistence under environmental conditions (Bradley et al., 2019). The deprivation of elements other than carbon will also induce a dormant state in mycobacteria; for example, phosphate limitation triggers persistence of *M. tuberculosis* within macrophages (Rifat et al., 2009). While no information is strictly available pertaining to this in the context of environmental persistence, phosphate availability in deer, cattle and sheep faeces decreased as the samples were subjected to drying under natural conditions (McDowell and Stewart, 2005). It is therefore suggested that the longer the faeces (and bacilli) reside environmentally, the more likely it would be for the mycobacteria to enter a dormant, and therefore VBNC state, prior to cell death.

The current evidence is weighed in support of the majority of faecally excreted *M. bovis* residing in a dormant or persistent state, making traditional culture an unsuitable method for detecting and accurately quantifying cells, as the method is limited by both VBNC cells and cell clumping. However, if the cells remained active

and viable during their passage through the badger digestive system, they would then be excreted into the environment. Faeces can contain up to 1.0 x 10^{12} bacteria per gram; for the purposes of culture, this high level of competition needs to be removed by decontamination to allow slow growing mycobacteria to both grow and be visible on the agar. However, the compounds used for the decontamination of samples containing mycobacteria rely on the fact that they are less toxic to mycobacteria than to other organisms (Corner et al., 1995). As a consequence, decontamination using sodium hydroxide and oxalic acid (the method used for APHA studies) results in high percentages of cell loss (Corner et al., 1995).

Given these problems with culture, the issue becomes: how do we replace it, and what do we measure new techniques against?

2.22 Viability dyes

While culture will not capture the true breadth of diversity, or provide a true measure of bacterial abundance, it has subsequently been shown that PCR based tests overestimate these figures in environmental samples due to the presence of 'relic' DNA (Carini et al., 2016). Viability dyes, such as ethidium monoazide, propidium iodide and propidium monoazide, are reshaping our current understanding of microbial diversity (Carini et al., 2016). When added to matrices containing cells, these fluorescent dyes penetrate those which have suffered sufficient injury so as to destabilise the polarity of the membrane. In the case of compounds containing an azide group, once within the cell, the dye will intercalate with the DNA and, when exposed to light of the appropriate wavelength, undergo photolysis to release a highly reactive nitrene radical. The radical forms covalent bonds with the cell's DNA, creating structural changes which subsequently inhibits the PCR reaction. This allows for the selective suppression of signal from cells which are no longer intact, and therefore, if using membrane integrity as a proxy for viability, are considered dead for the purposes of this assay (Emerson et al., 2017, Nocker et al., 2006). Viability dyes provide a rapid, specific and high through-put approach to community and organism analysis in the environmental context (Carini et al., 2016).

2.23 Dye selection

The dye most frequently associated with suppression of naked-DNA signals in recent literature is propidium monoazide, an azidified derivative of propidium iodide. Propidium monoazide is a development on the previously used ethidium monoazide (azidified ethidium bromide), and produces less false negative results and exhibits less cellular toxicity than its predecessor (Nocker et al., 2006, Nocker and Camper, 2006). A further development of this molecule is PMAxx™; a commercial dye marketed as a reformulation of PMA with greater powers of cellular penetration, while maintaining the selectivity for which PMA became the dye of choice. The study presented here focuses on the application of PMA and PMAxx™ to environmental samples, with comparison to PI only through fluorescence microscopy as PI lacks the nitrene radical producing azide group capable of covalently bonding with DNA (Taylor et al., 2014).

2.24 Aim

The work by Desneux *et al.* explored many of the factors influencing the efficacy of PMA for the enumeration of viable cells within environmental samples (in their case, piggery effluents). Their work explored the effects of the incubation and light-exposure periods on PMA efficacy, as well as addressing the question of required concentration (Desneux et al., 2015). The current study aimed to establish the effect of various parameters in badger faeces, namely the comparison of light sources, optimisation of concentrations specific for badger faeces, and the comparison of PMA with the company's follow on product, PMAxx™. The purpose was to create a rapid, high-throughput viability assay for the detection of intact *M. bovis* in potential environmental reservoir areas.

2.3 Methods

2.31 Growth *M. bovis* BCG Pasteur

Middlebrook 7H9 + 10% OADC + 0.05% Tween 20 (15 mL) was inoculated to OD 0.05 with a mid-exponential phase culture of *M. bovis* BCG (ATCC® 35734™). The culture was sealed and incubated at 37 ˚C, 130 rpm until the culture reached OD 0.6.

2.32 Killing of *M. bovis* BCG Pasteur

The culture was homogenised and divided into two equal parts. One part was heated at 65 ˚C ± 2 ˚C for 30 mins, frozen overnight at -20 ˚C, reheated and then returned to room temperature immediately prior to use.

2.33 Set-up

Known negative badger faeces (from captive badgers at the APHA, frozen at -20 ˚C and thawed at 4 ˚C) were weighed into 0.1 g aliquots in sterile 30 mL universal tubes and seeded with either 25 µL of *M. bovis* BCG Pasteur or 25 µL STW. Sterile 2% BSA + 0.05% Tween 20 was then added to a final volume of 1 mL, and vortexed for 20 s to create a faecal slurry.

2.34 PMA testing

To determine the optimum concentration of PMA, the appropriate volume of the 20 mM stock solution was added to the faecal slurry to achieve final concentrations ranging from 50 µM (as recommended by the manufacturer's instructions for pure culture) to 250 µM in increments of 50 µM. In addition, a control was included that was not treated with a viability dye. The slurry was vortexed for 10 s and incubated in the dark on a VXR-Basic-VIBRAX® platform shaker (IKA) set to approximately 75 rpm for 30 mins at 4 ˚C. The sample tubes were placed horizontally on top of a reflective foil base and attached with small pieces of tape at the poles. Finally, a 465 nm LED lamp was placed over the samples and the samples were exposed to the light for 45 mins at room temperature. The experimental set-up is demonstrated in Figure 2.1. Post exposure, the slurry was centrifuged at 3000 x g for 20 mins, the supernatant removed, and the pellet frozen at -15 ˚C ± 2 ˚C until DNA extraction.

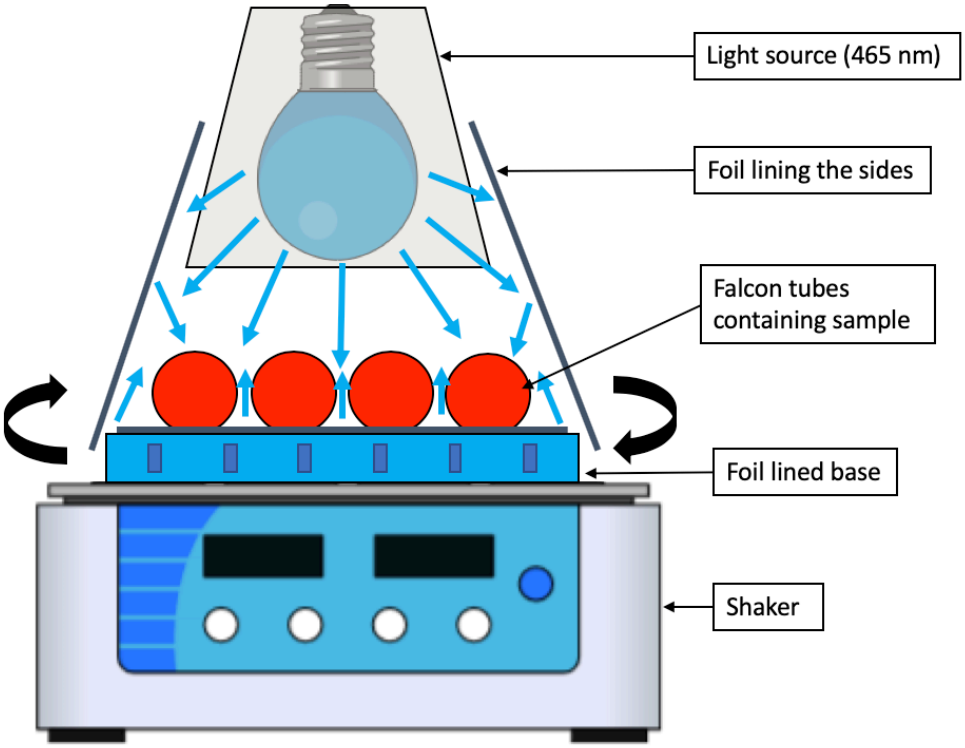

Figure 2.1: Experimental set up for the exposure of PMA added to samples to blue light with continual sample mixing. Figure partially created using the Mind the Graph platform available at www.mindthegraph.com.

2.35 Volume of diluent

An additional experiment aimed to determine whether the volume of diluent was an important aspect of PMAs affectivity. Final volumes of 700 µL, 1000 µL, 2000 µL and 3000 µL were created. All samples were then treated as above to result in a viability dye -treated pellet and frozen until DNA extraction.

2.36 DNA extraction

Total community DNA was extracted from the produced pellet using the FastDNA™ Spin Kit for Soil (MPbio) as per the manufacturer's instructions, with minor modifications. In brief, 0.1 g of the pellet, 978 µL of sodium phosphate buffer and 122 µL of MT buffer were added to Lysing Matrix E tubes. The tubes were vortexed and ribolysed at 6000 wibbles min^{-1} for 2 rounds of 40 s. The tubes were centrifuged at 13,000 x g for 12 mins and the supernatant transferred to microcentrifuge tubes

containing 250 µL PPS, inverted by hand 10 times and incubated at room temperature for 10 mins. The tubes were again centrifuged for 5 mins at 13,000 x g and the supernatant added to 7 mL universals containing 1 mL of binding matrix. The universals were inverted by hand for 2 mins, settled for 5 mins and then 400 µL of the top fraction discarded. The remaining mixture was resuspended and repeat aliquots of 650 µL transferred to a tube containing a spin filter and centrifuged for 3 mins at 13,000 x g and the flow-through discarded. Subsequently, the residue was eluted in 500 µL of SEWS-M and incubated for 5 mins at room temperature, centrifuged twice at 13,000 x g for 5 mins and the flow-through discarded. Finally, the spin basket was transferred to a new catch tube and air dried for 5 mins prior to gentle elution in 100 µL of DES. The tubes were incubated at 60 ˚C for 5 mins before being transferred to the centrifuge and spun at 13,000 g for 3 mins (twice). The spin baskets were discarded and the DNA frozen at -15 ˚C ± 2 ˚C until use.

2.37 qPCR Reaction

M. bovis BCG DNA was quantified using qPCR assays targeting the RD4 deletion region, unique in *M. bovis.* Samples were tested using an ABI 7500 Fast qPCR machine (ABI) with negative and positive controls. A panel of standards from 10^6 to 10^{-1} µL $^{-1}$ were included for the production of a standard curve. The qPCR reaction mix included 10 µL of either standard or total community DNA, 900 nM of RD4-forward primer $^{5'}$-TGTGAATTCATACAAGCCGTAGTCG-$^{3'}$, 900 nM of RD4-reverse primer $^{5'}$-CCCGTAGCGTTACTGAGAAATTGC-$^{3'}$, 250 nM probe AGCGCAACACTCTTGGAGTGGCCTAC-TMR, 1 mg/mL of BSA, 12.5 µL of Environmental Mastermix 2.0 (ABI), and made up to 25 µL with sterile, DNA-free water (Pontiroli et al., 2011). The conditions for the reaction were as follows; 50 ˚C for 2 mins, 95 ˚C for 10 mins, 40 cycles of 95 ˚C for 15 s and 58 ˚C for 1 min.

2.38 Staining with *Bac*Light

The LIVE/DEAD *Bac*Light bacterial viability kit (Invitrogen™) was used as per the manufacturer's instructions. In brief, equal volumes of component A and component B were mixed and 3 μL of the mixture added to 1 mL of cell culture suspension in a microcentrifuge tube (1.5 mL). The mixture was gently pipetted up and down, wrapped in foil and incubated in the dark at room temperature for 20 mins with flicking at 5 min intervals.

2.39 Staining with PMA or PMAxx™

A final concentration of either 50 μM of PMA or 25 μM PMAxx™ was established in a microcentrifuge tube (1.5 mL) containing an aliquot of *M. bovis* BCG culture. The culture and stain were homogenised, wrapped in foil and incubated for 20 mins at room temperature with flicking every 5 mins.

2.310 Visualisation of fluorescently stained cells

Post-incubation, the stained aliquot of culture was briefly vortexed and 5 μL of the suspension smeared onto a microscope slide and covered with an 18 mm square coverslip. A drop of immersion oil was placed on the coverslip and the cells were visualised and images captured on a Zeiss Axioskiop 2 Plus using the HCImage software (Hamamatsu) under the x100 objective. Total cell counts were established by counting cells on the brightfield filter setting, counts of cells containing DNA stained with SYTO9 established under the FITC filter, and cells stained with either PI, PMA or PMAxx™ established by cells fluorescing under the TRITC filter. Cellular counting was conducted using ImageJ software (NIH).

2.4 Results

2.41 Production of cells with compromised membranes

Heating and freezing the *M. bovis* BCG culture ('treated') resulted in a significant decrease in the number of detectable genome equivalents by qPCR (Figure 2.2). The samples decreased by 14.8% across three biological replicates (paired t-test: $t = 14.05$, $df = 16$, $p < 0.0001$). This suggested that it was not reliable to compare the non-treated cell counts with the treated cell counts by qPCR, as the heating-freezing method resulted in loss of DNA.

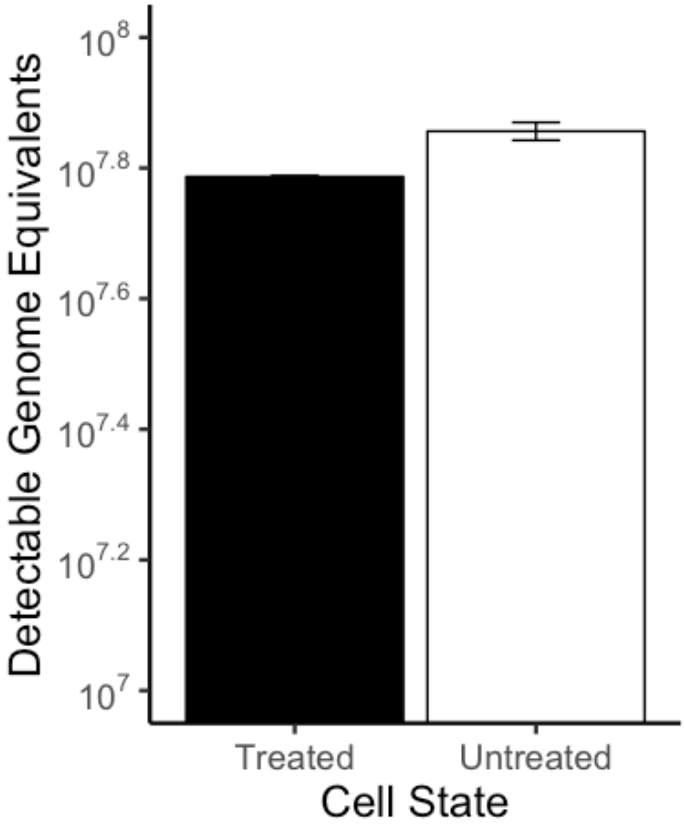

Figure 2.2: Effect of heat-freeze-thaw treatment on the mean number of detectable of genome equivalents (error bars representing the standard deviation).

2.42 Light source

Two light sources were tested to determine their suitability for providing sufficient energy for PMA to undergo photolysis. The light sources were compared by adding PMA (50 μM) to triplicate aliquots of pure culture for each light condition and subjecting these to photoactivation for the same time period under the same conditions. Both treatments resulted in similar levels of cellular detection as determined by qPCR, with no statistically significant difference detectable (Figure 2.3).

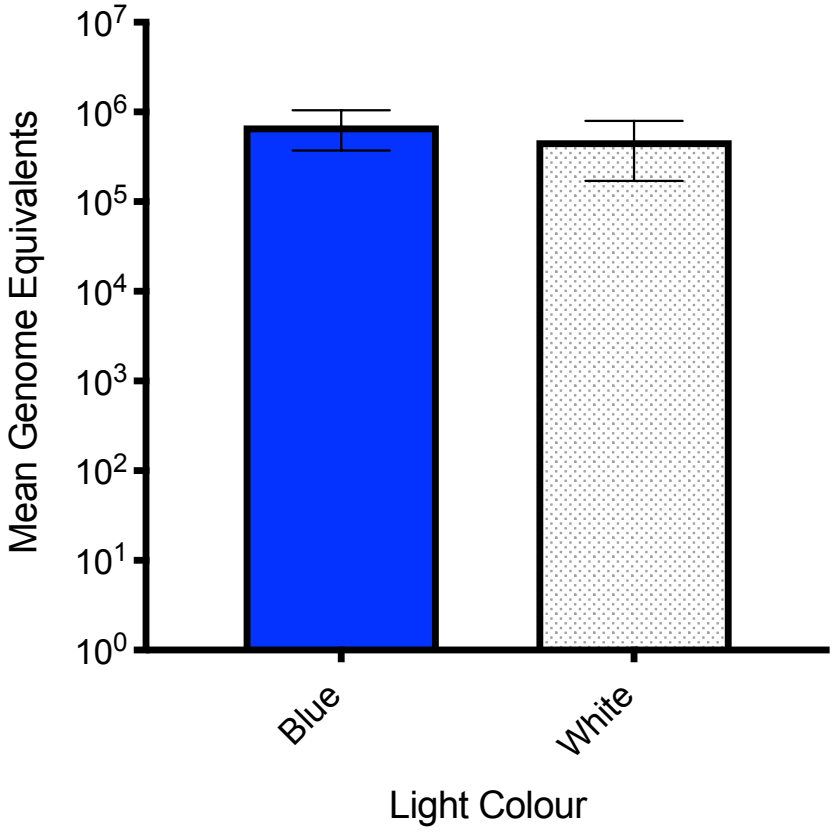

Figure 2.3: Comparison of the use of blue and white LEDs on the photoactivation of PMA, using the decrease in the number of genome equivalents detected as a proxy for successful photoactivation.

2.43 Optimisation of PMA concentration in badger faeces

Initial work on culture demonstrated a decrease in the detected number of genome equivalents; the use of PMA on pre-extracted DNA showed a complete suppression of signal. Once this had been confirmed, the method was applied to use on seeded badger faecal samples. Firstly, the concentration of PMA was inversely related to the detectable number of genome equivalents, with the maximal suppression observed at 200 μM of PMA (Figure 2.4). The number of detectable BCG genome equivalents in the non-treated samples was 5 x 10^8 which decreased to 1 x 10^7 genome equivalents upon exposure to PMA (98.0% decrease).

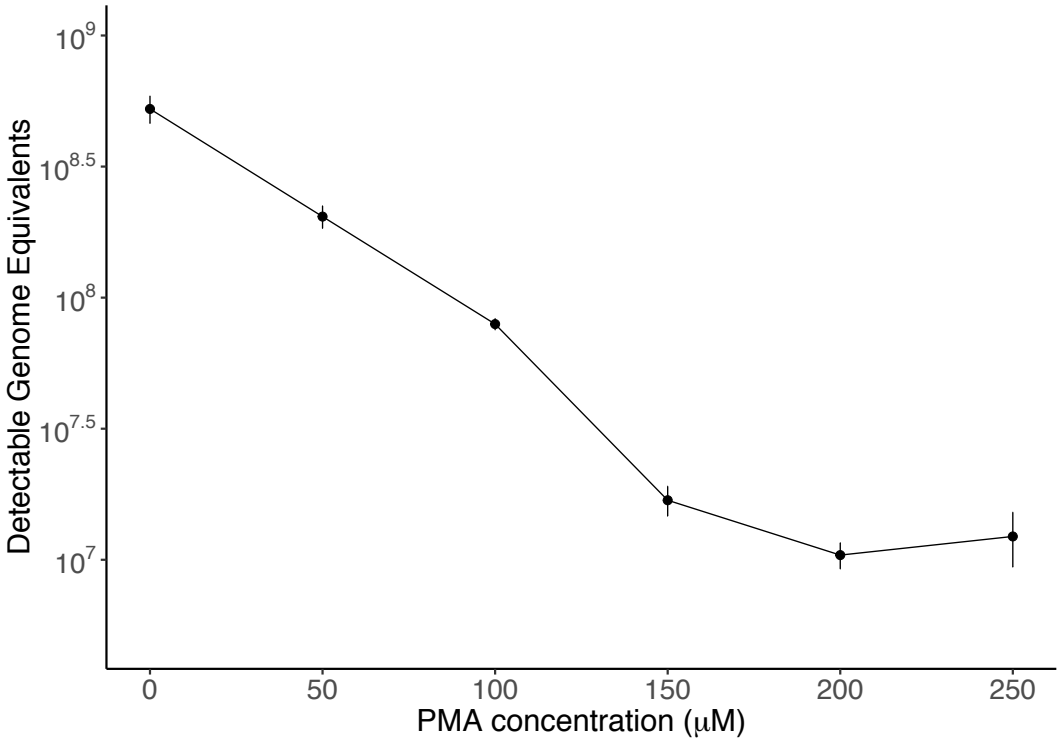

Figure 2.4: Decrease in detectable genome equivalents as concentration of PMA increases.

2.44 Reduction of faecal turbidity

Diluting the spiked sample also significantly impacted the suppression of qPCR signals. Faecal aliquots (0.1 g) were spiked with equivalent volumes of the same BCG culture and diluted to final volume of either 700 µL, 1 mL, 2 mL or 3 mL. The additional volume and reduction in turbidity of the sample resulted in a 99% decrease in the detected number of genome equivalents from 1.5 x 10^7 to 1.2 x 10^5 (Figure 2.5). However, it was impossible to determine whether the remaining detected genomes were from cells which had remained viable, were non-viable but had intact membranes (and thus remained impermeable to PMA) or from cells that had been penetrated by PMA but had not received sufficient light so as to cause the PMA to undergo photolysis and intercalation with the cells' DNA.

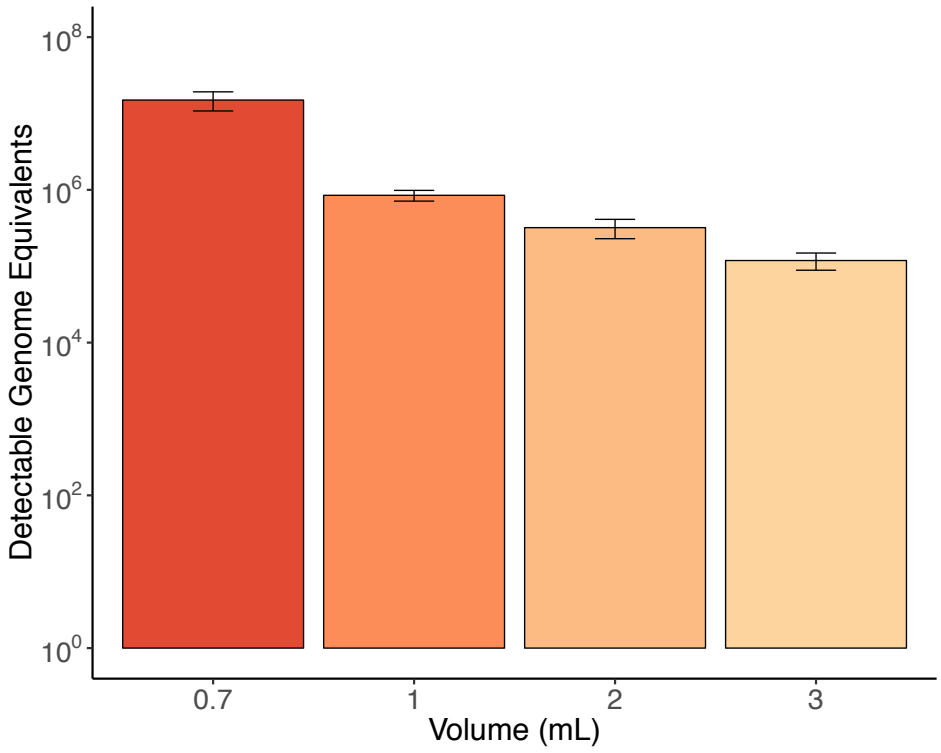

Figure 2.5: Effect of sample dilution on the efficacy of PMA photoactivation, using detectable genome equivalents as a proxy. Bars represent the mean of three replicates; error bars represent the standard deviation.

Cells were lost during sample processing due to the dilution of the sample and the latter repelleting to gather material and cells. Figure 2.6 demonstrates the mean number of cells 'lost' by diluting the sample up to a final volume of 2 mL and repelleting prior to DNA extraction. The diluted sample had a mean cell loss of 1 x 10^6 (8.62% of the value of the spike) across the 4 technical replicates, while the standard deviation across the undiluted sample across 4 technical replicates was 7.2 x 10^5 (6.21%). The variation between the effects of diluting and repelleting the sample and the normally anticipated value of the standard deviation was not statistically significant (paired t-test: t = 2.874, df = 2.862, p = 0.07), however, caution should be exercised and any future work with this method should aim to increase the number of cells pelleted post light exposure stage. It must also be noted that if there were cell-free DNA molecules within the sample, that these would have been lost when the supernatant was decanted due to centrifugation not pelleting the DNA material.

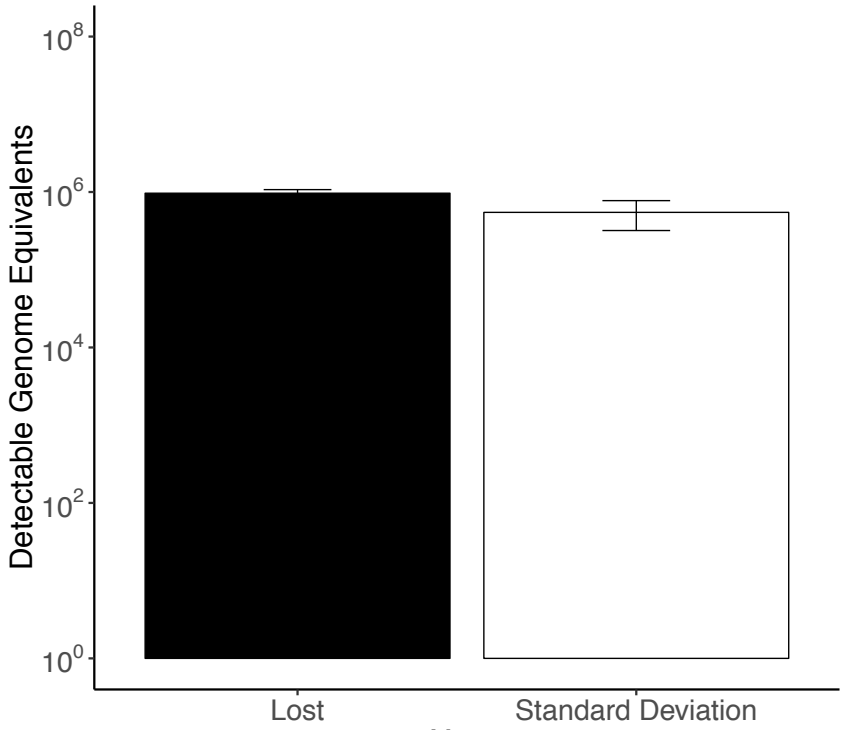

Figure 2.6: The standard deviation across 4 technical replicates when extracting from the same sample without performing the dilution step compared to the number of cells lost.

2.45 The penetration of cells by fluorescent dyes

Fluorescence microscopy was used to quantify the efficacy of cell penetration by various stains. A culture subjected to heat-treatment and freezing (twice) was thawed at room temperature, and the cells were counted both by brightfield microscopy and under the FITC filter using ImageJ. On the brightfield setting, a total of 1155 cells were counted; of these 1142 were also visible under the FITC filter (98.7%), suggesting that a significant number of cells across the 27 fields of view were not penetrated by the generic cell-DNA stain, SYTO9 (paired T-test: $t = 2.15$, $df = 51$, $p = 0.04$) (Figure 2.7).

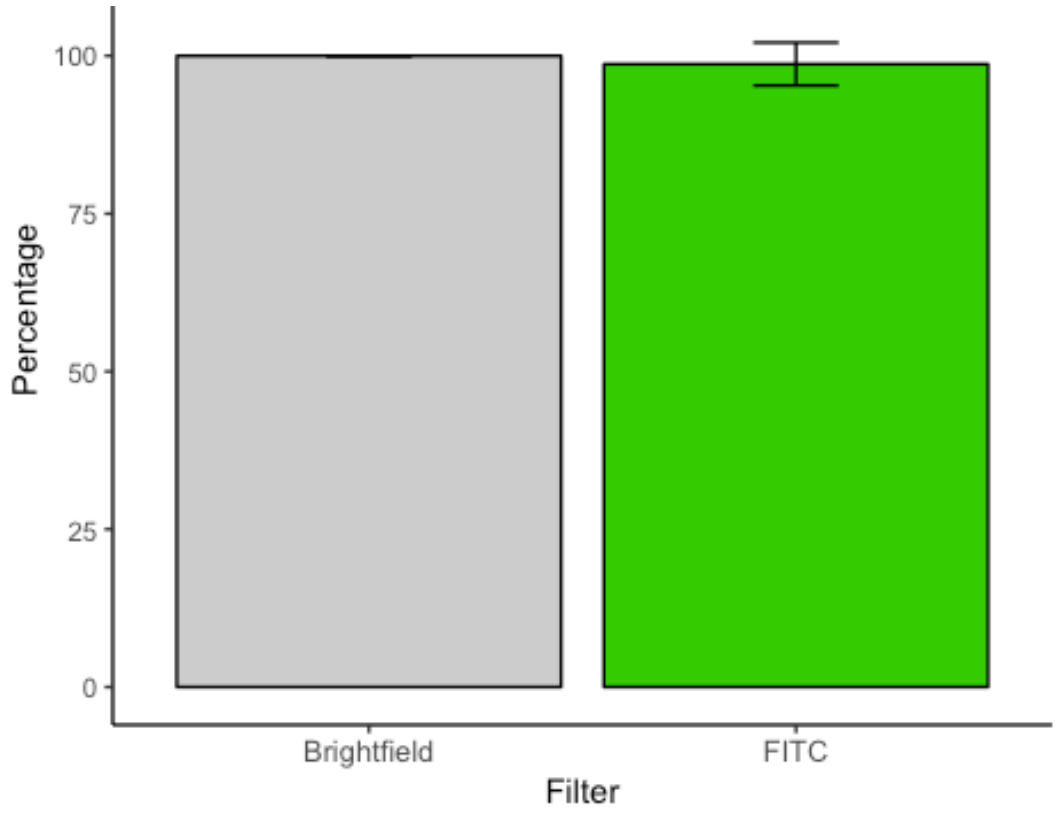

Figure 2.7: Comparison of cell counts in brightfield microscopy (grey) and cells stained by SYTO9 and visualised with FITC fluorescence microscopy (green).

The culture used above was also treated separately with three viability dyes for visualisation with fluorescent microscopy: the LIVE/DEAD *Bac*Light kit, PMA and PMAxx™. Of 1155 visualised cells treated with the *Bac*Light viability kit, 856 had been penetrated by PI and fluoresced under the TRITC filter. However, as noted above, some cells had taken up no stain despite appearing intact (Figure 2.8)

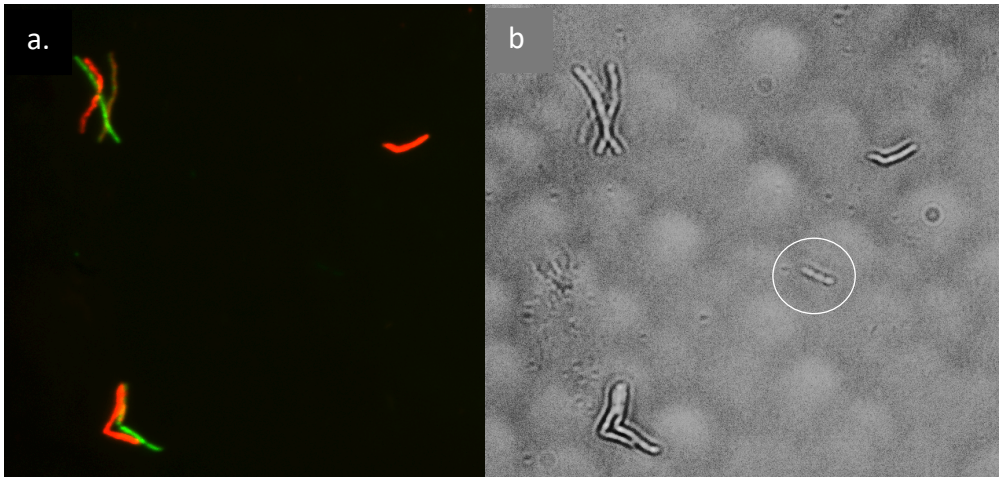

Figure 2.8: M. bovis BCG cells stained with BacLight and visualised with a. FITC/TRITC filters and b. with brightfield microscopy. Demonstration of a cell which has taken up neither the generic cell stain component of BacLight (SYTO9) or the dead cell stain (PI) circled in white in image b.

PMA had a slightly lower level of penetration with 691 of 916 cells fluorescing under the TRITC filter for this no-CFU culture, across 46 fields of view.

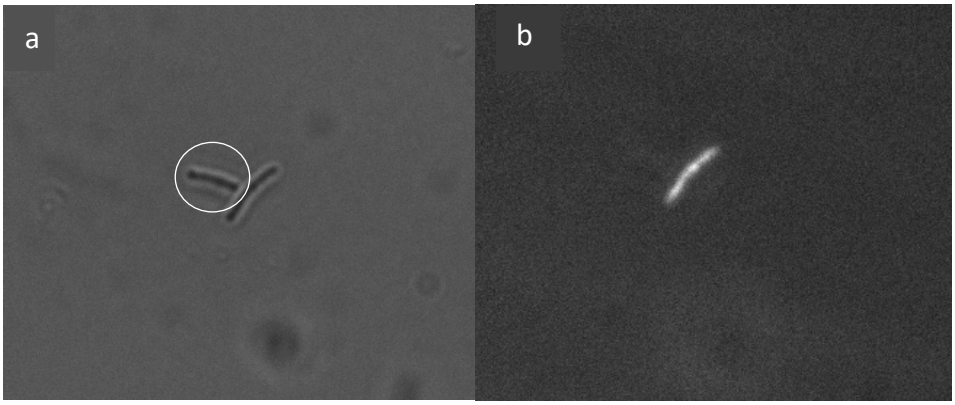

Figure 2.9: M. bovis BCG cells stained with PMA and visualised with a. brightfield microscopy and b. TRITC fluorescent microscopy. Demonstration of cell which has not taken up PMA within the circle on image a.

PMAxx™ demonstrated its improved efficacy and of 1342 cells, 1054 fluoresced under the TRITC filter across 56 fields of view.

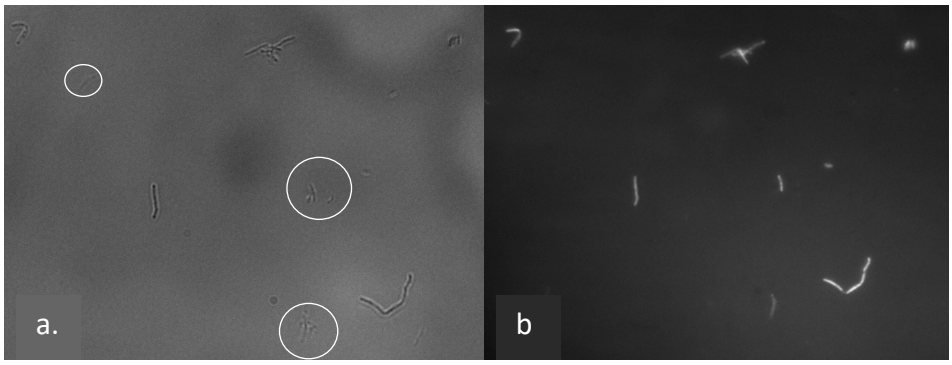

Figure 2.10: M. bovis BCG cells stained with PMA and visualised with a. brightfield microscopy and b. TRITC fluorescent microscopy. Demonstration of cell which has not taken up PMAxx™ within the circles on image a.

These data suggested that while there was no statistically significant difference between the efficacy of *Bac*Light and PMA (Wilcox: W = 940, p = 0.33), there was a statistically significant difference in performance between PMA and PMAxx™, (Wilcox: W = 485, p = 0.02) (Figure 2.11). This was to be expected as PMA is the azidified derivative of the PI component of the *Bac*Light kit. Total cell counts are summarised within Table 2.1.

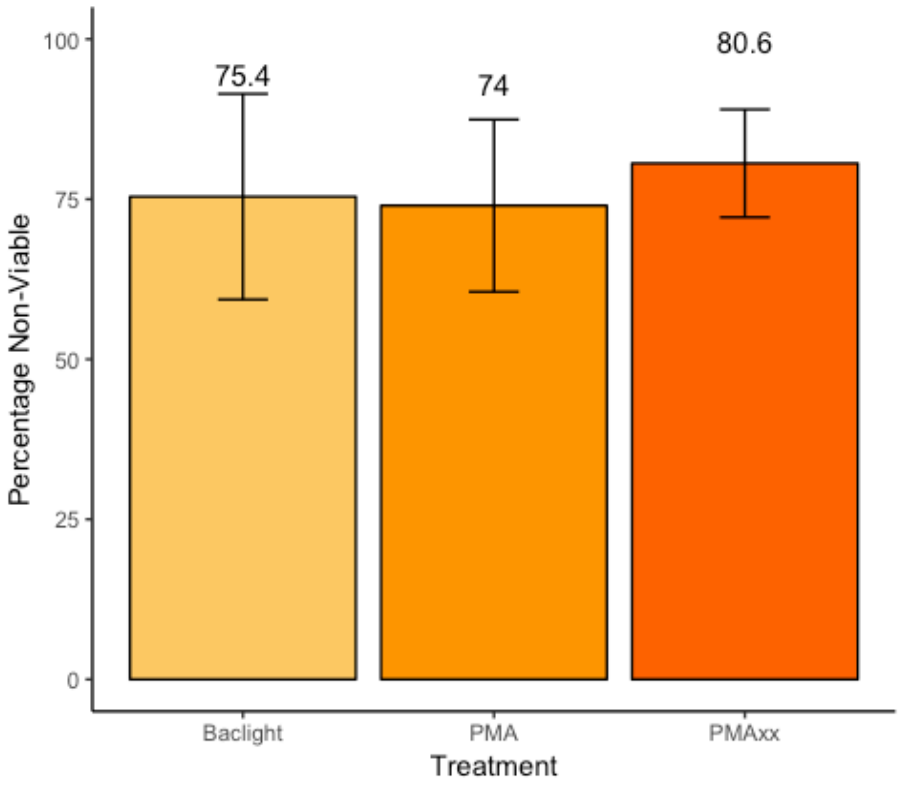

Figure 2.11: Comparison of cell penetration by commercially available viability dyes as determined by fluorescence microscopy. Performed on three M. bovis BCG *cultures subjected to heating and freezing to damage membranal integrity.*

Table 2.1: Summary of cells counted by microscopy under fluorescent TRITC filter to detect the number of 'dead' cells penetrated by the viability dye, and the total number of cells as determined by counts by Brightfield microscopy.

Treatment	Number of 'dead' cells	Total number of cells
Propidium Iodide	856	1155
PMA	691	916
PMAxx™	1054	1342

2.46 Comparison of PMA and PMAxx™ by qPCR

Triplicate cultures were treated separately (across three technical replicates) for their response to two treatments and a negative control. The three conditions were as follows: DNA extraction with no-prior treatment, DNA extraction post PMA-treatment, and DNA extraction post-PMAxx™ treatment. The untreated culture had an average of 1.4 x 10^7 detectable genome equivalents, standardised to 100% for usage in Figure 2.12 below. The PMA treated culture had an average of 3.5 x 10^6 cells detectable post treatment, and the PMAxx™ treated culture had 1.4 x 10^6 detectable GEs. This suggested that PMAxx™ has a greater ability to penetrate compromised cells and this resulted in a decrease in the detectable number of GEs during later qPCR analysis.

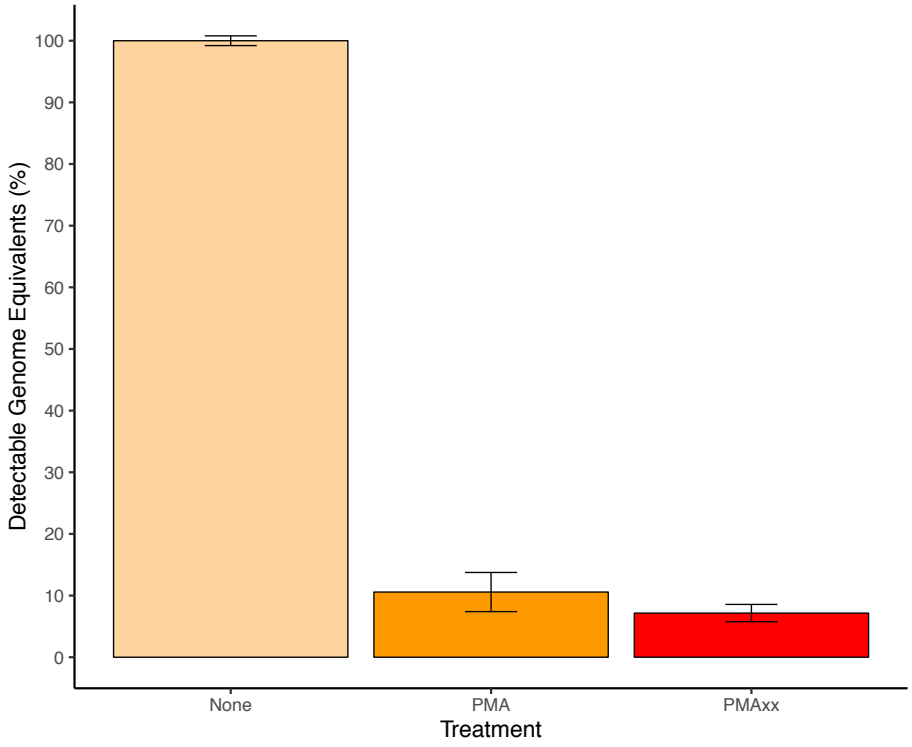

Figure 2.12: Comparison of genome detection when cells were treated with PMA and PMAxx™.

2.47 Optimisation of PMAxx™

Due to PMAxx™ consistently exhibiting greater penetration of non-viable cells, a curve to test the efficacy of PMAxx™ at increasing concentrations within seeded faecal samples was created (Figure 2.13). The results suggested that, as with PMA, increasing the concentration of PMAxx™ to 100 μM, decreased the number of detectable genome equivalents (paired t-test: $t = 10.39$, $df = 17.48$, $p < 0.0001$).

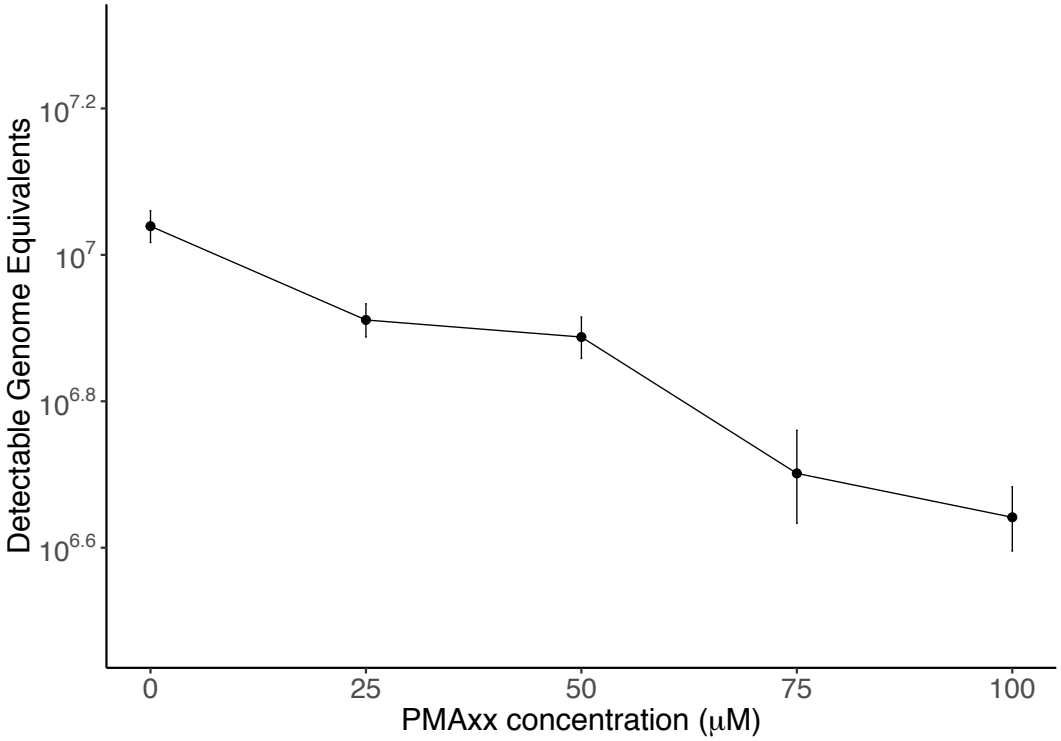

Figure 2.13: Effect on increasing PMAxx™ concentration on detectable genome equivalents; compilation of three technical and three biological replicates.

A ‘concentration of inhibitor versus response’ curve was constructed using a least squares regression model in Prism 8 (R^2 = 0.96, df = 34), with the decline in detectable GEs plotted against the increasing concentration of PMAxx™ (Figure 2.14).

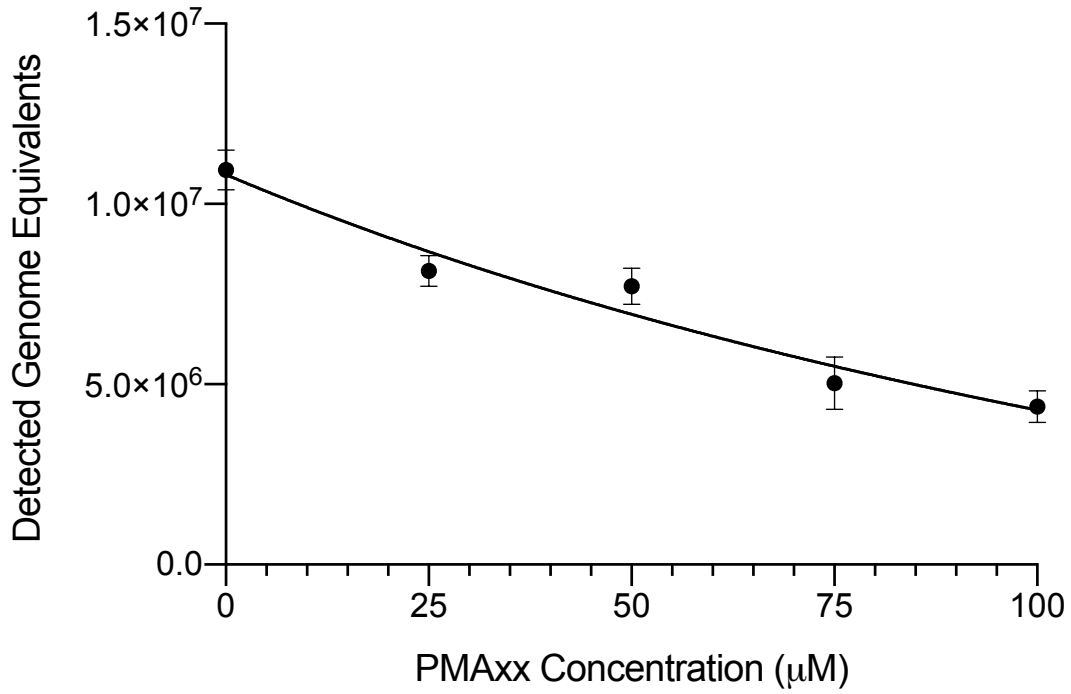

Figure 2.14: Response curve generated in order to predict the effect of increasing PMAxx™ concentration.

The regression line in Figure 2.14 gave the following equation to relate PMAxx™ concentration to detectable *M. bovis* BCG genome equivalents.

$$Y = \frac{10959836}{(1 + \left(\frac{X}{78.93}\right))}$$

Due to the expense of conducting further experiments at higher concentration of PMAxx™, the equation depicted above was solved to predict the likely detected number of genome equivalents if the concentration of PMAxx™ was increased to 125 µM. The equation suggested that there would be a decrease of 3.2% detectable genome equivalents (in this experiment, a decrease of 4.38 x 10^6 to 4.24 x 10^6) but this would be statistically insignificant when accounting for the predicted standard deviation (Figure 2.15). Thus, a final PMAxx™ concentration of 100 µM was deemed the final, optimised amount for use in badger faeces.

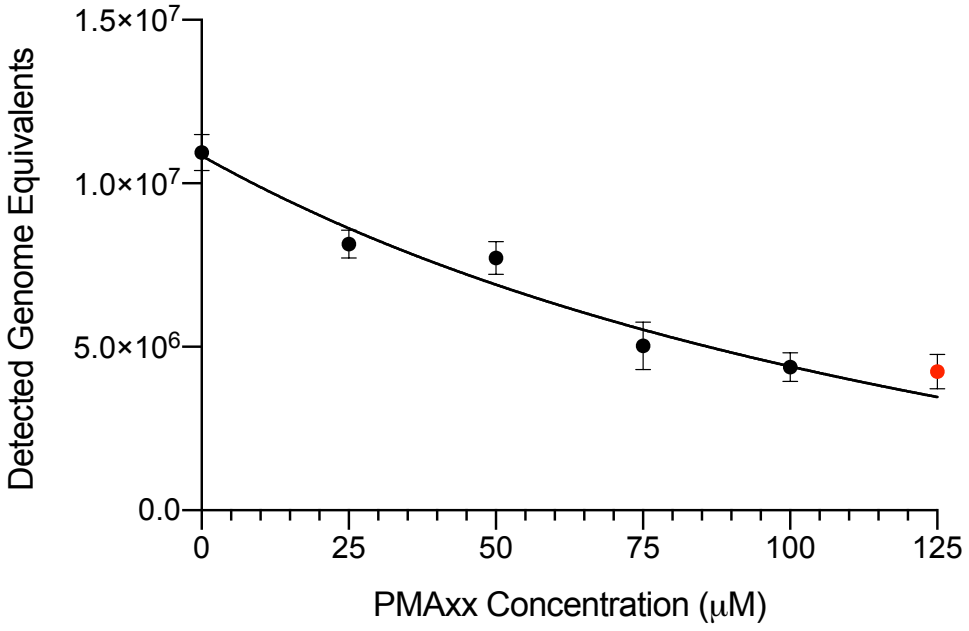

Figure 2.15: Development of Figure 2.14 to include the predicted point (red) of increasing PMAxx™ concentration.

2.48 Comparison of optimised methodologies for PMA and PMAxx™

In this final comparative test the optimised methodologies were employed; triplicate samples for each treatment were diluted to 2 mL to reduce turbidity and increase light penetration; viability dye was added to a final concentration of 200 µM of PMA, and 100 µM of PMAxx™ (Figure 2.16). Both PMA and PMAxx™ resulted in significant decline in the number of detectable cells in comparison to qPCR on untreated spiked samples ((PMA: $U = 0$, $p < 0.0001$); PMAxx™: $U = 0$, $p < 0.0001$)). As with microscopy, there was a statistically significant difference between the efficacy of PMA and PMAxx™ ($t = 16.66$, $df = 22$, $p < 0.0001$), with PMA reducing the detection to 25.35% of the untreated samples ($\sigma = 2.07\%$) and PMAxx™ reducing detection to 10.31% ($\sigma = 2.27\%$) suggesting only 10% of the cells within the spike contained intact membranes.

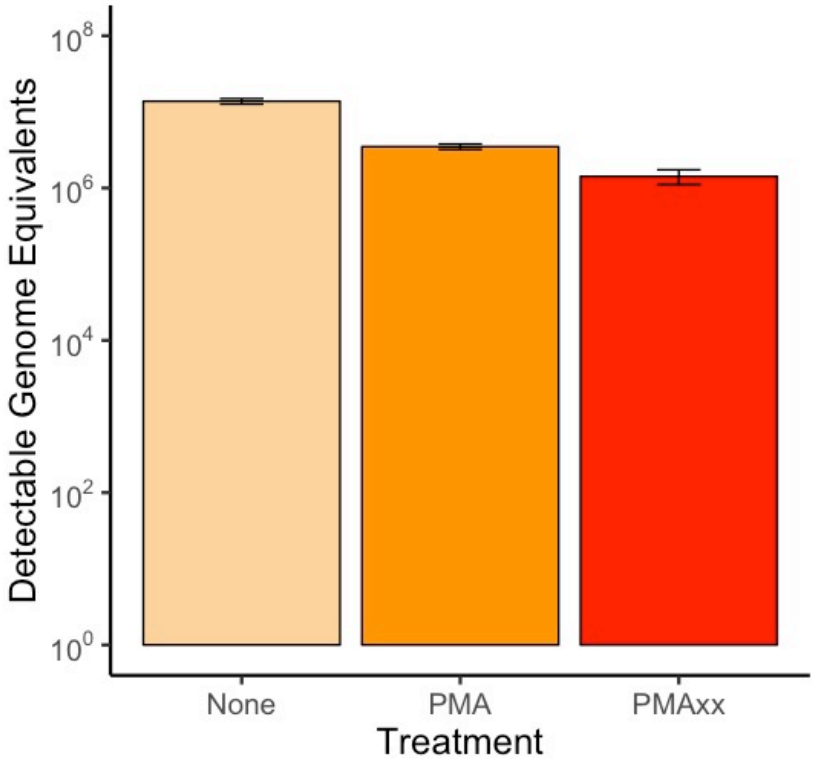

Figure 2.16: The efficacy of PMA and PMAxx™ when applied to seeded badger faeces using detectable genome equivalents as a proxy for efficacy.

2.5 Discussion

This research sought to optimise and establish a method for high-throughput environmental monitoring of *M. bovis* contamination and survival.

2.51 Light source

Two light sources are frequently referred to in the literature for photoactivating viability dyes, the first is the commercially available PMA-Lite™ LED Photolysis Device (Biotium) which was financially non-viable for the purpose of this project. The second is a 600-watt Halogen Lamp, a form of lighting which is being phased out due to energy inefficiency, the risk of skin burns and increased fire risk. Due to excessive heat production and its associated drawbacks (health and safety, monitoring requirements, sample rotation, ice block provision) the Halogen lamp method was deemed unsuitable as ultimately the method was aimed towards being conducted in Category 3 laboratories. Increased heat also decreases the half-life of PMA, as well as potentially compromising the membrane of cells and producing false negative results (Nocker et al., 2007).

Two alternative light sources were trialled, one white LED system with the equivalent light output of a 600W Halogen lamp; and a small blue LED set up within a standard desk-lamp. These two light sources were trialled on the same sample set, and no statistically significant difference was found. Due to the blue-light set up being considerably cheaper than the white LED set-up, this method was used for the remaining stages of optimisation. The efficiency of the blue-LED set-up is likely due to it producing light solely within the range of 465 – 470 nm, specifically targeting the wavelength at which PMA and PMAxx™ are excited.

2.52 PMA concentration optimisation

A working concentration of 50 µM of PMA is sufficient to completely suppress the amplification signal from non-viable cells according to the manufacturer's instructions. However, there is significant deviation in assays optimised for more complex sample types, as well as high variance between papers. Some key papers are summarised in Table 2.2.

Table 2.2: Summary of papers to demonstrate the range of PMA concentrations applied to complex sample types within the literature.

Author	Year	Matrix	[PMA] µM
Fujimoto *et al.*	2013	Faeces	50
Desneux *et al*	2015	Manure	55
Muzafar *et al.*	2015	Soil	400
Torremorrel	2015	Faeces	200
Carini *et al.*	2016	Soil	40
Fongaro *et al.*	2016	Manure/Soil	50
Gyawali *et al.*	2016	Ova floated from wastewater	100
Roth *et al.*	2017	Marine sediment	50-200
Perras *et al.*	2018	Human stool	100 (10-300)
Papanicolas *et al.*	2019	Human stool	100

There are many hypothetical reasons for why increased concentrations of PMA may be required for complex sample types. Firstly, in environmental sample types the dye could be absorbed by a large number of non-target organisms. In a culture of OD 1.0, as used in the manufacturer's instructions, there will be approximately 1 x 10^8 – 1 x 10^9 cells mL^{-1}. However, faeces is hypothesised to contain up to 1 x 10^{12} bacteria per g^{-1}, as well as additional cell types from fungi, protozoa, partially degraded food (plants, insects and meat) and shed host cells (Ott et al., 2004, Rose et al., 2015). The additional uptake of PMA by membrane-compromised non-target

cell types may reduce the availability to insufficient levels (Pan and Breidt, 2007, Fittipaldi et al., 2011).

Environmental matrices such as soil and faeces will contain high densities of cation exchange sites. These exchange sites may act as a sink for available PMA, thus limiting the concentration available to penetrate target cells (Desneux et al., 2015, Pisz et al., 2007, Taylor et al., 2014). From the concentration-response curve it was estimated that for badger faecal samples a working concentration of 200 μM of PMA was required, four times the concentration suitable for pure cultures of bacteria. This value was higher than the majority of reportedly used concentrations in the literature however it was not thought to compromise the reliability of the assay. Increasing concentrations of PMA did not result in further decreases in detectable genome equivalents and other studies demonstrate that PMA, unlike EMA, does not exhibit an antimicrobial effect and is effectively excluded from intact cells (Pan and Breidt, 2007).

2.53 Turbidity

The reduction of sample turbidity was an essential element of increasing the effectiveness of PMA and PMAxx™. This is likely due to the co-effects of the greater dilution facilitating the movement of PMA through the sample, and the lower turbidity permitting sufficient light penetration throughout the sample for PMA photolysis and subsequent intercalation to occur (Wagner et al., 2008, Varma et al., 2009, Taylor et al., 2014).

It was found that diluting 0.1 g of faeces to a final volume of 2 mL, and ensuring that the subsequent slurry was homogenous, produced the maximal reduction of DNA amplification. However, it is essential to consider the sample type and the DNA extraction method when applying to different sample types. This work focussed on the initial dilution, viability treatment and then repelleting of the material. This allowed an appropriate quantity of material to be used in downstream DNA extraction post-viability treatment. This repelleting stage permitted the recovery of cells from the diluted matrix; the variation fell largely within the

standard deviation established by repeat extraction of non-diluted samples, and therefore within the expected variation of the assay. This could be partly explained by the existence of 'free' DNA within the sample, which was not gathered within the repelleting stage, or simply due to the method intrinsically lacking in sensitivity.

The disadvantage of reducing sample turbidity, and therefore increasing the volume of the sample, is that the working concentration of viability dye also has to increase. The combination of the high working concentration and working with relatively large sample volumes, meant that for high throughput, large scale experiments, this would not be an affordable method. While it could have been possible to reduce the size of sample worked with, reducing the faecal weight from 0.1g would result in the requirement for revalidation of DNA extraction and qPCR to account for the reduced sensitivity. This would lower the tests reproducibility, as well as increasing the risk of encountering false negatives and increasing the level of risk to which a cattle herd would potentially be exposed to.

2.54 PMAxx™ versus PMA

PMA was described as a way of better discriminating live from dead cells than ethidium monoazide. The company, Biotium, has since built on the success of PMA with the release of PMAxx™, that they describe as a 'new and improved version of our popular viability dye PMA…'. Thus far, there is little information directly comparing the two dyes. This study, at the time of writing and to my knowledge, is the first of its kind comparing the two dyes, both for their cost and their effectivity. It was found that in side-by-side comparison PMAxx™ was cheaper than PMA when optimised as the dyes are equivalent in cost but PMAxx™ required half the volume of PMA in both the treatment of culture and in the faecal methods optimised within this study. It was also found that in the experiments conducted with the optimised treatments, PMAxx™ was more efficient at both penetrating the cells when visualised by microscopy, and in the reduction of the DNA amplification signal from qPCR.

2.55 Proxy rationale

A common counterargument to using membrane intactness as a proxy for viability is that bacteria are capable of self-repair when subjected to a mild degree of damage (Ray et al., 1971). However, repair has only been recorded when stress-inducing conditions have been replaced by optimum laboratory conditions; it is unknown how repair mechanisms perform under environmental conditions (Ray et al., 1971, Koseki and Yamamoto, 2006). A series of factors discussed in the following paragraphs suggest how environmental conditions may limit a cell's capacity to undergo repair beyond the remit of maintenance due to protein turnover (Kempes et al., 2017).

Mycobacteria possess a thick, lipid-rich cell wall which is essential for their shape and integrity and enhances resistance to antibiotics and environmental stresses (Primm et al., 2004, Dörr et al., 2016). In order for the plasma membrane to be compromised, it is likely that this barrier has been damaged rendering the cell more susceptible to desiccation, particularly relevant in an environmental system (Jarlier et al., 1994). A damaged plasma membrane underlying a damaged cell wall would theoretically increase the cells' susceptibility to immune attack, limiting their ability to establish an infection within the host, and removing their relevance in a disease transmission system (Jankute et al., 2015, Dörr et al., 2016).

Membrane repair (and presumably cell wall repair) is influenced by temperature with slower repair at lower temperatures, and cells being incapable of repair at 4 °C (Ray and Speck, 1973, Koseki and Yamamoto, 2006). It is unlikely that environmental bacteria in the UK will be exposed to consistent temperatures of 37 °C and therefore also unlikely that host adapted bacteria will be able to repair rapidly enough to prevent penetration by unfavourable compounds and leakage of cellular components (Tang and Marshall, 2017). The effect of temperature on factors such as membrane viscosity, enzyme activity and transport systems must also be considered (Sinensky, 1974, Galarza-Muñoz et al., 2011).

Bacteria in hosts or terrestrial and aquatic conditions are subjected to on-going nutrient deprivation (Matin et al., 1989, Rittershaus et al., 2013). Cells adapt by entering a non-replicating, resting state with nutrient starved mycobacteria

differentiating into small resting cell morphotypes and large resting cell morphotypes (Wu et al., 2016). If the cells are rapidly placed in unfavourable conditions then the cells freeze without undergoing further differentiation (Wu et al., 2016). It is unknown whether cells remodel the plasma membrane and cell wall while in this dormant state. However under conditions of nutrient deprivation, CwlM has been shown to downregulate *murA* and therefore subsequent peptidoglycan production; it is therefore hypothesised that repair is unlikely (Boutte et al., 2016). Furthermore, expression of ATP synthase subunits in *M. smegmatis* and *M. tuberculosis* were downregulated in *in vitro* and *in vivo* dormancy models (Shi et al., 2005, Koul et al., 2008). This downregulation resulted in ATP levels decreasing 5-10-fold in comparison with those of replicating tuberculous bacilli (Shi et al., 2005, Koul et al., 2008, Gengenbacher et al., 2010). While residual ATP activity persists in quiescent cells, this would presumably be sufficient for the costs of basal maintenance and not for the large levels of restructuring associated with complete cellular penetration (Scheffers and Pinho, 2005, Kempes et al., 2017). However, it must be acknowledged that data has been extrapolated from closed laboratory conditions testing the effects of individual components rather than investigations including a complex web of interacting factors. Furthermore, the cells used within the studies are taken from active, replicating cultures and rapidly inserted into these less adequate conditions. The sudden change in environment is likely to have compromised the ability for mycobacteria to adapt and survive and therefore be unlikely to be representative of environmental cells. (Wu et al., 2016).

In order to survive injury, it is hypothesised that the cell would have to repair within seconds, however bacterial turnover rate in soil is measured in days, as estimated from leucine and thymidine incorporation (Rousk and Bååth, 2011, Tang and Marshall, 2017). It is also suggested that even when the added mycobacterial inoculum is from replicating cultures, the cells still succumb to competition from cells which typically inhabit environmental matrices. Competition may be mediated by antibiotics, extracellular enzymes or secondary metabolites; all of which serve to increase the competitiveness of the organism (Feichtmayer et al., 2017). This is supported in the context of mycobacteria by the number of experiments conducted on sterile and non-sterile soil inoculated with *M. bovis* and found that *M. bovis* persists for longer periods in sterile soil (Stenhouse Williams and Hoy, 1930, Barbier

et al., 2017, Duffield and Young, 1985, Tanner and Michel, 1999). Therefore, it is hypothesised that the majority of environmental mycobacterial cells that have been damaged to a sufficient level so as to allow the penetration of PMA (and therefore other toxic compounds) prior to their repair, would not have sufficient time so as to repair the cell before succumbing to toxic compounds or loss of intracellular components (Tang and Marshall, 2017). It is also hypothesised that slow-growing *M. bovis* (even under optimum conditions) would be out-competed for resources by typically environmental microbes both due to generation time and adaptation.

To summarise, if the cells were able to extract sufficient nutrients from the environment, maintain suitable ATP levels above their basal metabolic rate, and sustain intracellular reactions at less-favourable temperatures, they would still have to self-repair in a small enough timescale so as to prevent excessive loss of mass, and prevent the influx of potentially toxic environmental compounds (Tang and Marshall, 2017). On the basis of the above information, membrane integrity was deemed to be a suitable proxy for viability in environmental mycobacteria.

2.56 Limitations

The ability of mycobacterial cells to persist within free-living protozoa further complicates the issue of both persistence and detection (Berk et al., 2006, Lamrabet et al., 2012). Not only has this been observed through co-culture under laboratory conditions, but acid-fast (AF) bacteria have been identified in amoebae within environmental soil samples collected from close proximity to badger setts (Adékambi et al., 2006, Mardare et al., 2013). The AF bacteria were not confirmed to species level within this study, as they were neither isolated or identified by PCR (Mardare et al., 2013). This lack of species confirmation may be due to low culture sensitivity attributable to both small numbers of cells and potential VBNC state of internalised cells, as well as non-optimised methods for DNA extraction and PCR. While the amoebae will protect internalised cells from external environmental stresses, they are not thought to be conducive to either the replication or the survival of mycobacteria (Cirillo et al., 1997, Ovrutsky et al., 2013). Internalised *M. bovis* declined by 2.5 log CFU over three months, while in pure culture by 1 log (Mardare et al., 2013). It is hypothesised that amoebae will not be a critical element in the

persistence of *M. bovis* within the UK, however their impact on v-qPCR should be acknowledged as internalised cells will not be reached by viability dyes as long as the host-amoeba is intact or encysted.

While membrane integrity is a well-established proxy for viability and the base principle of many commercialised and well cited products it does also have limitations. Firstly, it is unclear how long it takes for a membrane to degrade post cell death, as well as suggestions that a cells membrane can be damaged to the point that it can be permeated by dyes normally excluded by the cell, but still be able to meet other criteria for life i.e. metabolic activity and replication (Koseki and Yamamoto, 2006, Emerson et al., 2017). However, in some cases, such as in environmental samples, it may be difficult, too expensive or entirely impossible to optimise some of the methods applied to cells in single-cell-type cultures.

The final limitation of this method is the cost of undertaking it on a large number of samples as well as the lack of method scalability. At the time of writing, PMAxx™ (VWR) cost £157.00 per 20 mM in a 100 µL solution, and the optimised methodology required 10 µL in 2 mL of faecal slurry equating to an additional £15.70 per assay. Furthermore, due to the requirement of LED light exposure it was impossible for the method to be made high throughput, as a limited number of samples (n = 6) could be treated within the current set-up. This would have added a significant level of additional testing time, as well as sample storage optimisation to ensure there was no loss in detectable cells attributable to the storage of samples to account for the lower-throughput nature of the assay. Together, these impracticalities made the method unsuitable for its desired use within this thesis, but if accounted for early in the project design stages could be inbuilt into future surveys.

2.6 Conclusion

Once optimised, the method was financially unviable within the scope of this research for the application proposed; however, this does not mean that it could not be utilised in future work if financially accounted for prior to the project design stage. There is still a great deal we do not know about the community composition in faeces, both at the point of excretion and after environmental exposure. Future work should aim to draw comparisons between not only the method outlined within this chapter and the qPCR method on which it is based, but also with work relating to culture with and without the addition of cell resuscitating cytokines or supernatant from actively growing culture. This work would begin to outline the state of *M. bovis* within environmental samples and add weight to the argument of whether infectivity studies should be conducted using extracted cells. It would also be suggested that as a threat to human and public health, that an overestimation of risk by qPCR (if now more controlled with the incorporation of a viability step), would be a more desirable approach and reduce chances of exposure than it would be to use a low-sensitivity culture based method and underestimate the environmental risk (Taylor et al., 2014).

Chapter 3:

Spatial stability of environmental hotspots of *Mycobacterium bovis* due to a European badger (*Meles meles)* population on a chronically infected dairy farm in the High-Risk Area of England.

3.1 Abstract

Despite *M. bovis* having first been identified in a UK badger in the 1970s, there remains little known about the disease within local populations. It was demonstrated within this study that on a chronically infected dairy farm within the HRA there were large differences between the disease state of three sympatric badger social groups. Furthermore, it was shown that these differences in disease state were resistant to seasonal variation, remaining at consistent levels across the sampling/survey year. This has important consequences for the identification of disease hotspots and the targeting of biosecurity approaches. It also highlights the danger of assuming that all badgers are of equivocal disease risk on a farm and highlights the risk of culling induced perturbation or farm level persistence if the infected individuals are not within the 70 – 90% of badgers removed from the farm.

3.2 Introduction

3.21 The UK badger population

Ecologists have sought to quantify the population size of badgers since the 1980s, employing different methods including sett surveys, hair trapping and cull-sample matching (Anon., 2015c, Anon., 2018d). The most recent estimates are rooted in the designation of Land Class Groups (LCGs), with a subsample of 1 km^2 squares investigated for badger social group density and then applied across the UK and Ireland (Reid et al., 2012, Judge et al., 2014). There were variations in population density within LCGs as many factors impact the establishment and use of badger setts on a microscale, such as resource availability and soil type (Kruuk, 1989, Rosalino et al., 2005). Badger numbers are likely to fluctuate within and between years, with impacts including the timed birthing of young for the late winter period, the dispersal of males (and levels of road traffic collisions), as well as food availability in relation to seasonal and meteorological conditions as well as land usage (Kruuk, 1989, Macdonald et al., 2010). Combing these factors results in a heavily convoluted picture when it comes to elucidating badger numbers, however current estimates place the population in the region of 485,000, with approximately 5,000 killed in road traffic accidents (RTAs) each year (Giesler, 2018).

3.22 Control of bTB in badgers

In the UK, badgers are considered to be the primary environmental reservoir of *M. bovis* and have been implicated in the local maintenance of disease as well as transmitting the disease within and between local cattle populations (Krebs et al., 1997, Donnelly et al., 2003). Beyond reducing direct badger-cattle contact, there are two key methods employed for reducing the risk posed by *M. bovis* infected badgers. The first, and primary method employed by Defra, is licensed culling. Culling is based on research conducted between 1998 and 2005 on areas totalling over 3000 km^2 of South-West England during which 10,979 badgers were removed. This widespread badger removal was related to a 23% reduction in the number of new herd breakdowns within the target culling area. However, the overall efficacy of culling on reducing cattle bTB prevalence is disputed as this positive effect was offset by a 25% increase in herd breakdowns within the 2 km buffer (or

'perturbation') area (Bourne, 2007). Current cull zones must be at least equal in size to the areas used in the RBCT (100 km^2), with a minimum of 90% of the land being either accessible or within 200m of accessible land. During six weeks of intensive culling, badger populations are reduced to 5-30% of their estimated starting population and further annual culls maintain the lower population size for 3 years. In 2018, 32,601 badgers were killed across 30 zones in their first 4-year culling period, costing in excess of £9 million (Anon., 2018a, Anon., 2018e). Despite this large monetary investment and large loss of life, it is difficult to predict the effects that the current cull strategy will have on bTB breakdowns as the strategy deviates significantly from the trial from which the Independent Scientific Groups calculations and recommendations were derived.

The alternative strategy is vaccinating badgers with *M. bovis* BCG Danish 1331, administered at 10 times the dose administered to adult humans (Cymru, 2015). While more humane, vaccination requires a significantly more hands-on approach than that required for culling and has not been undertaken over large swathes of land. Vaccination has also been implemented less strategically than culls, with multiple charity led operations being in effect alongside the Government led Badger Edge Vaccination Scheme (BEV). Across England, 641 badgers were vaccinated in 2018, including 338 as a part of BEV (APHA, 2019b). Badger vaccination is still under investigation regarding its efficacy and few field studies have examined its impact on natural badger infection levels. Furthermore, it is difficult to draw comparisons between studies due to various approaches to vaccination and there being no agreed upon standard for establishing *M. bovis* positivity in badgers. One study conducted on a wild population of badgers in Gloucestershire was associated with a 73.8% ($p < 0.001$) reduction in positivity (as determined by Stat-Pak testing), and a 61.4% reduction as determined by the combined results of Stat-Pak and culture ($p < 0.01$) (Chambers et al., 2011). Captive populations inoculated and subsequently challenged with *M. bovis* demonstrated a reduction in positive clinical samples compared to their non-inoculated counterparts as well as a significant reduction in the excretion of *M. bovis* in faeces and urine (Chambers et al., 2011, Lesellier et al., 2011). As yet, no field trial has published results regarding the effect that badger vaccination has on local cattle breakdown rates; however, as there is a significant

reduction in environmental excretion it could be a method of breaking that aspect of the transmission cycle.

3.23 National disease distribution

Despite the large financial and legal burden placed on land holders, there are no measures in place to establish the pre-intervention infection level of a farm's badger population, or to measure the impact which interventions have on this infection level. Defra have stated that establishing badger disease prevalence is as an objective of the current cull work "-as it had been many years since regular surveillance was carried out"; tracking these changes in TB prevalence throughout the cull could lead the formation of an "exit strategy" (Anon., 2019c). Unfortunately, due to strong spatial and temporal sampling bias, unsuitable storage methods (83% of carcasses suffered high levels of decomposition and all carcasses were subsequently frozen - reducing the sensitivity of culture methods), and difficulties processing the carcasses due to necrosis, the APHA were unable to accurately establish the prevalence of infection in badgers from the HRA (Anon., 2019c). However, the methodologies employed by APHA have recently been improved and successfully applied to the newly established Area 32 (Cumbria) in which 20.9% of badgers removed from the main target area (or minimum infective area, MIA) tested positive for *M.* bovis by culture at *post mortem* (37/177). A further 1.7% (3/173) of badgers from outside of the MIA but still within the intervention area tested positive by the same protocol (Anon., 2019d). Though the studies are not directly comparable, this is an increase of 20.74% from the positivity rate identified within this region between 1972 and 1990 (Atkins and Robinson, 2013, Barron et al., 2018). The increase in the prevalence of *M. bovis* in badger populations is mirrored in the local cattle population, with the Cumbrian herd prevalence recorded as increasing from 2% in 2002 to 13% in 2017 (Taylor, 2010, APHA, 2017b, Barron et al., 2018). However, due to the aforementioned lack of ongoing surveillance and despite knowing the large changes that have occurred in both the local cattle populations and the newly assessed Cheshire badger population, historic infection levels for the HRA have been used for the application of policy in recent years. Within South-West England, badger TB prevalence in 2005 was 6.3-33.9% in 2005 in endemic areas, with large variations between counties (mean = 16.6%) and years (Bourne, 2007). Therefore,

it is suggested that basing our current disease control strategies on historic data is not appropriate and efforts should be focussed on developing disease surveillance protocols to inform strategy design as well as to quantitively assess the outcomes of the strategy.

3.24 Optimisation and application of qPCR as a non-invasive method for *M. bovis* detection

Previous research has employed the RD4-qPCR method to determine the level of threat which individual social groups pose to wildlife and cattle populations (Pontiroli et al., 2011, Travis et al., 2011, King et al., 2015a). In comparison to the IFN- γ assay, the positive predictive value (PPV) of qPCR for the identification of an individual infected badger was 36-59%; this is low for use as a diagnostic test though was expected due to qPCR only detecting individuals which are shedding faecally (King et al., 2015b). However extensive sampling and analysis was conducted in order to establish the method for the identification of infected social groups at 95% certainty. The method is based on the likelihood of collecting a positive faecal sample from a latrine associated with a social group, based on the known disease prevalence of said social group (Figure 3.1). However, for routine purposes, the method dictates that 20 faecal samples should be collected in order to detect the top 83.34% of shedding social groups (King et al., 2015b). This method was used to investigate the disease prevalence within a high-density badger population within the protected Woodchester Park site in Gloucestershire, UK. Twelve social groups were tested, all of which were positive by qPCR faecal testing, though to hugely varying degrees (Figure 3.2).

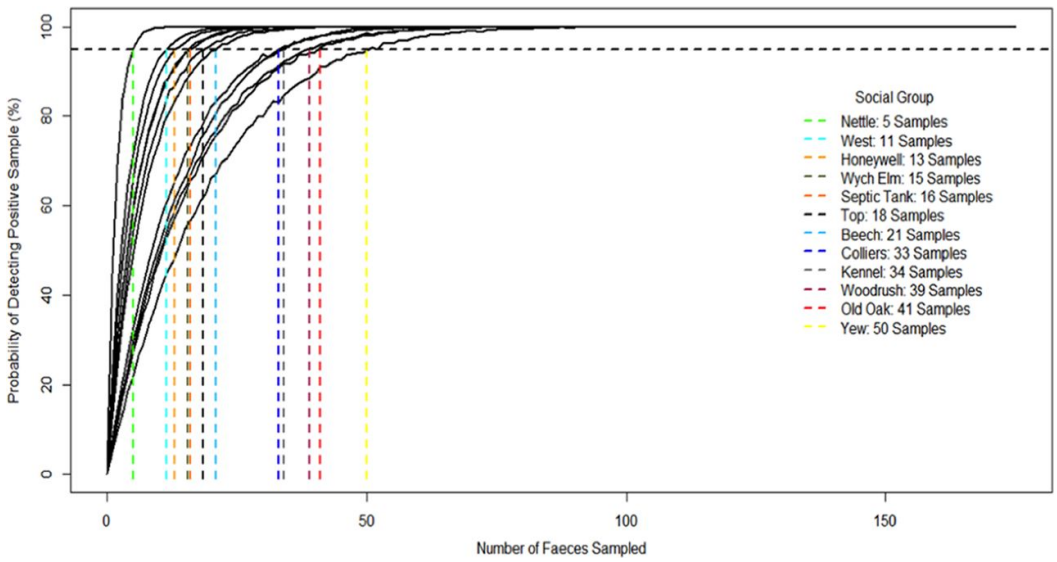

Figure 3.1: Number of faecal samples required to detect social group level infection. Used with permission (King et al., 2015b).

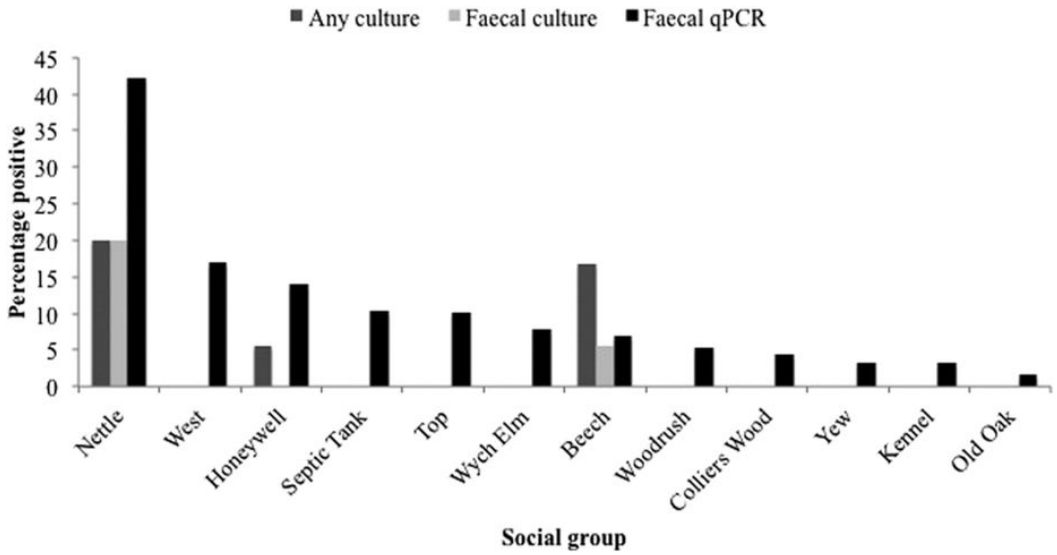

Figure 3.2: Percentage positive badgers per social group determined by any culture positive (tracheal or faecal) or faecal culture compared with positives by faecal qPCR. Reproduced with permission (King et al., 2015a).

3.25 Aim

The work presented here aimed to improve our current understanding of local spatial infection distributions and the environmental disease burden attributable to the badger population of an active and chronically infected dairy farm in the HRA. It was hypothesised that there would be variable disease status of badger social groups and that these would be detectable via qPCR testing. It was also hypothesised that different times of year may be more suitable for the application of this methodology to include *M. bovis* detection as a part of a farm's wildlife associated biosecurity strategies.

3.3 Methods

3.31 Site establishment

Field sites required historical and continuous badger occupancy, with a high likelihood of collecting sufficient numbers of *M. bovis* positive samples due to the necessity of achieving suitable levels of power for this study, as well as samples being required for the 'exclosure'/survival study. After reviewing factors related to multiple field sites, a new fieldwork site was established on a dairy farm in Gloucestershire due to the following factors: the known presence of a stable badger population; the site's exclusion from the cull zone and the promise of this remaining so; the farmer being willing to make no crop alterations that may have a significant impact on the badger population (both in terms of numbers, location and stability); the chronic nature of the farm's bTB breakdown (ongoing since 21/04/2015); and finally the ability to establish an exclosure study site in close proximity.

An initial survey was conducted in December 2017 to ensure the site's suitability, followed up in March 2018 and finally with a total sett survey in May 2018 (spring) immediately prior to commencing sampling (data provided in Appendix A, Table 1). This total sett survey took place later than intended due to the extended winter period, with heavy snow making the roads impassable in March 2018 when sampling was initially scheduled. The total sett survey was conducted with the landowner's permission over approximately 1.74 km^2 of mixed-use land. The land types included well-established, broadleaved woodland (semi-natural), mixed woodland (semi-natural), coniferous woodland (plantation), and calcareous grassland, as well as fields used for the growth of wheat.

3.32 Sample collection

Samples were collected from latrines frequently used by badgers, as determined by the number and freshness of samples found during the initial survey. Using disposable, single-use wooden spatulas, faecal samples were placed into sterile 30 mL universal tubes and labelled with an identifier number. The latrine number, location, social group ID, date of sample collection, the presence of anal secretion, and sample's identifier number were logged both on paper and digitally. The samples

were placed within a Ziploc bag, in an appropriately labelled cool bag on ice (to remain unfrozen) within 2 hours of collection from the field. They were then transferred to the nearby site of the exclosure experiment, at a separate location away from badgers, wildlife and the general public. Samples were then placed into the exclosure set-up, with a subsample transferred to a 2.0 mL microcentrifuge tube labelled with the identifier number and date for storage at -15 ± 2 °C until processing.

Sample collection occurred in May 2018 (spring/summer), August 2018 (summer/autumn), and November 2018 (autumn/winter). Surveys took place over a 14 to 16-day period, with latrine sampling occurring on alternate days. The initial survey undertaken in December 2017 was intended to be the start of the seasonal work, however heavy snow and heavy winds resulted in the formation of large snowdrifts, making it impossible to complete the work due to travel restrictions, ice, and difficult snow-covered terrain. The spring sampling period was also postponed until later in the year due to heavy snowfall and adverse conditions. The autumn 2018 survey was unseasonably cold for the first week, with the first snowfall of the winter occurring at the start of the survey period. It was deemed unnecessary to re-establish a winter sampling period due to the uncertainty surrounding the weather, the suitability of the sampled period for identifying variations between conditions, and the decreased chance of badger-cattle contact due to cattle being housed over that period.

3.33 DNA extraction

Total community DNA was extracted from the samples within the CL3 facility using the FastDNA™ Spin Kit for Soil (MPbio) as per the manufacturer's instructions, with minor modifications. Each set of extractions included a faecal sample spiked with *M. bovis* BCG and a negative control with an equivalent volume of water added. In brief, 0.1 g of sample, 978 µL of sodium phosphate buffer and 122 µL of MT buffer were added to Lysing Matrix E tubes. The tubes were vortexed and ribolysed at 6000 wibbles min^{-1} for 2 rounds of 40 s. The tubes were centrifuged at 13,000 x g for 12 mins and the supernatant transferred to microcentrifuge tubes containing 250 µL PPS, inverted by hand 10 times and incubated at room temperature for 10 mins.

The tubes were again centrifuged for 5 mins at 13,000 x g and the supernatant added to 7 mL Universals containing 1 mL of binding matrix. The universals were inverted by hand for 2 mins, settled for 5 mins before 400 µL of the top fraction was discarded. The remaining mixture was resuspended and repeat aliquots of 650 µL transferred to a tube containing a spin filter and centrifuged for 3 mins at 13,000 x g and the flow-through discarded. Subsequently, the residue was eluted in 500 µL of SEWS-M and incubated for 5 mins at room temperature, centrifuged twice at 13,000 x g for 5 mins and the flow-through discarded. Finally, the spin basket was transferred to a new catch tube and air dried for 5 mins prior to gentle elution in 100 µL of DES. The tubes were incubated at 60 ˚C for 5 mins before being transferred to the centrifuge and spun at 13,000 g for 3 mins (twice). The spin baskets were discarded and the tubes wiped with 5% Chemgene prior to being placed in a sterile 10 x 10 storage box. The exterior of the box was again wiped with 5% Chemgene and the box transferred to the CL1 laboratory where the DNA was stored at -15 ˚C ± 2 ˚C until use.

3.34 qPCR Reaction

M. bovis BCG DNA was quantified using qPCR assays targeting the RD4 deletion region, unique in *M. bovis.* Samples were tested using an ABI 7500 Fast qPCR machine (ABI) with negative and positive controls. A panel of standards from 10^6 to 10^{-1} ul^{-1} were included for the production of a standard curve alongside a negative control of the mastermix made up to 25 µL with sterile di-H_20. The qPCR reaction mix included 10 µL of either standard or total community DNA, 900 nM of RD4-forward primer $^{5'}$-TGTGAATTCATACAAGCCGTAGTCG-$^{3'}$, 900 nM of RD4-reverse primer $^{5'}$-CCCGTAGCGTTACTGAGAAATTGC-$^{3'}$, 250 nM probe AGCGCAACACTCTTGGAGTGGCCTAC-TMR, 1 mg/mL of BSA, 12.5 µL of Environmental Mastermix 2.0 (ABI), and made up to 25 µL with sterile, DNA-free water (Pontiroli *et al.,* 2011). The conditions for the reaction were as follows; 50 ˚C for 2 mins, 95 ˚C for 10 mins, 40 cycles of 95 ˚C for 15 s and 58 ˚C for 1 min. Mastermix was made immediately prior to the addition to the 96-well plate in a designated PCR-clean room within a UV sterilised PCR workstation to ensure no cross contamination of reagents. The plates would then be transferred to the category

1 laboratory where the DNA for that reaction would be added in a separate UV sterilised PCR workstation. All reagents were made into stock solutions and aliquoted to limit the likelihood of cross contamination. When the reaction was successfully completed, the sample numbers were unblinded in order to link the sample back to the social group and latrine of origin.

3.35 Bait marking

Bait marking is used to identify the territories of a badger social group or, as in this case, to identify latrine usage patterns. This component was not initially planned and thus was undertaken in the autumn rather than the spring where there is considered to be a second smaller peak in activity (Roper, 1994, Delahay et al., 2000).

Bait was prepared by mixing peanuts, plastic pellets (NHBS ltd.) and a commercially available brand of golden syrup. The beads were purchased in various colours to allow the distribution of different colours between hypothesised main setts that had been occupied throughout the spring and summer of 2018. Suspected 'main' setts were visited every 2 days for eight days, with the bait placed both in the sett entrance and into 10 shallow pits in close proximity to the sett; each pit contained circa 100 mL of bait. The bait was then shielded by a rock to prevent interference by rain, and to limit the number of non-specific mammals and birds that could opportunistically feed upon it.

Bead identification was conducted in conjunction with the *M. bovis* exclosure work, with the samples being checked for the presence of coloured beads during transfer into the exclosure, and conclusions drawn regarding the use of that latrine by a particular social group/sett.

3.36 Power analysis

The samples collected in spring 2018 were used as a preliminary guide to the expected variance in positivity within social group and were the basis upon which the subsequent power analyses were conducted (see Table 3.1 below).

Table 3.1: Mean percentage positivity of samples collected from latrine associated with each of the three social groups in spring 2018, and levels of variation between the latrines.

Group	Percentage Positive (%)	Standard Deviation
1	65.8	21.1
2	25.7	18.9
3	5.6	2.0

Firstly, the pooled standardised difference must be calculated in order to account for inherent variability within the social groups regarding the number of positive samples retrieved from each group-associated-latrine using the following equation:

$$SD_{pooled} = \sqrt{\frac{SD1^2 + SD2^2}{2}}$$

Secondly, the proposed levels of the significance level (α) and the effect size (1 - β) were derived from Table 3.2. While it is important to ensure significance levels are representative of the stringency which you wish to apply to the experiment, it is also important to ensure that realistic sample numbers are set. For this work, α was defined as 0.01 and $(1 - \beta)$ was 0.10; the function of these values ($f(\alpha, \beta)$) was identified from Table 3.2 below as being 14.9.

Table 3.2: f(α,β) for the most commonly used values of α and β

	β			
α	0.05	0.1	0.2	0.5
0.05	13.0	10.5	7.9	3.8
0.01	17.8	14.9	11.7	6.6

The value for $f(\alpha, \beta)$ was inserted into the equation below, with SD_{pooled} as calculated above in equation X, and δ being the smallest difference in the mean to be considered important.

$$n = f(\alpha, \beta) \cdot \frac{2({SD_{pooled}}^2)}{\delta^2}$$

Power analysis was conducted retrospectively for each season in order to determine whether the variation witnessed is due to a truly significant difference between the social groups. The spring analysis suggested that in order to determine a 30% difference between Social Group 2 and Social Group 1, sixteen samples would need to be collected from each site. To determine a 15% difference between Social Group 2 and Social Group 3, eighteen samples would need to be collected from each social group. Finally, in order to detect a 50% difference between Social Group 1 and Social Group 3, three samples would need to be collected from each social group. The above values relate to a study with a power level of 90% and with a significance level of 0.01%. These values were set as minimum sample collection numbers for the subsequent sampling periods.

3.37 Data analysis

Data was recorded using workbooks in the field and transferred to Excel 2018 on the same day. R version 3.4.1 was used for analysis and graphical representation using the 'dplyr', 'ggplot2', 'Hmisc' and 'ggmap' packages. Satellite images were obtained from Google.

3.4 Results

3.41 Total sett survey

Four hypothesised main setts were identified and clustered into 4 approximate groups on the basis of proximity and badger activity (Figure 3.3). The setts were all well established and exhibited high levels of activity in close proximity to the sett as well as additional satellite setts in the territory surrounding them.

Figure 3.3: Map of proposed main setts (red circle), key annex setts (orange circle), and latrines (yellow diamond).

In the autumn of 2018, bead baiting was used to confirm the number of badger social groups present on the farm. The results of bead baiting combined with field surveys once the vegetation had retreated suggested the presence of three badger social groups on the farm, with the two eastern social groups being resolved into a singular group; this has been used for placing the latter analysis in context. Distribution of the retrieved marked samples and the setts which were baited are demonstrated in Figure 3.4 below.

Figure 3.4: Main setts marked by large circles and the colour of beads used in baiting, latrines marked by diamonds filled either with the colour of the retrieved beads, the mix of the colours if more than one colour was retrieved or greyed out if no beads were retrieved.

3.42 Sample collection

Surveying and sampling were conducted at the end of spring, summer and autumn 2018. The number of active latrines identified per social group in each seasonal survey is demonstrated in Figure 3.5. The warmer months in spring/summer and summer/autumn exhibited the highest number of active latrines, with a significant decrease in latrine numbers seen in the autumn/winter period (t-test: μ = -40%, p = 0.02).

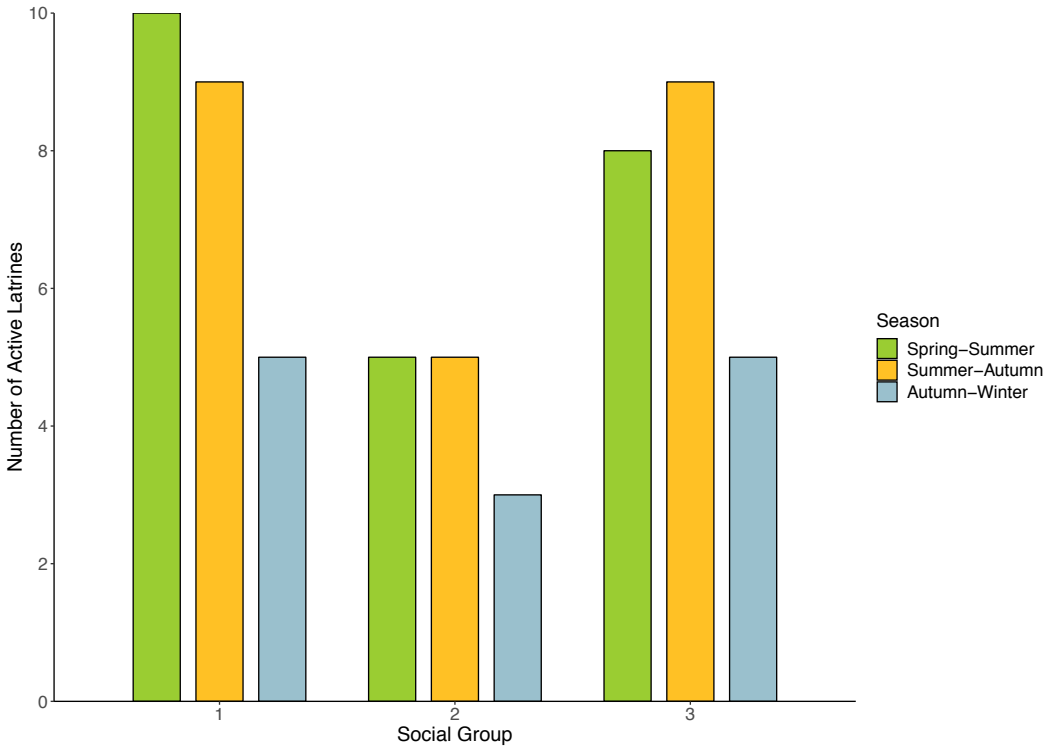

Figure 3.5: Number of active latrines located per social group, per season.

The number of samples collected (Figure 3.6) did not reflect the changes in active latrine numbers and showed no seasonality across the three social groups (ANOVA: $p = 0.8$). It should also be noted that the recent remains of three badgers (two adults and one juvenile) were found in the territory associated with Social Group 1 in the summer/autumn sampling season.

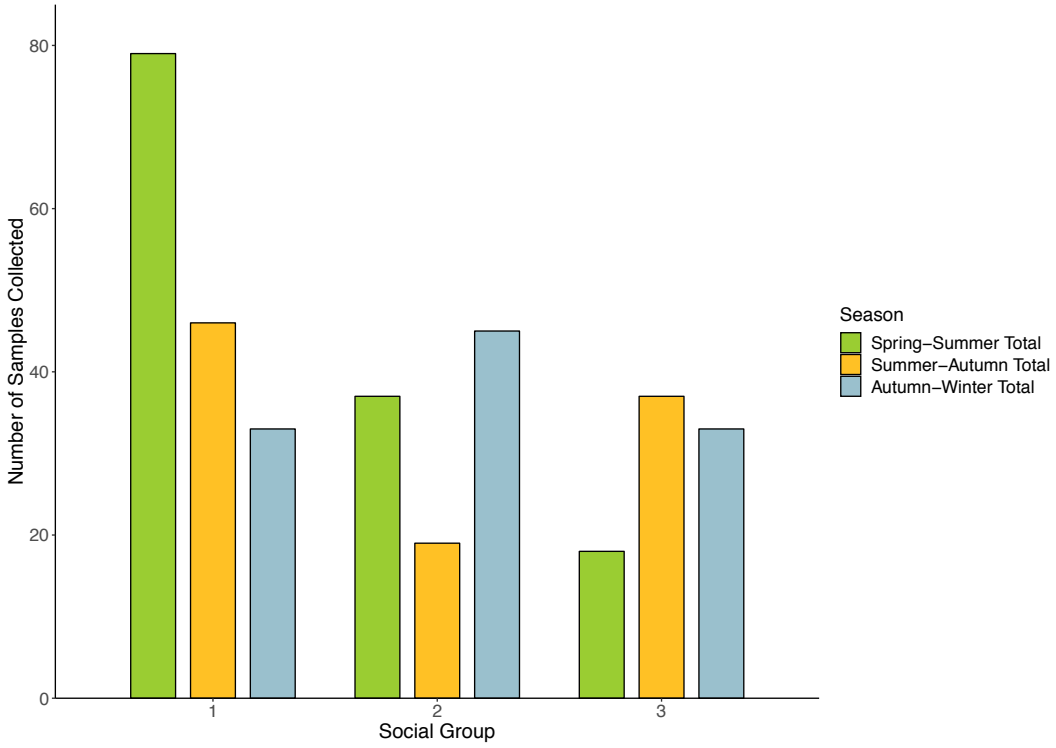

Figure 3.6: Number of faecal samples collected per social group, per season.

3.43 The stability of social group positivity

Within each social group, seasonality did not have a statistically significant effect on the mean percentage positivity of the latrines associated with a social group. Social Groups 2 and 3 maintained relatively consistent levels of positivity, but Group 1 saw a large decrease in the percentage of samples containing *M. bovis* DNA between the summer/autumn and autumn/winter sampling periods, though this did not test as significant (Figure 3.7, Table 3.3). There was found to be no statistically significant difference in terms of the latrine percentage positivity in relation to the season in which the samples were collected; this statement is true for each of the three social groups (1: $F(2,21) = 1.676$, $p = 0.211$; 2: $F(2,9) = 0.188$, $p = 0.831$; 3: $F(2,13) = 0.575$, $p = 0.577$).

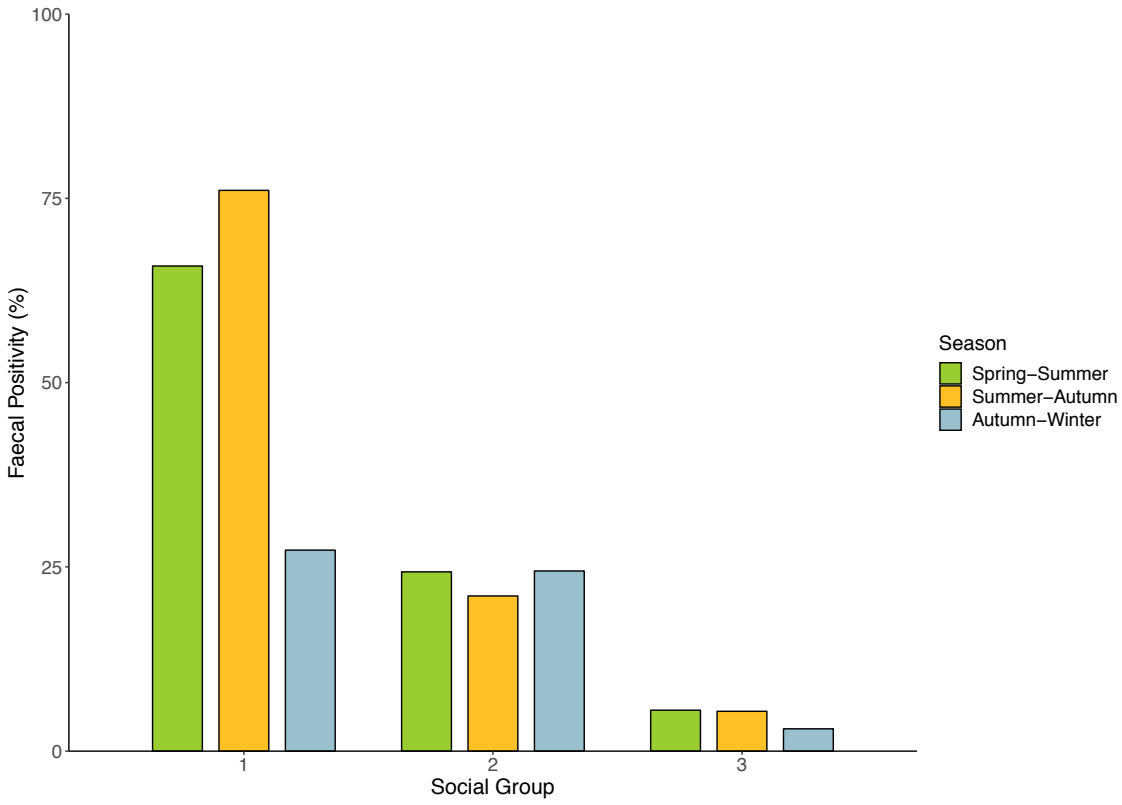

Figure 3.7: Total percentage of samples testing positive for M. bovis per social group, per season

A total of 347 faecal samples were collected across the seasons of which 123 tested positive for *M. bovis* by PA-PCR. The summary of sample positivity, seasons and social groups is shown in Table 3.3.

Table 3.3: Summary of total faecal numbers collected, and the total that tested positive by qPCR per season.

Social Group	Sampling Period					
	Spring-Summer		Summer-Autumn		Autumn-Winter	
	Total	Positives	Total	Positives	Total	Positives
1	79	52	46	35	33	9
2	37	9	19	4	45	11
3	18	1	37	2	33	0

The overall levels of percentage positivity exhibited little variation across the seasons (Figure 3.8) when collected from the main latrines associated with the social group, defined as those that remained in continual, frequent use in close proximity to the main sett. Despite the large decrease in faecal positivity demonstrated in Figure 3.7 for Social Group 1, samples retrieved from the main latrines retained consistent levels of positivity across the sampling year.

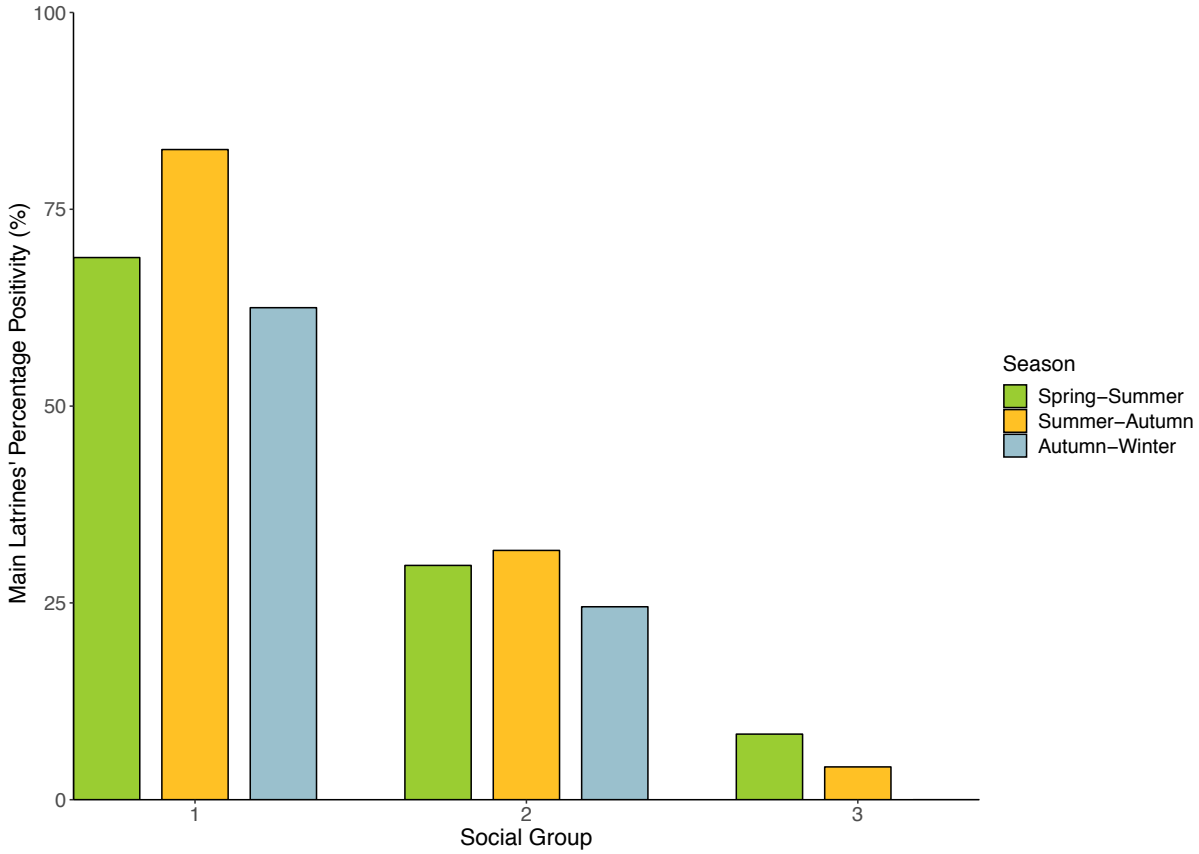

Figure 3.8: The percentage of faeces recovered from the 'main' latrines associated with a social group that tested positive by qPCR across the period of sampling.

The total number of samples retrieved from the main latrines is given in Table 3.4 and associated with Figure 3.8, above. The samples were collected from different numbers of main latrines which may be linked to the size of the respective social group; there were four main latrines associated with Group 1, three main latrines for Group 2, and 2 main latrines for Group 3.

Table 3.4: The number of samples collected from the main latrines associated with each social group across the seasons.

Social Group	Spring-Summer	Summer-Autumn	Autumn-Winter
1	40	23	20
2	26	13	31
3	11	21	17

Not all samples that tested positive for *M. bovis* by PA-qPCR were taken forward for quantification. However, of the samples that were quantified as a part of the exclosure experiment due to exhibiting the highest CT counts during PA-qPCR, there was a statistically significant difference between the counts obtained during the spring and the summer ($W = 92$, $p < 0.001$), as well as the spring and the autumn ($W = 88$, $p = 0.002$). There was no statistically significant difference between the values detected within the summer and the autumn samples ($W = 69$, $p = 0.166$). Available values are presented in Table 3.5.

Table 3.5: Summary of the variation in the ten samples containing the highest numbers of detectable genome equivalents per gram of faeces across the three sampling periods.

Season	Mean	Median	Range
Spring	2.4×10^5	1.65×10^5	$1.31 \times 10^5 – 4.46 \times 10^5$
Summer	6.5×10^4	3.99×10^4	$1.59 \times 10^4 – 1.51 \times 10^5$
Autumn	6.1×10^4	1.82×10^4	$4.28 \times 10^3 – 2.09 \times 10^5$

3.44 Mapping to inform biosecurity

Seasonal maps were produced to present the percentage positivity of latrines across the farm. In the spring-summer of 2018, a total of 144 samples were collected from 22 latrines (Figure 3.9). Of these latrines, for Group 1, 100% of the latrines (n = 10) had a positive sample retrieved; for Group 2, 60% of the latrines (n = 5) had a positive sample retrieved, for Group 3, 33% of the latrines (n = 6) had a positive sample retrieved. Latrines were excluded from the analysis of group positivity levels if they were thought to be associated with more than one social group, as determined by bead-baiting and surveying for badger runs, but the boundary latrine between Groups 1 and 2 also tested positive.

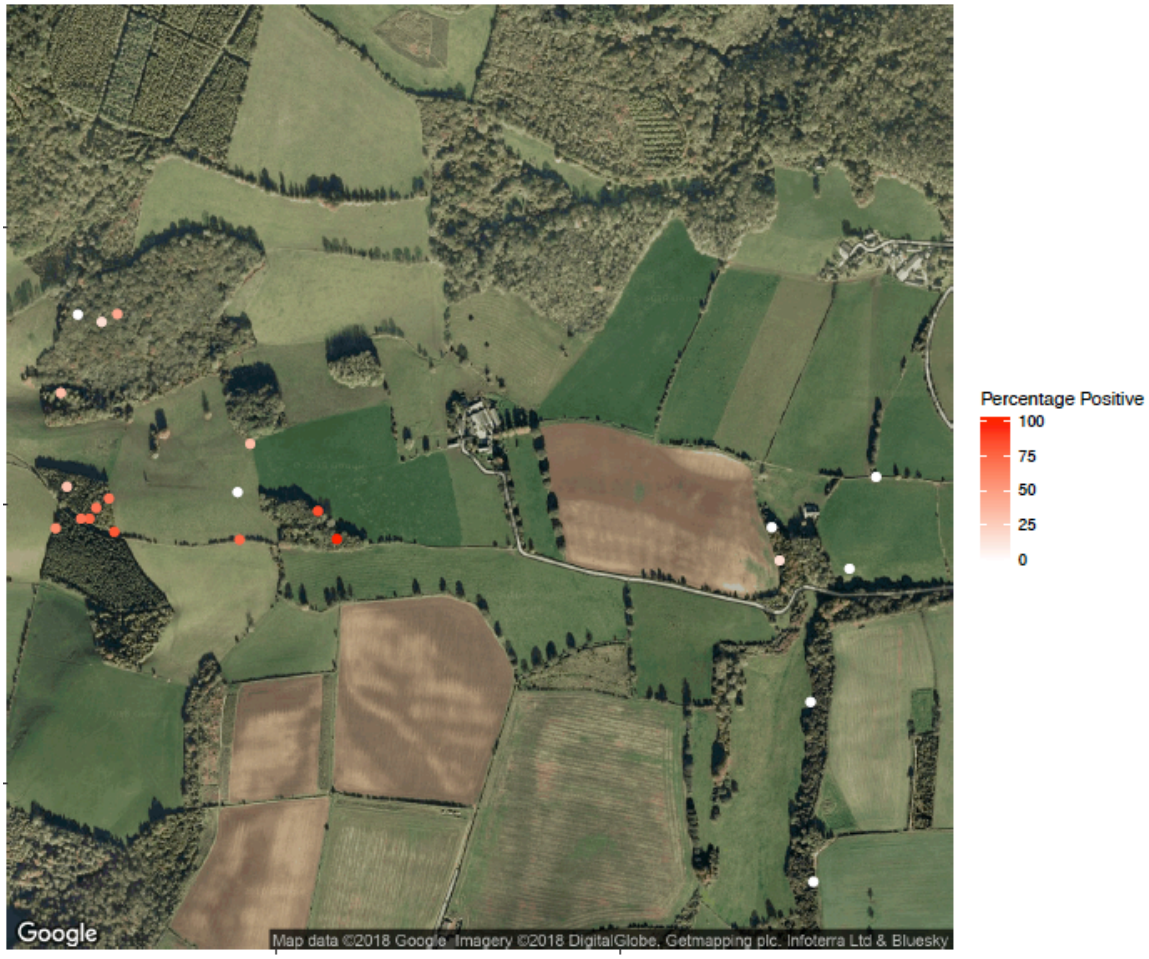

Figure 3.9: Map produced for the spring/summer survey representing the spatial distribution of latrines across the farm, along with their percentage positivity; higher latrine positivity represented by increasingly red tones.

In the summer/autumn of 2018, a total of 140 samples were collected from 24 frequently used latrines (Figure 3.10). Patterns were similar to that of spring/summer, with 100% of the latrines associated with Group 1 (n = 9) had a positive sample retrieved; for Group 2, 50% of the latrines (n = 4) had a positive sample retrieved, for Group 3, 33% of the latrines (n = 4) had a positive sample retrieved. The boundary latrine between Groups 1 and 2 also tested positive.

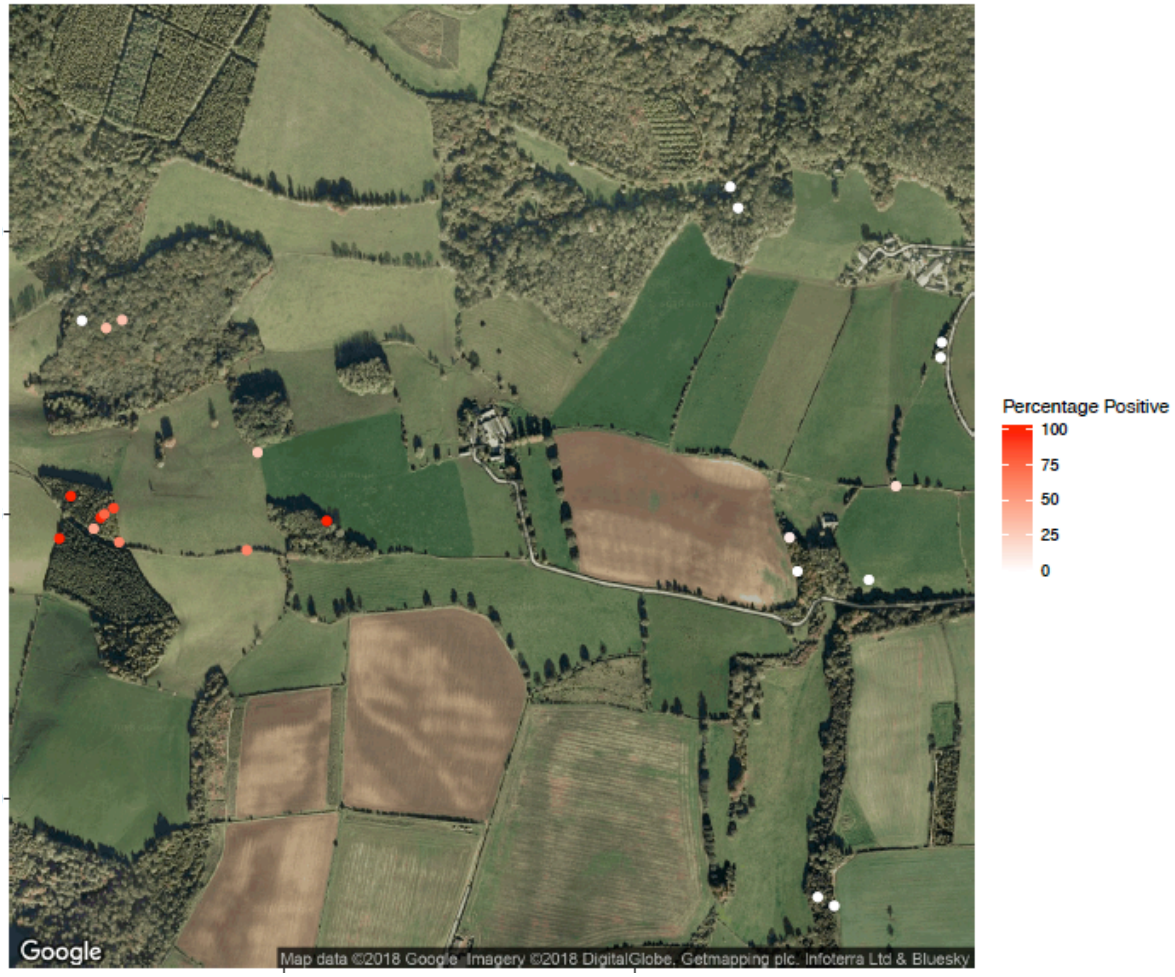

Figure 3.10: Map produced for the summer/autumn survey representing the spatial distribution of latrines across the farm, along with their percentage positivity; higher latrine positivity represented by increasingly red tones.

In the autumn/winter of 2018, a total of 140 samples were collected from 13 latrines (Figure 3.11) with a slight shift in patterns observed with 80% of the latrines (n = 5) associated with Group 1 having a positive sample retrieved; for Group 2, 100% of the latrines (n = 3) had a positive sample retrieved, for Group 3, 25% of the latrines (n = 4) had a positive sample retrieved, and a boundary latrine between Groups 1 and 2 also tested positive.

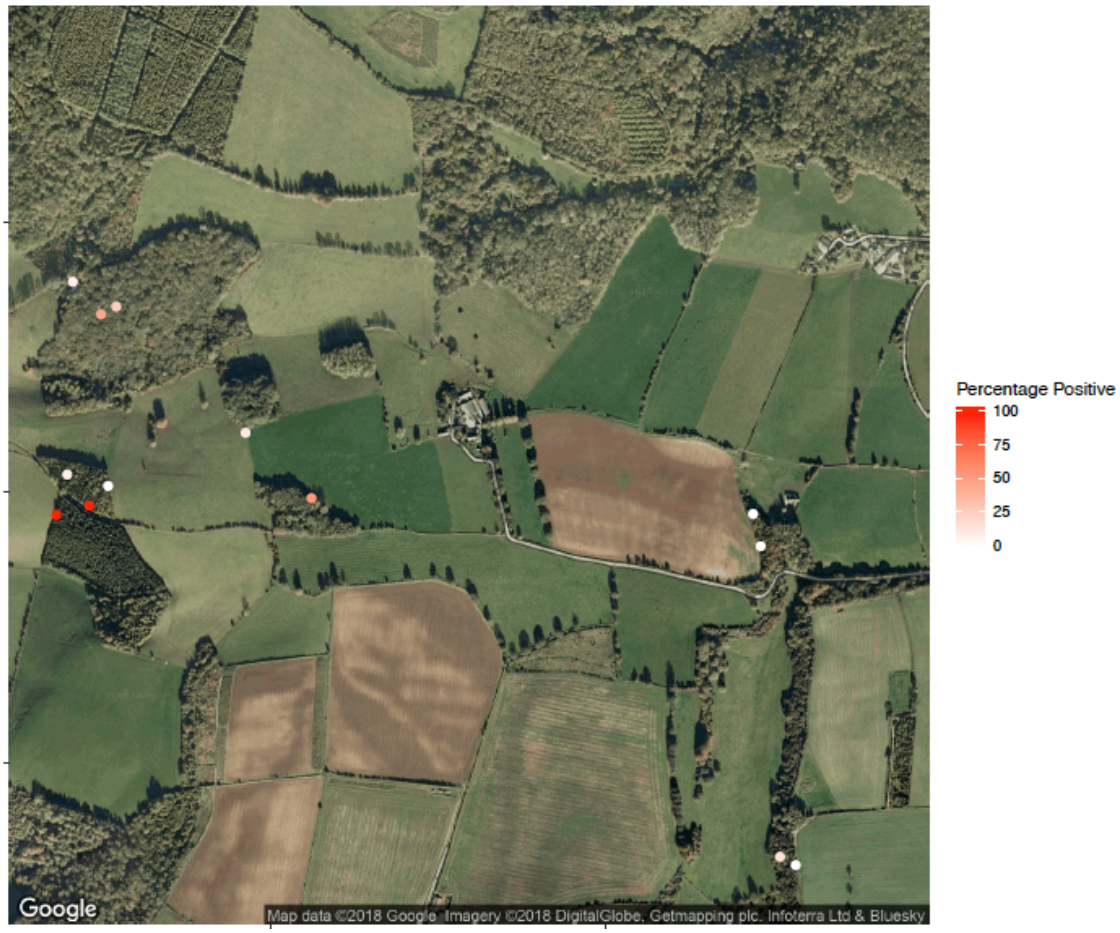

Figure 3.11: Map produced for the autumn/winter survey representing the spatial distribution of latrines across the farm, along with their percentage positivity; higher latrine positivity represented by increasingly red tones.

3.45 Disease clustering

Three potential factors were isolated: the number of samples that tested positive, the distance between the main setts and the latrine being tested, and the social group to which the latrine was assigned.

Tests of normalcy determined that these data were not normally distributed. Non-parametric rank correlations were conducted using Kendall's Tau and demonstrated that while 'percentage positivity' was not correlated with distance between latrines ($z = -0.058$, $p = 0.954$), nor with distance between the main setts' of social groups ($z = -0.937$, $p = 0.348$), that the percentage of positive samples retrieved was related to the social group from which the samples originated ($z = -4.434$, $p < 0.001$).

From spatial analysis it was possible to identify clusters of disease, particularly a divide between the Eastern and Western sides of the farm. Within the sampling period (May until November 2018), the latrines associated with Social Group 1 demonstrated a mean positivity rate of 70.7% ($n = 158$, $\sigma = 9.6$), Social Group 2 of 16.2% ($n = 101$, $\sigma = 3.9\%$) and Social Group 3 of 1.6% ($n = 88$, $\sigma = 1.4\%$). For this analysis, the mean latrine percentage positivity of each social group was used to avoid biases from certain latrines with higher or lower contamination rates presenting with greater numbers of samples; graphical representation of the results is demonstrated in Figure 3.12 below.

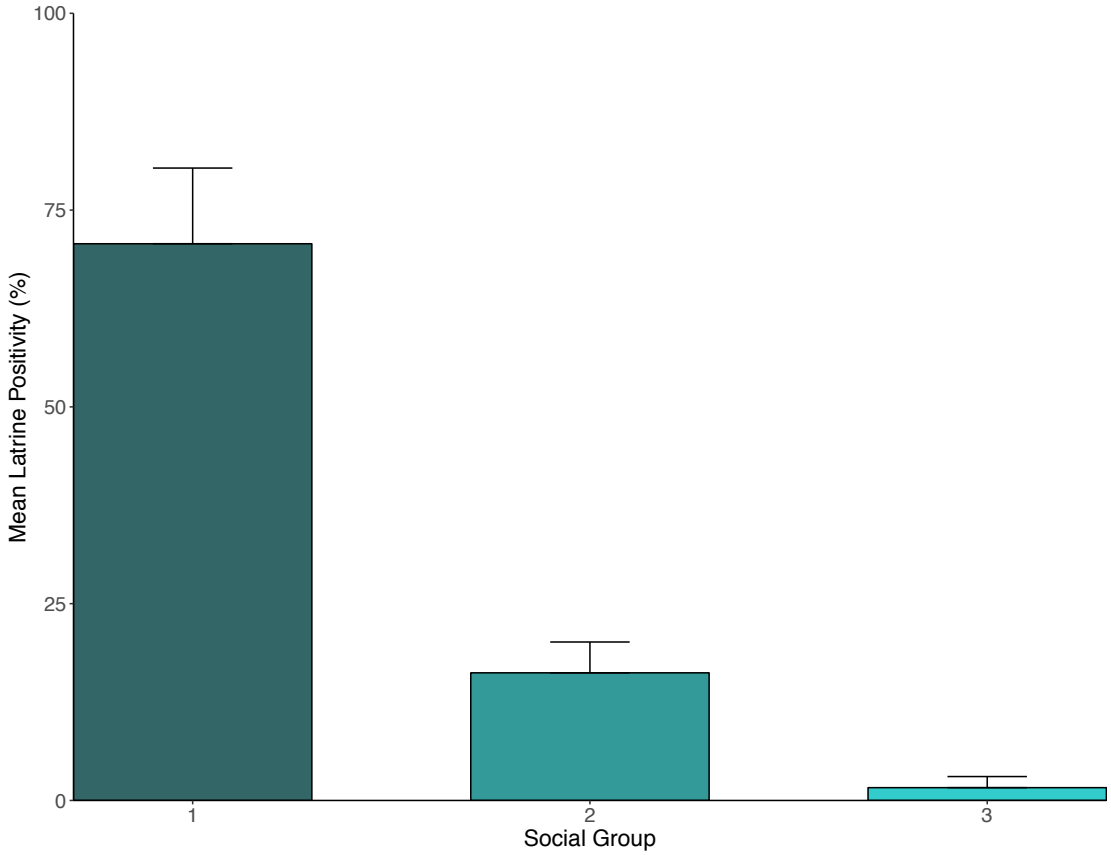

Figure 3.12: Mean percentage of faecal samples determined to be M. bovis positive by qPCR from each latrine associated with the three badger social groups across the sampling period (May – November 2018, inclusive) with error bars representing the standard deviation.

Power analyses were carried out as indicated above in Section 3.36 and the results are given in Table 3.6, with number of required samples being stated for 90% power at both 95% ($f(\alpha, \beta)$) = 10.5) and 99% ($f(\alpha, \beta)$) = 14.9) confidence. The number of samples collected for the 2 social groups being compared is then in the 'Actual N' column.

Table 3.6: Number of samples required from each social group in order to achieve a confidence level of 95 and 99% compared to the number of samples actually collected. The number of samples required for each group at the two different power levels are indicated in the column entitled 'Required N'. The number of samples actually collected for the groups (as indicated in the column entitled 'Groups' is then represented in the 'Actual N' column. Sufficient sample numbers were not achieved for 90% power at 95 or 99% confidence where marked with '' but were sufficient for 90% power at 90% confidence or 80% power at 99% confidence in the singular case.*

Season	**Spring-Summer**			**Summer-Autumn**			**Autumn-Winter**		
Confidence (%)	95	99	**Actual N**	95	99	**Actual N**	95	99	**Actual N**
Groups	**Required N**			**Required N**			**Required N**		
1/2	21	29	79/37	22	30	46/19*	31	43	33/45
1/3	13	18	79/18	15	21	46/37	22	31	33/33
2/3	11	16	37/18	11	15	19/37	9	12	45/33

3.5 Discussion

Most work investigating the level of disease in badgers has focussed around *post mortem* examination of badgers removed from cull zones, or on badger carcasses from RTAs (Barron et al., 2018). While the cull related research could provide information regarding disease status on a local scale, data has been summarised in terms of area or zone when the data has been generated at all (Anon., 2019c). Data generated by RTA studies does not provide information over a small land-scale, with carcasses being retrieved and data collated by county or area. Carcasses are also only retrievable (largely) as individual cases rather than as part of an in depth study of disease prevalence in local populations (Bourne, 2007, Goodchild et al., 2012, Barron et al., 2018).

This study sought to elucidate disease dynamics within badger social groups on a local scale via non-invasive methodologies. The site was an active, chronically infected dairy-farm set over 5 km from the edge of the nearest cull zone; the stability of the populations was therefore hypothesised to be at a natural level, uninfluenced by culling activity (Woodroffe et al., 2006, Pope et al., 2007). The landowner undertook no large alterations in practice before or during the study so as to ensure social group stability (Winkler and Mathews, 2015).

3.51 Pros of qPCR

There are three defined states when testing humans for TB infection: the unexposed state in which the individual's T-cells are naïve to tuberculous bacteria; the active infection state which is characterised by classical tuberculous symptoms and excretion of bacilli; the latent state, in which the individuals will test positive to skin tests, but not be suffering any symptoms of disease or excreting bacilli (Sarrazin et al., 2009, Pai and Sotgiu, 2016). The latter is the most common state in non-immunocompromised humans, with a third of the world's population hypothesised to be infected with TB and the huge majority only demonstrating latent disease (Dye et al., 1999, NHS, 2016). Arguably, we should be more concerned by the presence of individuals presenting with active disease and actively excreting bacilli. In humans, different tests are better suited for the identification of disease in these different states;

the same is true in badgers (Daera, 2013, Pai and Sotgiu, 2016). As faecal excretion of bacilli is associated with active, highly progressed disease, faecal qPCR is unsuitable as a diagnostic method on the individual as the limits of detection may be too low for badgers with less progressed pathology and will not detect individuals who are not shedding bacilli (Gallagher et al., 1998, Corner et al., 2011, Corner et al., 2012, King et al., 2015b). However, for research and monitoring purposes, incorporating qPCR as a method of assessing the infectiousness of a badger population could eliminate the risk of sample degradation while awaiting transportation. It is far easier to store a badger faecal sample (as only 0.1 g is required) than an entire badger carcass. This form of testing also allows rapid testing-turnaround, and therefore quick, informative results for the landowner either whilst making the culling/vaccination/biosecurity enhancement decision, or to ensure that the environmental disease burden is decreasing or redistributed when measures have been undertaken. Furthermore, it provides a non-invasive sampling technique that eliminates the requirement for the time and expense of employing a badger license holder to trap and perform invasive, stress-inducing procedures on a live badger.

3.52 Differences in social group shedding over a small landscale

The study presented above achieved the appropriate statistical power and detected meaningful differences in *M. bovis* excretion levels between social groups; these differences were maintained across the sampling period (May-November). Social Groups 1 and 2 were closer in terms of both spatial proximity and shedding status (high and intermediate) than to Group 3 which was essentially bTB-free, excreting very few bacilli per gram. It is unknown whether Groups 1 and 2 had higher levels of shedding (and theoretically infection) due to transmission either directly or indirectly between their social groups due to mating, the usage of boundary latrines, co-foraging or movement of individuals between social groups (Rogers et al., 1998). Furthermore, the pasture which was the primary foraging ground of Social Group 1 and had runs linking to Social Group 2 and was also the location of reactor cattle that were removed shortly after the summer sampling took place, with disease hypothesised to pass between the two species on this land (personal communication from landowner). Indirect contact had been observed between the two species: cattle were observed head rubbing and licking fenceposts next to the main run from Social Group 1 to the pasture;

head rubbing on soil next to where the run entered the pasture; and a high percentage of cowpats were 'flipped' by badgers which forage for insects and earthworms beneath them, particularly in hot dry weather such as that of the summer of 2018 (Olsen, 2013, Bacher et al., 2018).

Small, high-intensity spatial analysis had not previously been undertaken using environmental detection methods for the identification of hubs of infection related to latrines on farmland. However, clustering of badgers social groups infected with *M. bovis* has been detected by *post mortem* analysis of culled badgers in studies conducted in Ireland and South-West England (Jenkins, 2007, Byrne et al., 2015). It is therefore suggested that this is not a local phenomenon and that qPCR testing of badger faeces could be used as a simple, non-invasive method for surveying a social group's bTB risk (King et al., 2015a).

3.53 Seasonal and spatial analysis

In Woodchester Park, the seasonal prevalence of *M. bovis* in badger faeces was assessed across 12 social groups. These data demonstrated that within an atypically high-density badger population there were fluctuations in social group level percentage positivity (King et al., 2015a). This differed from the findings of the current research in which the percentage of positive samples retrieved from a social group were consistent across the seasons with the exception of winter samples from Social Group 1 (suggested to be related to badger death and behavioural change related to an alteration in main sett usage). This is hypothesised to be related to increased levels of social-group connectedness due to the atypically high density of badgers at Woodchester Park. It is likely that areas of high badger density promoting movements related to dominance hierarchies, mating or increasing sick badger mortality or movement from the area due to high competition for resources (Rogers et al., 1998).

3.54 Application

Results of this assay could be used to 'diagnose' the land, providing the landowner with knowledge of environmental hotspots to help direct biosecurity protocols and aid in the decision-making process as to whether or not to engage in active strategies to

tackle the environmental reservoir. Season was not a significant factor in predicting social group positivity, and therefore sampling could be undertaken at any time of the year. However, latrine locations are easier to find in the spring prior to vegetation growth and if the surveyor/sampler was not familiar with the badger population this should be considered when planning sampling (Anon., 2015b).

3.541 Monitoring purposes in response to vaccination

Current vaccination strategies are either being undertaken by the BEV scheme or by charitable organisations such as the Wildlife Trust and Badger Trust. Due to the small-scale approach to vaccination, it is hard to predict the effects and to make any assumptions based on local cattle populations bTB levels. There are currently no monitoring protocols in place to assess the effectivity of vaccination. The qPCR-based assay could provide charities with a quick method of initially screening a badger population, and then conducting follow up analysis to ensure that vaccination is successfully reducing the faecal excretion of *M. bovis*. As suggested before, *M. bovis* is likely to be related to an enhanced disease state; meanwhile vaccination, while not yet demonstrated to be preventative in badgers, is known to reduce the progression of disease and results in less bacteria excreted faecally under experimental systems (Chambers et al., 2011). The inclusion of qPCR in vaccination schemes could provide evidence for the reduction of excretion under environmental systems, providing more direct evidence for the benefits of vaccination and allowing the assessment of whether vaccination is a suitable method for reducing the disease burden in sympatric cattle populations, or whether this route is not as significant as hypothesised.

3.542 In the context of the current culling strategy

Current applications for the licensing of a cull area must meet strict eligibility criteria, and applications are only considered for areas within the HRA or in the EA which are of increasing risk. Groups of land holders (or the tenant farmer with permission of the landowner, if applicable) form a ‘company’ who bare financial, logistical and legal responsibility for the proposed cull. Under current policy**,** pressure is placed on nearby landowners to cooperate with culling operations, both socially and due to non-culling

farms being at greater risk post culling due to the perturbation effect (Bourne, 2007, Carter et al., 2007).

Culling places a large financial burden on the landowner; the total estimated cost to farmers per cull area is £140,000 for four years of active badger control (Anon., 2018a). Furthermore, licensee must compensate and protect any 'Relevant Authority' from 'all claims, proceedings, actions, damages, legal costs, expenses and any other liabilities whatsoever arising out of or in connection with the License or this Agreement…'. This means that not only is the licensed company responsible for the majority of the upfront cost, but also takes on the risk posed of incurring future costs (Anon., 2018b).

If a large enough dataset were to be developed across multiple sites demonstrating social group shedding variations over a small scale it would indicate the potential for a trial designed to remove specific social groups as determined by their *M. bovis* faecal excretion status. Not only could testing faecal samples prove to be ethically more favourable, but with further trials could prove to be more cost effective. This method is intrinsically non-invasive and could be conducted by landowners during routine field inspections if samples were collected appropriately and in adequate volumes, negating the requirement for trained and licensed ecologists as well as anti-social hours of work associated with trap-side testing. The samples from a single social group (n = 20, recommended) can be tested within 24 hours once within the laboratory environment, offering a quick and specific approach to screening badger population for bTB before landowners commit to a costly, time-consuming and ethically questionable cull (Travis et al., 2011, King et al., 2015a). At the time of writing, and using local-costings, for materials only it would cost approximately £120 to test the required 20 faecal samples for determining social group positivity via PA-qPCR, and an additional £2.12 per positive sample for a confirmatory qPCR run (assuming a full plate of 26 samples being loaded). Considering the large financial outgoings for landholders/tenants implementing a cull strategy, if applied to the dairy farm used for this work as a model system, for three social groups with a high sample positivity rate, it would have cost circa £360 in materials for the initial PA-PCR testing sweep, followed by £60 in follow-up qPCR costs. For the purpose of screening a farm prior to cull engagement, this has the potential to save time and money, as well as the social

and mental well-being benefits for being able to justify non-participation, or conversely, participation.

3.543 Monitoring of employed strategies

Due to the cheapness and simplicity of the method it has the potential to be implemented for measuring the impact of current control strategies on environmental contamination levels. Current strategies for controlling disease within the badger population include culling and vaccination; neither of these strategies are (currently) directly monitored over much of the UK. While some attempt has been made in recent years, problems with appropriate methods of sample storage has hampered widespread implementation.

Farmers do not bare the entire cost of badger culling; further financial implications are placed on taxpayers via APHA, Natural England and Defra which total £258 thousand for four years of culling in one licenced area, based on figures from 2017 (Anon., 2018a). A further cost unaccounted for in the original estimates was that of policing the culls due to large numbers of protestors; thus far policing has increased costings by approximately £210 thousand across the 4 years (based on figures from 2016 and 2017). In total, for four years of culling in one target area, the farmers and government share a £0.6 million bill in the hope of reducing the number of farms under restriction within that area (Anon., 2018a). With the strategy being highly controversial and ethically questionable, the introduction of a simple sampling measure in order to demonstrate to the taxpayer that their funds are contributing to a meaningful reduction in disease in badgers as well as in cattle would be a favourable outcome. The surveying undertaken within this research demonstrates that the method can be applied to a working dairy farm demonstrating a typical badger sett density for the HRA of England.

3.544 Targeted reactive culling

Culling in response to cattle herd infection was trialled as a component of the RBCT as a method of reacting to wildlife reservoirs while minimising the number of badgers removed. Under this initiative, badgers were culled in small (5.3km^2) areas in response

to an outbreak of infection in local cattle populations over nine 100km^2 study areas (Donnelly et al., 2007). The results of this aspect of the trial were less successful than those of proactive culling as the study areas saw increases in the numbers of herd under restrictions, as well as a large increase (mean = 29%) in the 2 km perturbation zone surrounding the trial area. In contrast, the proactive cull zones saw a decrease of 19% after the initial cull and 23% after the follow up culls; though the benefits of this were offset by a 29% increase in herd breakdowns in the perturbation areas, reducing to 22% post-secondary cull. The small scale, reactive culls were therefore deemed an unsuitable method of controlling the disease, presumably due to the larger edge effects associated with culling over a small area, with the badgers ranges expanding due to the reduced population density and emigrating to areas of lower density (O'Corry-Crowe, 1996, Eves, 1999, Carter et al., 2007). These culls have also been shown to increase the spatial spread of both infected and non-infected badgers, therefore posing a risk to cattle and wildlife that may previously have been unexposed (Jenkins, 2007). Badger movements from one area into neighbouring territories disrupts the stability of social groups in these neighbouring areas. Increased badger movement in one year is strongly associated with an increase in TB in badgers in the next year, even under non-cull conditions (Rogers et al., 1998). It is likely that the increased incidence of badger tuberculosis then has a spill over effect into the local cattle populations as culling (and therefore increased badger movement) is associated with an increase in herd breakdowns in the perturbation zone (Donnelly et al., 2007, Jenkins, 2007, Jenkins et al., 2008).

As a proposed alternative to the reactive cull, badger social groups would be non-invasively screened to determine their shedding status; the number of positive samples per social group would be used as a proxy for the level of threat which they pose to the local cattle population. These results would be used to inform a targeted cull protocol aiming to remove badgers associated with the social groups of animals that are actively excreting bacilli and therefore likely to be in the latter stages of disease progression (Chambers et al., 2011, Lesellier et al., 2011). While social group level culling is still likely to involve the culling of healthy badgers, this would be to a lesser extent than what is hypothesised to be occurring during the current cull strategy and would prevent the removal of healthy badger social groups that could be replaced by infected individuals. However, under current legislation this is strictly forbidden as

guaranteeing the complete removal of a social group is not thought to be possible under known-to-be-humane methods of euthanasia. Furthermore, it is likely that the area and latrines related to the sett would demonstrate high levels of contamination with *M. bovis* and therefore must also be decontaminated or removed so as to prevent infection of wildlife that colonise the site and rendering culling meaningless. It is illegal to damage or destroy a badger sett under the Wildlife and Countryside Act and therefore, under current legislation it is recommended that the method is used to target the improvement of biosecurity measures towards these areas which are known to pose a high infection risk to cattle.

3.545 Biosecurity

The recommended approach for using this method is to survey farmland for areas of increased disease risk and use the results to target the implementation of biosecurity protocols, as largescale alterations to farm structure and practice can be difficult or costly to implement. Knowledge of the local badger disease status would allow farmers to directly target areas at most risk from wildlife associated disease transmission, or in the absence of high risk posed by wildlife, target improvement of husbandry practices and cattle related biosecurity measures. Examples of this include: the movement of cattle away from areas of increased disease prevalence when possible; inclusion of extra fencing to increase segregation distances and potentially lower the contact rates; badger-proofing feed and water troughs; clearing of latrines on pasture and the use of Defra approved disinfectants to reduce the risk of transmission.

3.6 Conclusion

It is likely that the natural distribution of disease is heterogenous, and average levels of infection can result in an overestimation or underestimation of the risk which specific social groups pose. In terms of informing farm-level biosecurity strategies this poses problems at either end of the spectrum. If we over-estimate the level of disease, then calculations will suggest the removal of unnecessarily high numbers to reduce levels to a more manageable amount and reducing the likelihood for the disease to persist. While many operate on the 'better safe than sorry' principle, there are ethical issues related to culling healthy badgers which, outside of licensed areas and periods, retain their protected species status.

The above research highlights local scale variation in badger disease prevalence and offers a rapid method for non-invasively monitoring and targeting infectious social groups. Research should be conducted over a larger land scale and could be performed in line with the current culling strategies to appropriately established at badger *post mortem* and compare to the RD4-qPCR results. This method has the potential to not only provide a scale of measurement for the effectivity of control methods, but also to influence the current policy if able to prove that disease clustering is not a localised phenomenon and is detectable via non-invasive sampling. A change in policy resulting in the pre-testing of any proposed cull has the potential to reduce the costs associated with culling due to only targeting infectious social groups, as well as potentially reducing knock-on ecosystem effects.

Chapter 4:

An Examination of the Short-Term Survival of *M. bovis* in Naturally Infected Badger Faeces

4.1 Abstract

Studies on the survival of *M. bovis* have largely focussed on the length of time for which bacilli can be detected by the presence-absence of cells by standard culture-based techniques. However, studies have neglected to examine the initial decline of bacilli within naturally infected material when first excreted from the host which would have consequences on any models of survival constructed for epidemiological purposes. The findings of this study firstly demonstrate the biphasic nature of *M. bovis* decay in naturally infected faeces and that this is linked to the season in which the cells are excreted into the environment. The shape of the curve is suggestive of the persistence of cells with high capabilities to survive environmental desiccation and if findings from persistence *M. tuberculosis* cells can be translated to *M*. bovis, then are unlikely to be captured by typical culture-based techniques employed by the APHA. Furthermore, there are significant interactions between the meteorological conditions and the persistence of *M. bovis*, with soil temperature being the best predictor of survival and UV and air temperature hypothesised to be the key factors underlying *M. bovis* persistence. It is suggested that the British climate is conducive to the long-term survival of *M. bovis* in badger latrines, and that these areas should be considered contaminated until visible clear of faecal material by natural conditions.

4.2 Introduction

The understanding of disease transmission mechanisms and analyses of their risks are convoluted by the presence of wildlife reservoir species (Tompkins et al., 2011). In the case of bovine tuberculosis in the UK, the badger (*Meles meles)*, a secondary maintenance host, is implicated in the transmission and maintenance of the disease in cattle due to their high infection rate and abundance on farmland (Judge et al., 2013). Approximately 5.7% (CI 0.9-25%) of bTB breakdowns are attributable to direct badger-to-cattle transmission, while a further 15% is attributable to environmental contamination and subsequent transmission, as determined by model prediction (Brooks-Pollock et al., 2014, Donnelly and Nouvellet, 2013). However, research suggests direct transmission is unlikely to be a key driver for intra-specific transmission as badgers largely avoid cattle, remaining at a distance of 10-15 m (Benham and Broom, 1989). They are therefore unlikely to come within the hypothesised distance (1.5 m) required for the direct transmission of *M. bovis* (Benham and Broom, 1989, Sauter and Morris, 1995, Allen et al., 2011, Woodroffe et al., 2016). However, badgers spend much of their above ground time on pasture, and will preferentially forage on this land class offering opportunities for indirect transmission events (Kruuk et al., 1979, Woodroffe et al., 2016).

Incidences of *M. bovis* being excreted into the environment by badgers are well recorded in faeces, urine, pus, saliva and sputum (Allen et al., 2011, Clifton-Hadley et al., 1993). Specifically, high levels of bacilli have been recovered from the latrines of infected badger clans in which faeces and urine deposition is concentrated into a small geographic space of a maximum of a few meters (Travis et al., 2011). Badgers have been shown to faecally excrete *M. bovis* for 165-1305 days before death, at typical concentrations of up to 4×10^5 g^{-1} of faeces as estimated by qPCR, but values of up to 1×10^8 g^{-1} of faeces have been reported (Little et al., 1982a, Travis et al., 2011, King et al., 2015b). Cattle will largely avoid grazing plots contaminated with badger excreta for up to 28 days when other suitable areas were available (Benham and Broom, 1991). However, during a behavioural study, 2 of the 240 strong cohort were willing to graze close to faeces, and 7 to graze close to urine. It was also noted that some cattle responded to urine and faeces with greater amounts of sniffing and demonstrated notable interest in the areas (Benham and Broom, 1991).

The next factor to consider in environmental transmission is the persistence of bacilli under natural environmental conditions in the UK. Studies on this topic are sparse and widely vary in design which limits the strength of comparisons (Stenhouse Williams and Hoy, 1930, Maddock, 1933, Anon., 1979, Fine et al., 2011a). The earliest published work was conducted on cattle faeces and found that one fifth of naturally infected faeces remained infectious 1-month post spreading onto pasture (Stenhouse Williams and Hoy, 1930). Follow-up work artificially inoculated cattle faeces with 2.5 x 10^5 bacilli g^{-1} with environmental survival monitored from September, November, May and August with rates of 4 months, 3 months, 5 months and 2 months respectively. There were limitations to this study such as the relatively high level of bacilli and, for the purpose of badger to cattle transmission that cattle faeces is likely to differ compositionally. Furthermore, the use of guinea-pig infection models is difficult to attribute solely to *M. bovis* due to the large number of environmental mycobacteria which could also have been within the feed and presented with lesions containing acid-fast bacilli (Aronson and Whitney, 1930, Feldman, 1936, Ordway et al., 2008, Silva-Gomes et al., 2015).

Similar work conducted in Kruger National Park, South Africa, left naturally infected lung and lymph node tissue and spiked faecal matter within plastic cups in shallow pits in the environment with culture confirmation of *M. bovis* (Tanner and Michel, 1999). Again, persistence was highest in the winter, and lowest in summer, though their timescales were measured in days (maximum mean was 27 days in the winter) rather than in months as found in the earlier, UK based study (Stenhouse Williams and Hoy, 1930). This is likely to be largely due to large differences in the climate under which this study was conducted.

In relation to badger excreta, a study undertaken by the Ministry of Agriculture, Fisheries and Food examined and monitored the culturability of *M. bovis* from naturally infected faeces and urine on pasture (Anon., 1979). *M. bovis* was cultured from urine for a maximum of 3 days in the summer and 4 weeks in winter. Naturally infected faeces retained culture positivity for longer than urine but tested negative after 2 weeks in the summer and after 1 month in the winter. The high bacterial counts (up to 3 x 10^6 in urine and unstated in faeces besides being describes as ‘lower’, possibly

due to harsher decontamination methods) were concluded to have been a key factor in the persistent detection of bacilli in urine (Anon., 1979).

The ability to predict the duration of environmental contamination with viable bacilli from an infected badger clan is imperative for the implementation of appropriate biosecurity measures to reduce the likelihood of cattle interacting with infected material. It is also important to ensure that when undertaking a Government led cull that badgers do not return to the land until it is sufficiently sterilised to prevent establishing infection in a recolonising population. Despite environmental persistence and subsequent infectivity of *M. bovis* being researched as early as the 1930s, the variability between methods and objectives, as well as the complex nature of the organism, make drawing conclusions regarding survival time difficult (Stenhouse Williams and Hoy, 1930, Maddock, 1933, King et al., 2015b). Most environmental persistence studies have used soil which will vary compositionally from faecal matter; furthermore sterilisation is undertaken prior to artificial inoculation, leading to greater persistence of the inoculated organism than might otherwise be expected (Wray, 1975, Tanner and Michel, 1999, Ghodbane et al., 2014, Barbier et al., 2017). This is hypothesised to be due to the typical microbiota outcompeting the slow growing and un-adapted *M. bovis* for nutrients (Hibbing et al., 2010). However, it may also be due to the decreased requirement for harsh decontamination methods prior to culture increasing the method's sensitivity (Corner et al., 1995). Even when sterilisation has not occurred prior to inoculation, culture studies on artificially infected samples should be interpreted with caution due to the potential for the cells to be in a better physiological state than those excreted from a host and therefore better able to both survive and adapt to the change in environment (i.e. host induced dormancy) (Mukamolova et al., 2010).

4.21 Aim

The purpose of this research was to elucidate the interactions between the environment and the persistence of faecal *M. bovis* and how these were affected by the season and the meteorology. The study used qPCR in order to detect copies of the RD4 gene in faecal samples collected from a local dairy farm and transferred to an exclosure in which continuous subsampling was undertaken. The aims of the work were as follows:

1. To understand how seasonal variation affects the persistence of *M. bovis* in badger faeces.
2. To establish the effect of meteorological parameters on environmental survival.
3. Discuss the key parameters relating to *M. bovis* survival and how these can be incorporated into farm biosecurity practices.

4.3 Methods

This work was conducted to minimise the risk of transmission to wildlife or human populations, with respective landowners' permissions and approved by the University of Warwick GMBSC committee.

4.31 Sample collection

Using single-use wooden spatulas, faecal samples were placed into sterile 30 mL Universal tubes, labelled with a latrine identification number and sample number. The latrine number, sample numbers, location, social group ID, date of collection and the presence of subcaudal secretion (compromised of unsaturated fatty acids and bacteria for scent marking) were logged both on paper and digitally. Tubes were placed in a Ziploc bag, in an appropriately labelled cool bag on ice within 2 hours of collection from the field to be transferred to the exclosure experiment site. A total of 134 faecal samples were collected in the Spring (62% positive), 102 samples in the Summer (40.2% positive) and 111 samples in the Winter (18.0% positive).

Sample collection was divided into three sets of seasonal sampling, commencing in May 2018 (spring/summer), August 2018 (summer/autumn), and October 2018 (autumn/winter). These timings were chosen to reflect the period when cattle are generally grazed on pasture and therefore most at risk from indirect transmission from badger latrines. The initial sett survey was undertaken in October 2017, and contamination survey conducted in December 2017, with the winter period discounted as an option for seasonal monitoring due to adverse weather conditions making reliable sampling impossible as well as the applicability of the data being minimal.

4.32 Establishing the 'exclosure'

The exclosure was set on private land in Gloucestershire, UK with the landowner's permission and away from the location of the farm and public rights of way. The exclosure consisted of a modified small-animal-pen with an additional wire boundary, security bolts, and staked into the ground. A camera trap (Ltl Acorn 5210A) was placed at the site two weeks prior to the placement of faeces to ensure there was no

animal contact with the exclosure. A tarpaulin sheet was used to shield one half of the exclosure from the direct effects of weather, with the other side left exposed to the elements besides the presence of wire and transient shadows cast by the wooden framework.

Samples from the Gloucestershire farm site were transferred to the location of the exclosure and were divided in half at random and placed either in the 'unshielded' section of the exclosure or beneath the covered side. The samples had plastic markers with their identifier number. Sample positions were logged both on paper and electronically.

4.33 Continual sub-sampling

Sub-sampling commenced on 'Day 0' (the day of collection) and continued for the duration of the experiment unless the sample degraded to an unsamplable state. The exclosure site was revisited 1-month post completion of the initial, daily 'short-term' sampling for the purpose of the 'sunlight and shade' study. Approximately 1 g of faeces was placed into a 2 mL microcentrifuge tube labelled with the day number, and the sample's identifier number (e.g. 5.12 for 'day 5' and 'sample number 12'). Subsamples were frozen immediately post-collection at -15 °C. Samples were transferred to the CL3 facility at the University of Warwick and remained frozen until DNA extraction.

4.34 DNA extraction

Total community DNA was extracted from the samples and a positive control (badger faeces spiked with *M. bovis* BCG) and negative control (badger faeces with equivalent volume of di-H_2O) using the FastDNA™ Spin Kit for Soil (MPbio) as per the manufacturer's instructions, with minor modifications. In brief, 0.1 g of faeces, 978 µL of sodium phosphate buffer and 122 µL of MT buffer were added to Lysing Matrix E tubes. The tubes were vortexed and ribolysed at 6000 wibbles min^{-1} for 2 rounds of 40 s. The tubes were centrifuged at 13,000 x g for 12 mins and the supernatant transferred to microcentrifuge tubes containing 250 µL PPS, inverted 10 times and

incubated at room temperature for 10 mins. The tubes were again centrifuged for 5 mins at 13,000 x g and the supernatant added to 7 mL bijoux tubes containing 1 mL of binding matrix, inverted for 2 mins, settled for 5 mins before 400 µL of the top fraction was discarded. The remaining mixture was resuspended and repeat aliquots of 650 µL transferred to a tube containing a spin filter and centrifuged for 3 mins at 13,000 x g and the flow-through discarded. The residue was eluted in 500 µL of SEWS-M and incubated for 5 mins at room temperature, centrifuged twice at 13,000 x g for 5 mins and the flow-through discarded. Finally, the spin basket was transferred to a new catch tube and air dried for 5 mins prior to gentle elution in 100 µL of DES. The tubes were incubated at 60 ˚C for 5 mins before being transferred to the centrifuge and spun at 13,000 g for 3 mins (twice). The spin baskets were discarded and the DNA frozen at -15 ˚C ± 2 ˚C until use.

4.35 PA-PCR reaction

An initial screening stage targeting the RD4 deletion region which is unique to *M. bovis* was included in order to identify samples which contain sufficiently large numbers of GEs g^{-1} for a decline to be detectable along the length of the experiment. For this, samples were tested in duplicate using an ABI 7500 Fast qPCR machine (ABI) with negative and positive controls included from the extraction process. A 10^3/µL standard and a negative control for the PCR reaction (of mastermix and additional di-H_2O) were included. The PCR reaction mix included 10 µL of either standard or total community DNA, 900 nM RD4-forward primer $^{5'}$-TGTGAATTCATACAAGCCGTAGTCG-$^{3'}$, 900 nM RD4-reverse primer $^{5'}$-CCCGTAGCGTTACTGAGAAATTGC-$^{3'}$, 250 nM probe AGCGCAACACTCTTGGAGTGGCCTAC-TMR, 1 mg/mL of BSA, 12.5 µL of Environmental Mastermix 2.0 (ABI), and made up to 25 µL with sterile, DNA-free water (Pontiroli et al., 2011). The conditions for the reaction were as follows; 50 ˚C for 2 mins, 95 ˚C for 10 mins, 40 cycles of 95 ˚C for 15 s and 58 ˚C for 1 min.

4.36 qPCR reaction

For samples which demonstrated amplification in both wells of the PA-PCR screening step, the quantity of *M. bovis* DNA was established using qPCR assays targeting the RD4 deletion region. The method was as illustrated above but included a panel of standards for the production of a standard curve and subsequent quantification. The standards ranged from 10^{6} to 10^{-1} genome equivalents per μL and these, as well as each DNA sample were quantified in triplicate. A negative control of mastermix with additional di-H_2O was run alongside the standards in triplicate.

4.37 Sample numbers

Sample numbers were difficult to establish due to reliance on the badgers producing faecal matter, and these samples containing suitably high numbers of *M. bovis* to be detected by the qPCR assay. Samples were omitted from the continual exclosure DNA extraction process if only one well of the duplicates run during the PA-qPCR screening component amplified, or if the CT was above 35. It was hoped to collect at least 10 suitable samples per season to increase on the number of replicates used within related studies within the literature, and also to cover a range of 'faecal types' due to wide variation in consistency and content.

4.38 Meteorological data

Data for the local weather station (approximately 1 mile from the exclosure site) was obtained from the Met Office Integrated Data Archive System (MIDAS) with permission from The Natural Environment Research Council's Data Repository for Atmospheric Science and Earth Observations (MIDAS, 2018).

4.39 Data analysis

This research focussed on the impact of temperature (air, soil and dew point), humidity, shortwave radiation and precipitation. Data was analysed in R using the 'stats' package as well as the 'lubridate', 'chron', 'plyr', 'dplyr', 'Hmisc', 'caret' and 'splines' packages, and visualised using 'corrplot', 'ggplot2' and 'KernSmooth'

statistical packages, as well as Prism 8 software for the production of the dual linear models representing biphasic decline.

4.4 Results

The data presented in this section will demonstrate the rate at which *M. bovis* counts decline in badger faeces as determined by qPCR. Further exploration will seek to determine whether this rate can be attributed to time since excretion from the host; the season in which the sampling occurred; various meteorological parameters; exposure to direct sunshine.

4.41 Sample numbers and positivity

A total of 32 latrines were identified across the seasons with 26 of these being sampled from during the study. In the spring-summer, 22 latrines were sampled from, 24 sampled from in the summer-autumn, and 13 in the autumn-winter. A total of 347 faecal samples were screened by PA-qPCR, of which 123 tested positive (35.45%).

4.42 Visual depiction of the samples

Large variation was seen in faecal consistency, content and volume (Figure 4.1) of which the content (and therefore consistency) was partially relatable to the social group of origin and their proximity to specific food sources as well as the season of collection (observation). In brief, samples collected in the spring and summer seasons were of drier consistency than those collected in the winter. It was also observed that the faeces of Social Group 1 contained far higher ratios of pine-nuts and wheat, and little of what would be described as wet faecal matter; these samples became difficult to sample from as the study progressed.

Figure 4.2: Example of faecal diversity.

4.43 Sample screening

The selection of samples for monitoring qPCR positivity over the initial environmental exposure was established with an initial PA-qPCR screen and follow-up quantification qPCR of positive samples. Samples were deemed suitable for follow-up based on sufficiently high *M. bovis* GE values to allow observation of decline of these values over time, and eluted DNA being visually clear and free of contaminants. The summary of seasonal samples *M. bovis* content is depicted in Table 4.1. *M. bovis* GEs shed in the spring/summer season were significantly higher than in the summer/autumn ($W = 357$, $p < 0.001$) and autumn/winter ($W = 184$, $p = 0.01$) sampling seasons. There was no statistical difference between the numbers of detected genomes in the summer/autumn and the autumn/winter sampling periods ($W = 133$, $p = 0.99$). Sample numbers were sufficient so as to include a range of the faecal consistencies demonstrated in Figure 4.1.

Table 4.1: Summary of the initial M. bovis content of naturally infected faecal samples used within the exclosure experiment. N_0 represents the number of samples at day 0.

Season	Mean	Median	Range	N_0
Spring-Summer	1.37×10^5	9.26×10^4	7.88×10^3 - 4.46×10^5	16
Summer-Autumn	2.47×10^5	6.53×10^4	5.45×10^3 - 1.96×10^6	21
Autumn-Winter	8.54×10^4	6.75×10^4	1.65×10^3 - 2.09×10^5	10

4.44 Seasonal and temporal decline of *M. bovis* in naturally infected samples

As a preliminary investigation of the effect of environmental exposure on the number of *M. bovis* detectable within faeces, the mean number of genome equivalents were plotted against the number of days since the sample had been excreted from the host (Figure 4.2). While there are large declines in the numbers of bacteria, as demonstrated by Figures 4.2 and 4.3, high numbers remained detectable within the samples. After one week of environmental exposure, the faecal samples contained a mean of 1.86 x 10^4 cells g^{-1} (SE 7.4 x 10^3 cells g^{-1}, all season data). With badger faeces weighing circa 50 g (mean = 53.3 g; minimum = 37.0 g; maximum = 87.05 g; n = 7) this content poses a significant transmission risk.

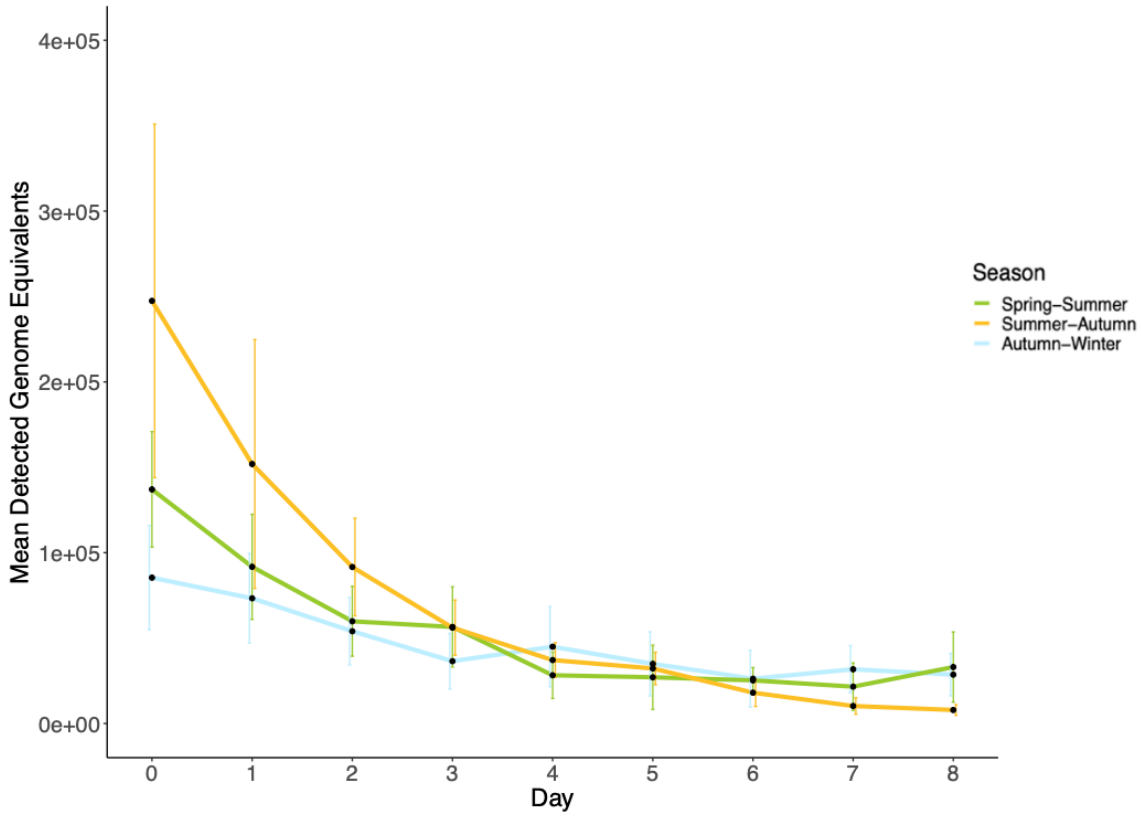

Figure 4.2: Decrease in detected genome equivalents in badger faeces as detected by qPCR across the three sampling seasons. Error bars represent the standard error on the mean number of genome equivalents detected. LOD 10^3 genome equivalents.

The mean percentage of remaining cells across the samples were plotted against time in days (Figure 4.3). This demonstrated a rapid rate of decline over the first three days of environmental exposure. As anticipated, the percentage decline within the spring-summer and summer-autumn periods was more rapid than in the autumn-winter.

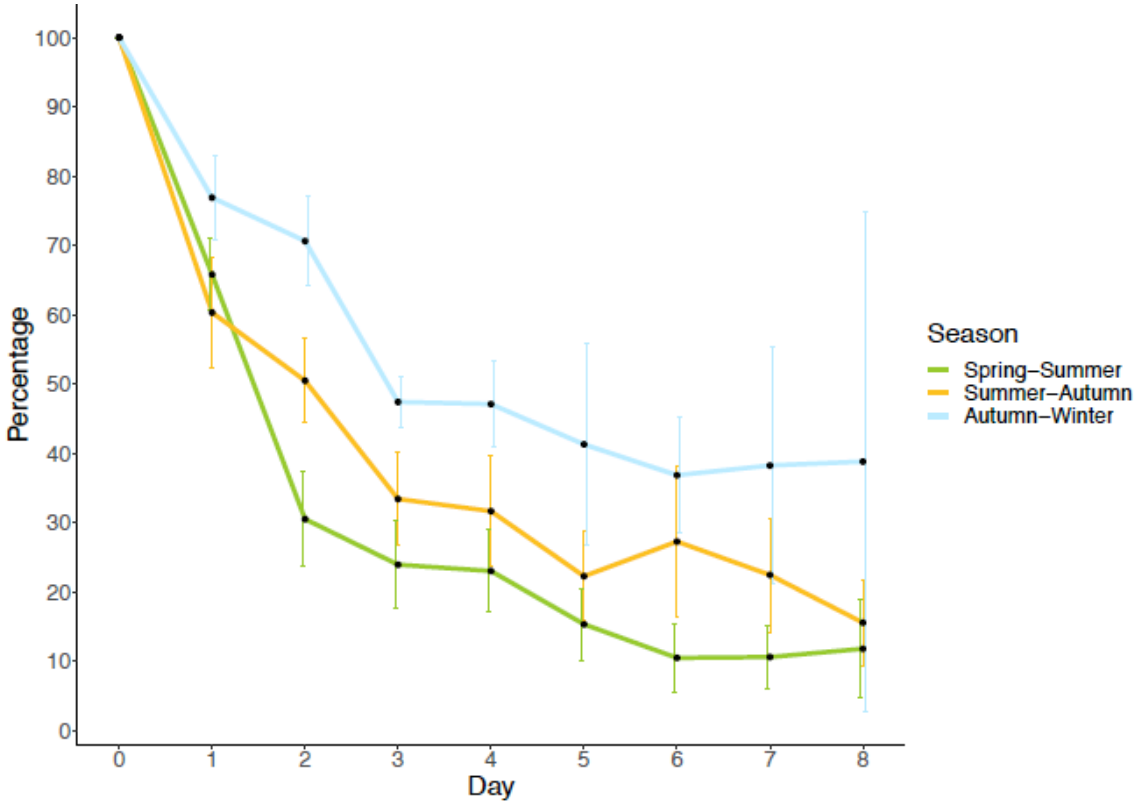

Figure 4.3: The mean percentage of M. bovis cells detectable daily by qPCR, standardised against the initial values (day 0). Data presented for 3 sampling sessions, with error bars representing the standard error.

For each sample, the percentage remaining of the initial value was calculated per day and the mean of this value taken across the range of samples (Figure 4.4). While there is significant variation for any given day, the prevailing trend remains as in Figure 4.3 with spring/summer and summer/autumn exhibiting a more rapid decline in GEs than seen in the autumn/winter sampling period. While the biphasic model prevails, the autumn/winter samples do not appear to have reached the stationary phase indicated within the previous two figures.

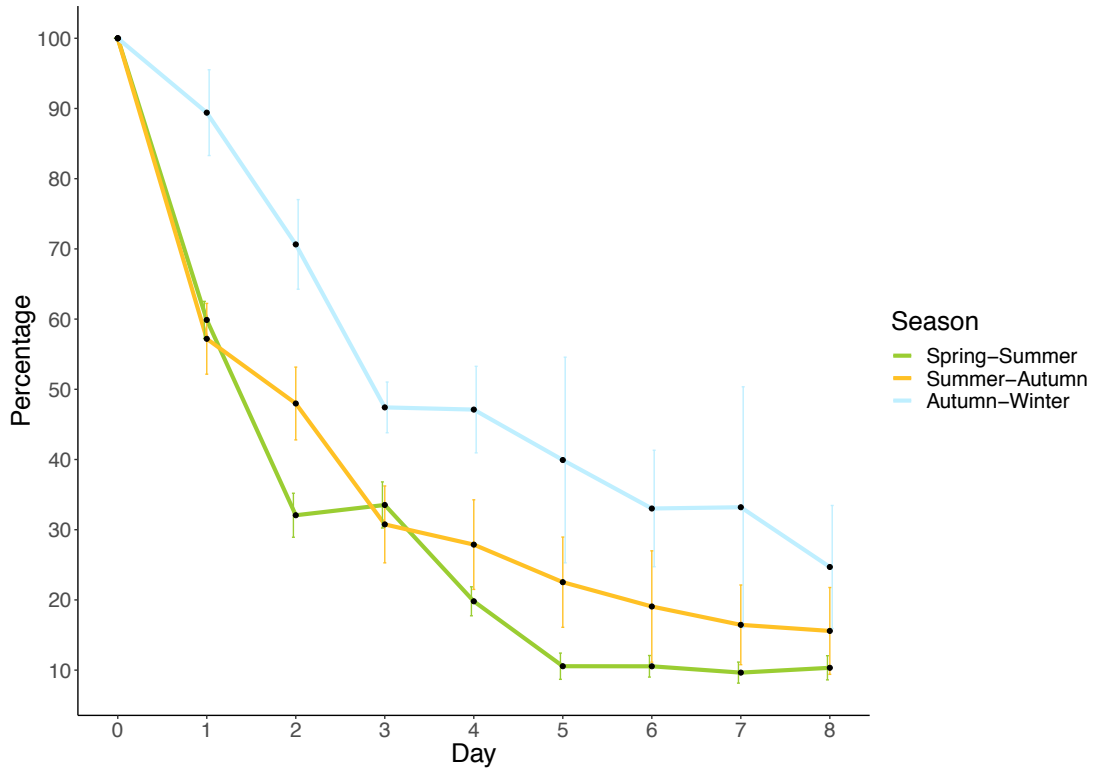

Figure 4.4: The mean of the 'within samples' percentages remaining as calculated by the mean value across all samples' demonstrated declines. Error bars represent the standard error.

As an alternative measure of variance, a box and whisker plot was also produced (Figure 4.5) This was produced from the raw data and to illustrate the variation around the median, with persistence of *M. bovis* found to be substantially higher in outlier samples. However, the prevailing trend remaining as in Figures 4.3 and 4.4.

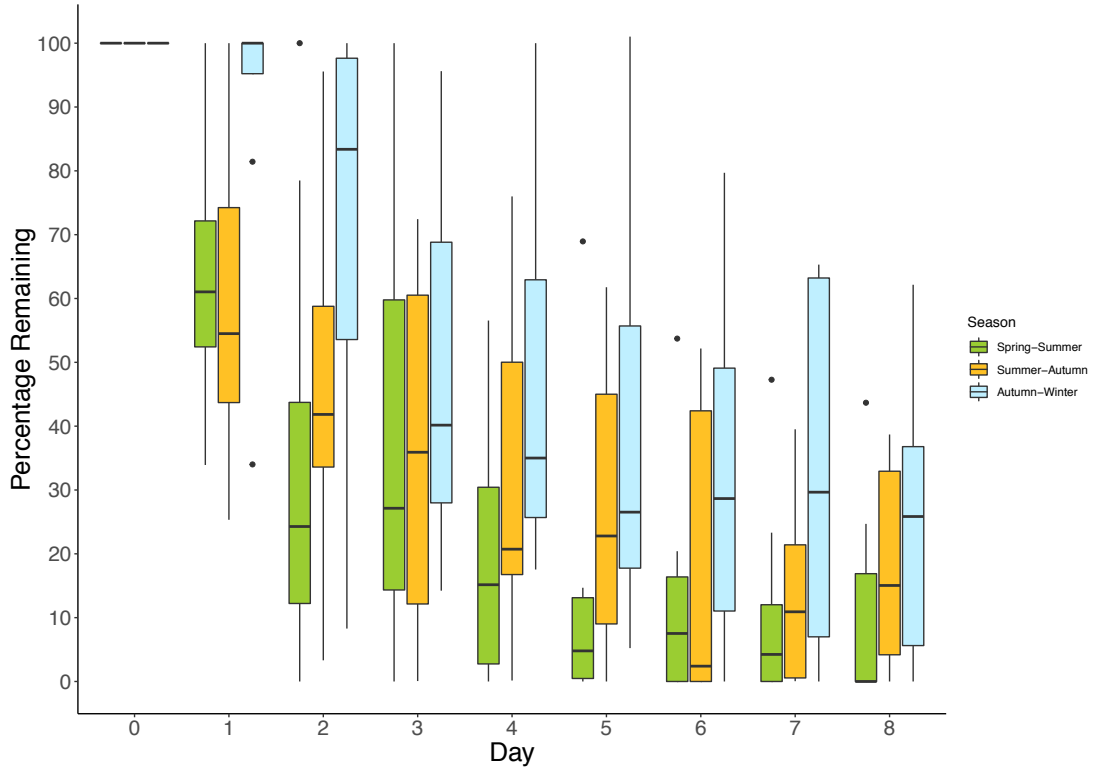

Figure 4.5: Box and whisker plot demonstrating the median percentage of M. bovis cells that remain detectable daily (central line), the interquartile range (the upper and lower limits of the box), the range represented by whiskers and statistical outliers.

4.45 Modelling *M. bovis* decline in faecal samples

Three seasonal datasets were formed to examine the relationship between individual seasons and *M. bovis* persistence. A polynomial regression model was produced for the data of the raw counts of *M. bovis* across the sampling period demonstrated correlation between the decline in the raw counts of *M. bovis* within the faecal samples and time (Figure 4.7). However, the relationship was obscured by high variance (2 order of magnitude) in the initial raw counts from naturally infected samples (RMSE = 1.2 x 10^5, $R^2 = 0.18$).

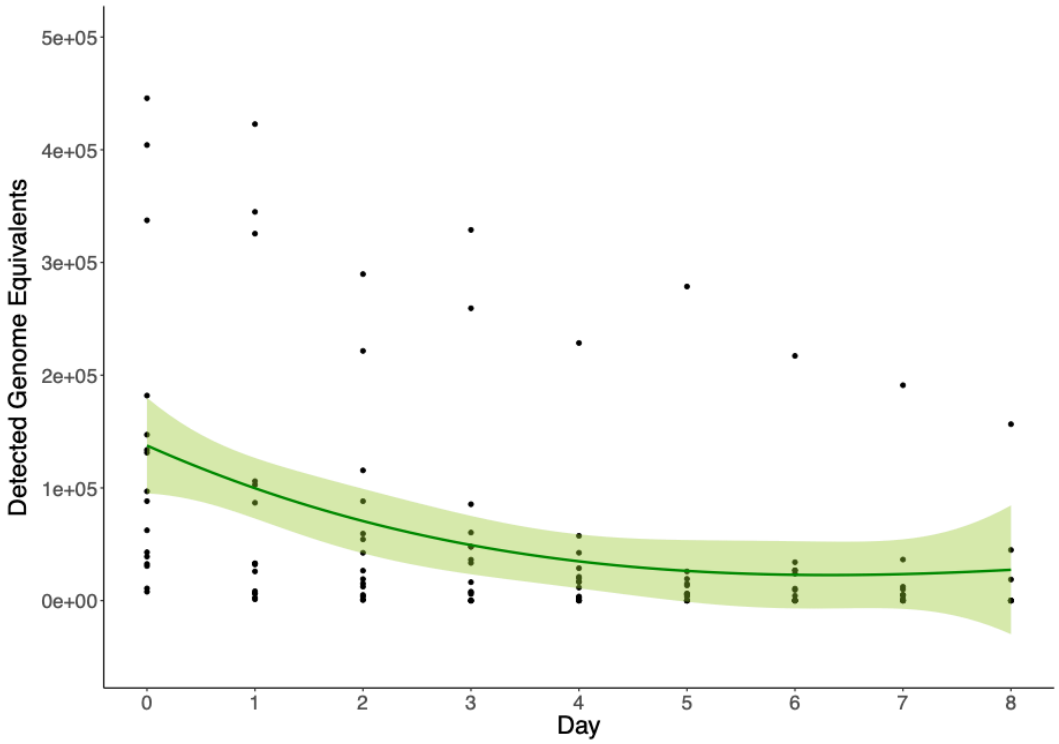

Figure 4.6: Cubic polynomial regression model and 95% CI of the decline in detected genome equivalents during spring-summer, LOD 10^3 genome equivalents.

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The relationship between environmental exposure and persistence expressed as a percentage of initial values in the spring dataset was modelled using a cubic spline regression model to attempt to eliminate some of the variation within the datapoints to establish the strength of the relationship. The model had a RMSE = 20.43, and a R^2 = 0.56 suggesting that the number of exposure days is a good predictor for *M. bovis* decline under environmentally relevant conditions (Figure 4.6).

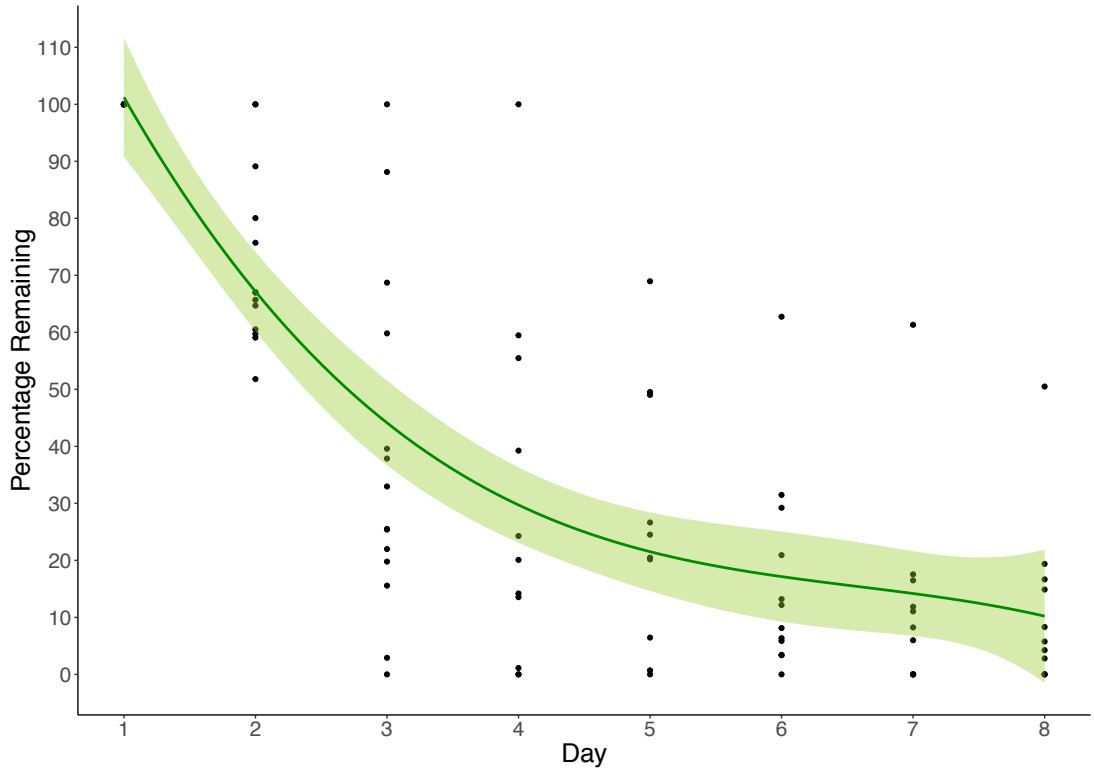

Figure 4.7: Cubic polynomial regression model and 95% CI of the decline in detectable M. bovis cells over an 8-day period in spring-summer.

Further analysis of the model suggested the existence of an initial period of more rapid decline during the first 3 days post-excretion (linear model for initial decay: RMSE = 25.23, and $R^2 = 0.52$). This was simulated in the form of two linear models plotted in Figure 4.8 using the following equations.

$$Y = -23.2(X) + 95.4$$
$$Y = -6.5(X) + 51.8$$

Running an ANCOVA of the two linear models suggested a statistically significant difference between slopes ($F = 19.05$, $dfn = 1$, $dfd = 113$, $p < 0.0001$), with the rate of decline decreasing by 72.12%.

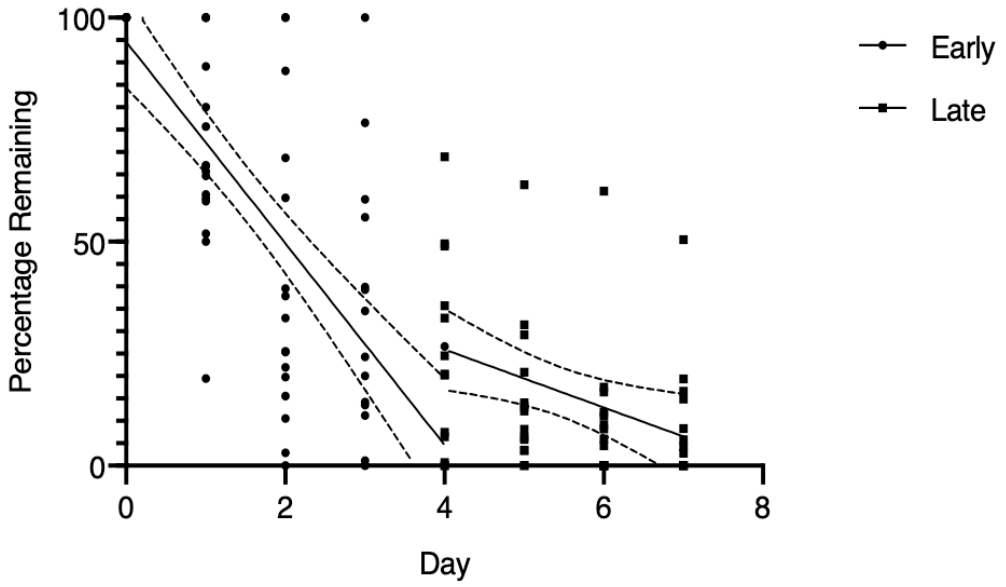

Figure 4.8: Demonstration of the biphasic decay of M. bovis in faecal samples in late spring through two linear models and dashed lines show the 95% confidence interval.

The approach used for the spring/summer dataset was replicated across the two further sampling seasons. The regression model of bacterial counts across the summer/autumn sampling period (Figure 4.10) demonstrated a slight, but not explanatory, relationship between time and decline of *M. bovis* (RMSE = 1.7 x 10^5, R^2 = 0.12).

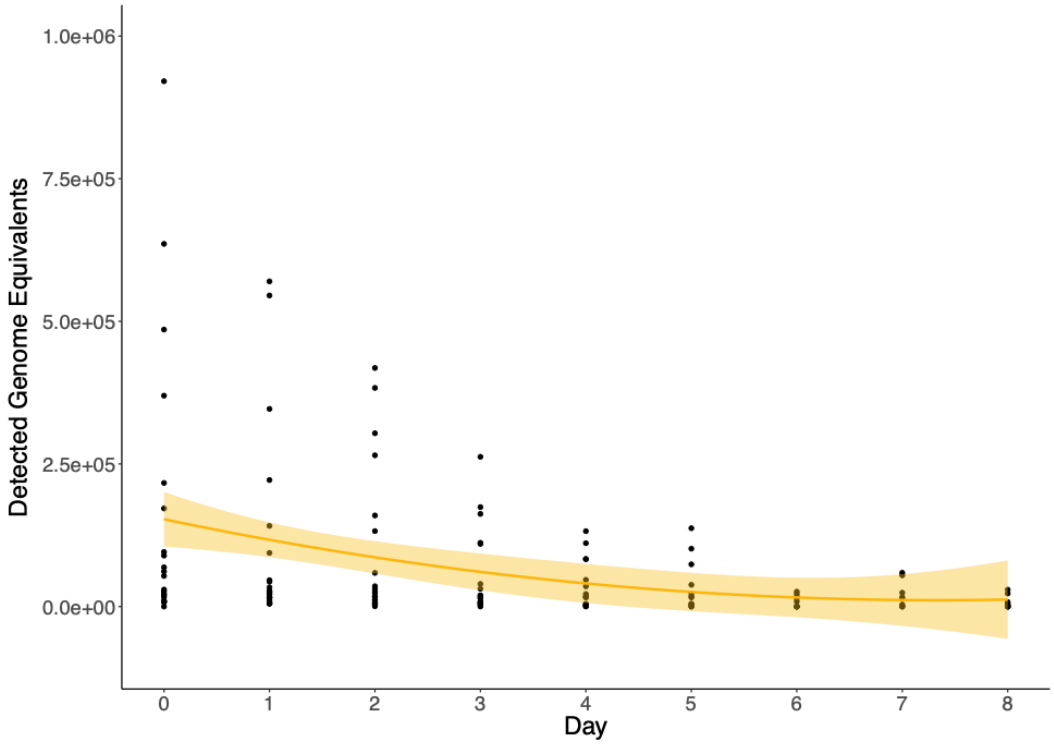

Figure 4.9: Cubic polynomial regression model and 95% CI of detected genome equivalents during summer-autumn sampling. LOD 10^3 genome equivalents.

Once again, when the dataset was transformed into percentage data, and the summer/autumn dataset modelled using a cubic spline regression model. This model produced an RMSE = 20.43, and $R^2 = 0.58$ suggesting that, as in the spring/summer, the number of exposure days is a reasonable predictor for *M. bovis* decline under these conditions (Figure 4.9).

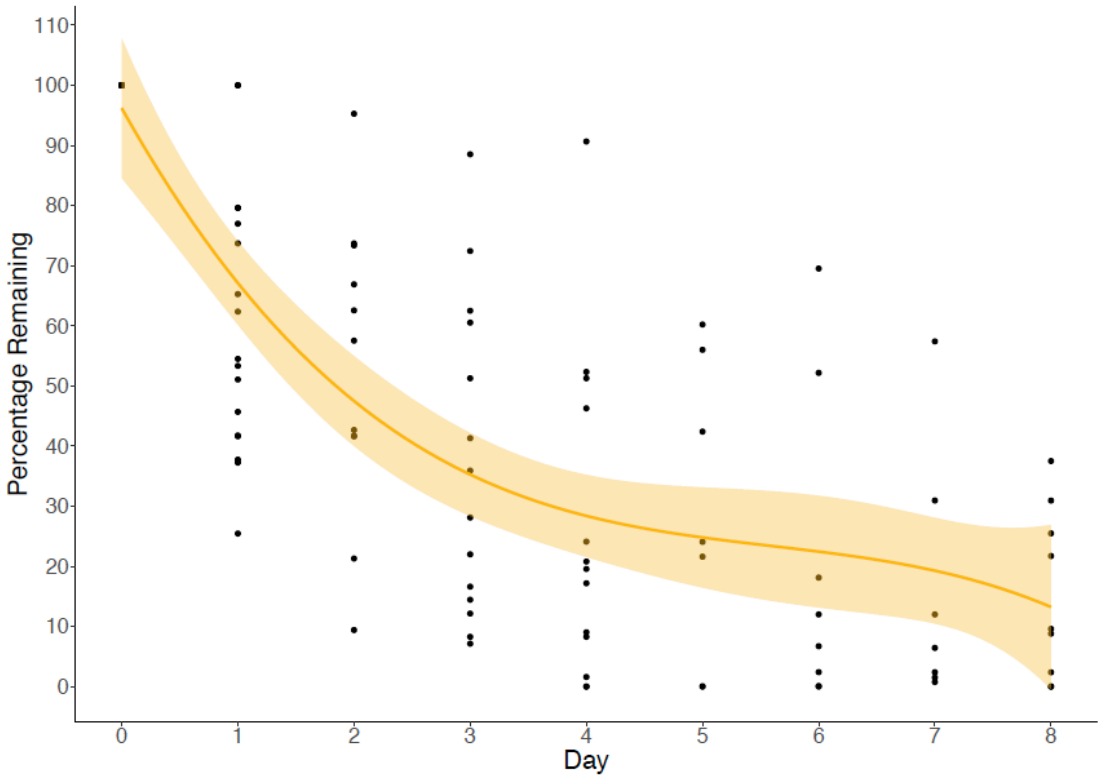

Figure 4.10: Cubic polynomial regression model and 95% CI of the decline in detectable M. bovis cells over an 8-day period in summer-autumn.

The summer/autumn model demonstrated a biphasic decline to that in spring/summer. The two decay rate equations calculated for the summer/autumn model are given below and the initial decay model demonstrated RMSE = 21.55, and $R^2 = 0.53$.

$$Y = -22.5(X) + 94.7$$
$$Y = -6.5(X) + 51.8$$

There was a statistically significant difference between the slopes of initial and later decline (ANCOVA; F =17.05, dfn = 1, dfn2 = 11, $p < 0.0001$), with the gradient decreasing by 71.24% (Figure 4.11).

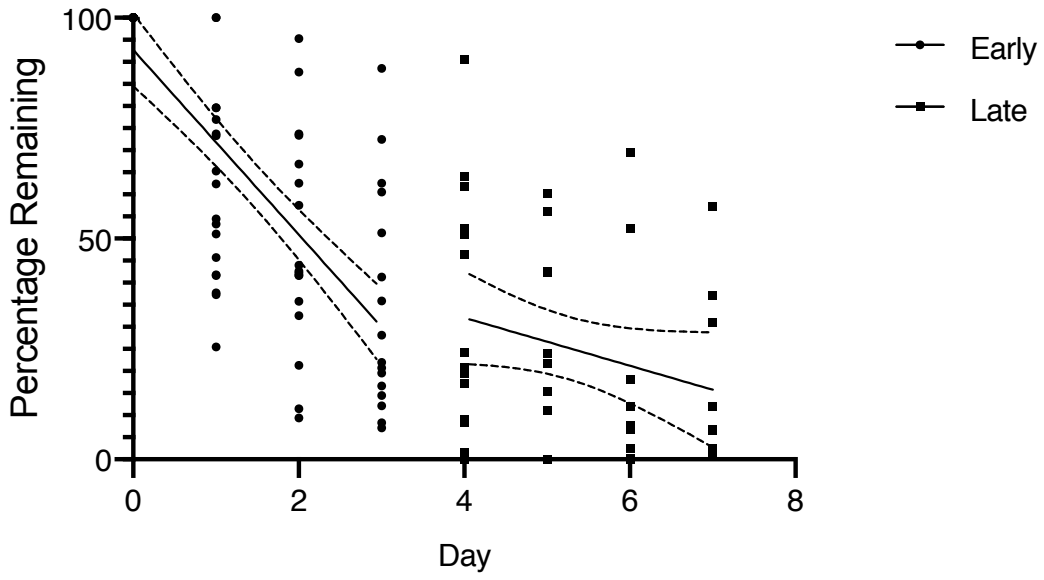

Figure 4.11: Demonstration of the biphasic decay of M. bovis in faecal samples in late summer through two linear models and dashed lines show the 95% confidence interval.

Finally, across the samples there was a linear decline in the absolute numbers of *M. bovis* (Figure 4.13) in the autumn/winter sampling period, with the regression model demonstrating a slight relationship between the dependent and independent variable (RMSE = 1.3 x 10^5, $R^2 = 0.13$).

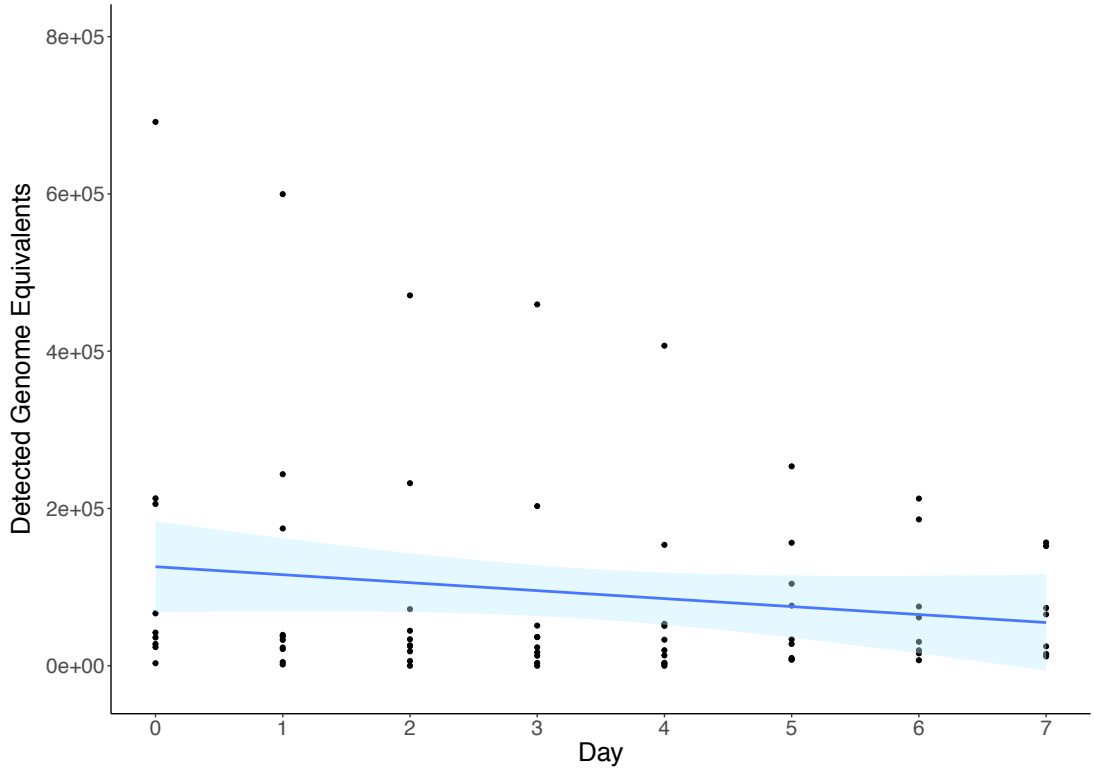

Figure 4.12: Linear regression model and 95% CI of decline in detected genome equivalents over the autumn-winter sampling. LOD 10^3 genome equivalents.

The data from the winter exclosure were transformed into 'percentage remaining of the initial count data' and modelled using a cubic polynomial regression. The resultant model (Figure 4.12) had an RMSE of 31.58 and an R^2 of 0.22. This large error value, wide 95% confidence interval and low R^2 suggests that in the autumn/winter, time is not as strong of an explanatory variable as it was in the summer/autumn and spring/summer.

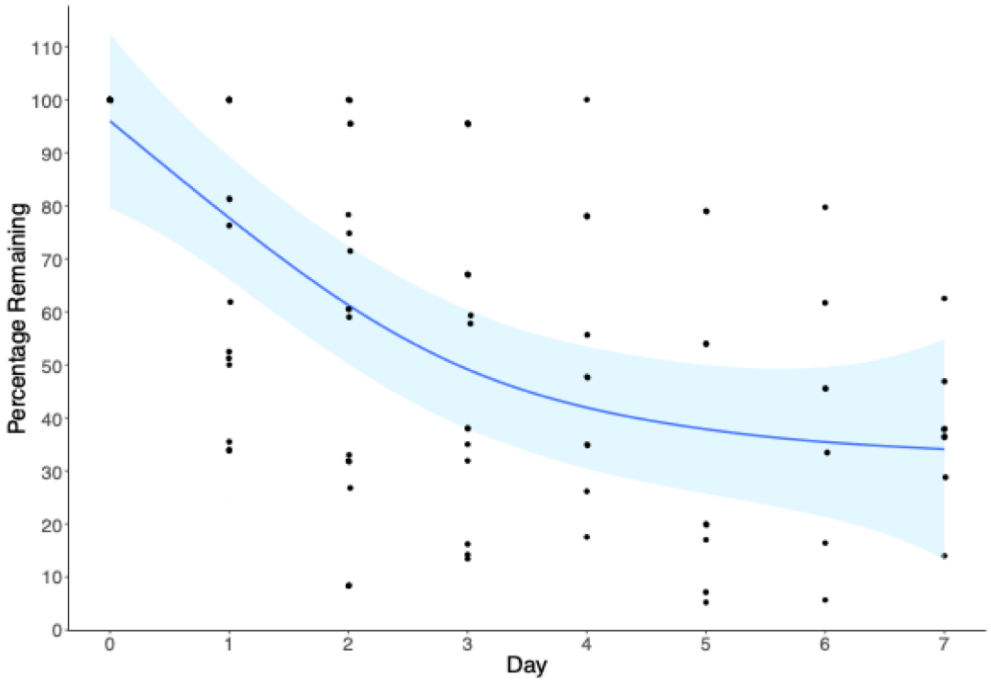

Figure 4.13: Regression model and 95% CI of decline in detectable M. bovis cells over a 7-day period over the autumn-winter sampling.

The biphasic model, constructed of two linear models, was produced with measurements from the first 3 days modelled (RMSE = 24.87, and R^2 = 0.46) separately to the remaining days.

$$Y = -20.4(X) + 101.7$$
$$Y = -3.7(X) + 59.7$$

These equations were used to produce the figure below to demonstrate the biphasic decline as the slopes differed significantly (F = 4.59, dfn = 1, dfd= 45, p = 0.04). Despite demonstrating an 81.8% decrease in gradient, the change in rate of decay was not as significant as in spring/summer and summer/autumn; this is probably attributable to the high variation level within the dataset and the smaller sample size.

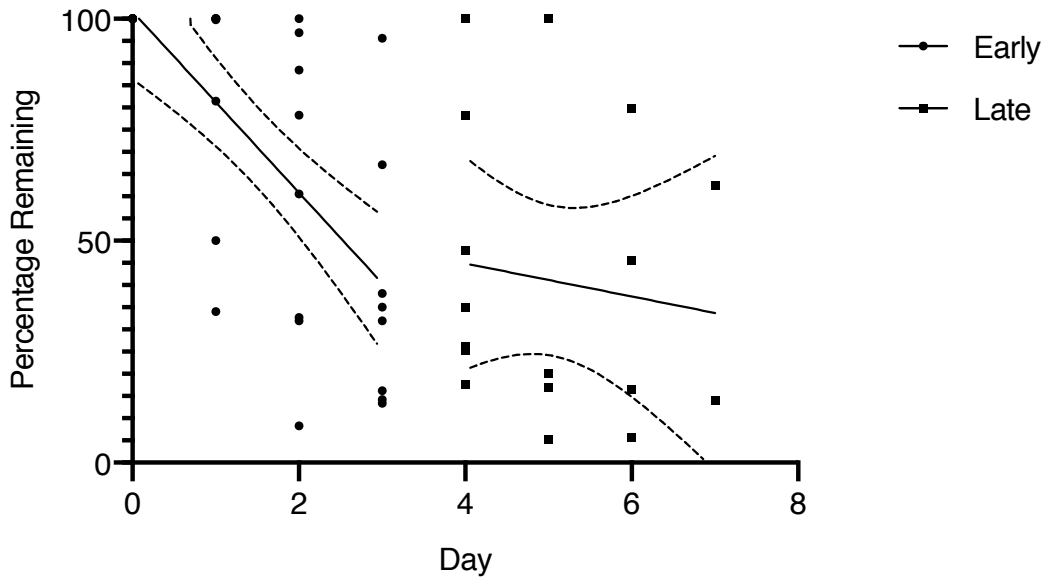

Figure 4.14: Demonstration of the biphasic decay of M. bovis in faecal samples in late autumn through two linear models, dashed lines show the 95% confidence interval.

Across the three periods there was no statistically significant difference between the gradients of the initial slopes ($F = 0.27$, $dfN = 2$, $dfD = 159$, $p = 0.76$) or in their calculated intercepts ($F = 2.43$, $dfN = 2$, $dfD = 161$, $p = 0.09$) for the models when their intercepts were constrained to $c = 100$. Pairwise comparisons were run between the seasons, again with no statistical difference identified between the slopes of the seasons. However, when the initial decline models were not constrained to an intercept of $c = 100$, the difference in the intercepts of the spring and winter tended towards significance ($F = 3.77$, $dfN = 1$, $dfD = 92$, $p = 0.05$); as did the intercepts of the summer and winter ($F = 4.09$, $dfN = 1$, $dfD = 99$, $p = 0.05$).

4.45 Second decline phase

There was no statistically significant difference in the rate of decay demonstrated between the three sampling periods after day 4 of environmental exposure ($F = 0.106$. $dfN = 2$, $dfD = 110$, $p = 0.90$); however, there was a statistically significant difference in the intercepts of the lines ($F = 5.85$. $dfN = 2$, $dfD = 112$, $p = 0.004$).

Pairwise comparisons were also run, demonstrating no statistically significant difference between the gradient of the slopes. As suggested by the comparison of all the models above, the intercepts were significantly different between the spring and winter ($F = 11.24$, $dfN = 1$, $dfD = 68$, $p = 0.001$), and between the summer and winter ($F = 3.9$, $dfN = 1$, $dfD = 59$, $p = 0.05$).

4.46 The effect of meteorological parameters on the persistence of *M. bovis*

Parameters of interest were identified from the MIDAS dataset and relevant data extracted using R. Data relating to weather were split by season and standard descriptive statistical analyses are presented in Table 4.2. Graphical representation giving greater detail on the daily weather are presented in Appendix B (Figures B1-B8). The meteorological parameters were calculated from 24 hours of data, from 3pm the previous day until 3pm on the day of interest (the time of sampling from the exclosure).

Table 4.2: Hourly data was summarised with the mean daily value, the minimum daily average, maximum daily average, median of the daily average and the mean of the daily average for each season.

Parameter	Spring	Summer	Winter	Spring	Summer	Winter	Spring	Summer	Winter	Spring	Summer	Winter
	Min (daily mean)			Max (daily mean)			Median (daily mean)			Mean (daily mean)		
Temperature (°C)	9.4	10.7	0.83	19.3	20.1	11.4	14.3	14.5	7.8	14.0	14.8	6.8
Relative Humidity (%)	55.1	61.5	78.0	97.6	96.6	99.9	78.9	80.6	92.8	77.9	79.8	99.6
Rainfall (mm)	0	0	0	42.6	16.6	39.6	1.0	1.0	2.6	4.2	3.2	6.1
Dew Point (°C)	9.8	11.1	0.21	15.3	15.6	10.7	10.0	10.6	5.9	9.8	11.1	5.5
Soil Temperature (°C)	12.2	14.6	3.4	22.5	26.1	10.2	17.7	17.3	7.3	17.5	17.8	7.5
Sunshine Duration (mins)	6	6	0	924	816	528	477	222	114	465.6	282	159.6
UV (W/m^2)	89.3	85.5	20.9	359.1	296.6	97.0	282.7	174.3	43.5	256.4	174.9	47.3

The decline in *M. bovis* was expressed as a percentage of the detected counts on the prior day and a correlation matrix produced (n =282 faecal sampling incidents) using Pearson's product moment correlation. The correlation coefficients were extracted and used to formulate a correlogram (Figure 4.15) to demonstrate the strength of the relationship between variables.

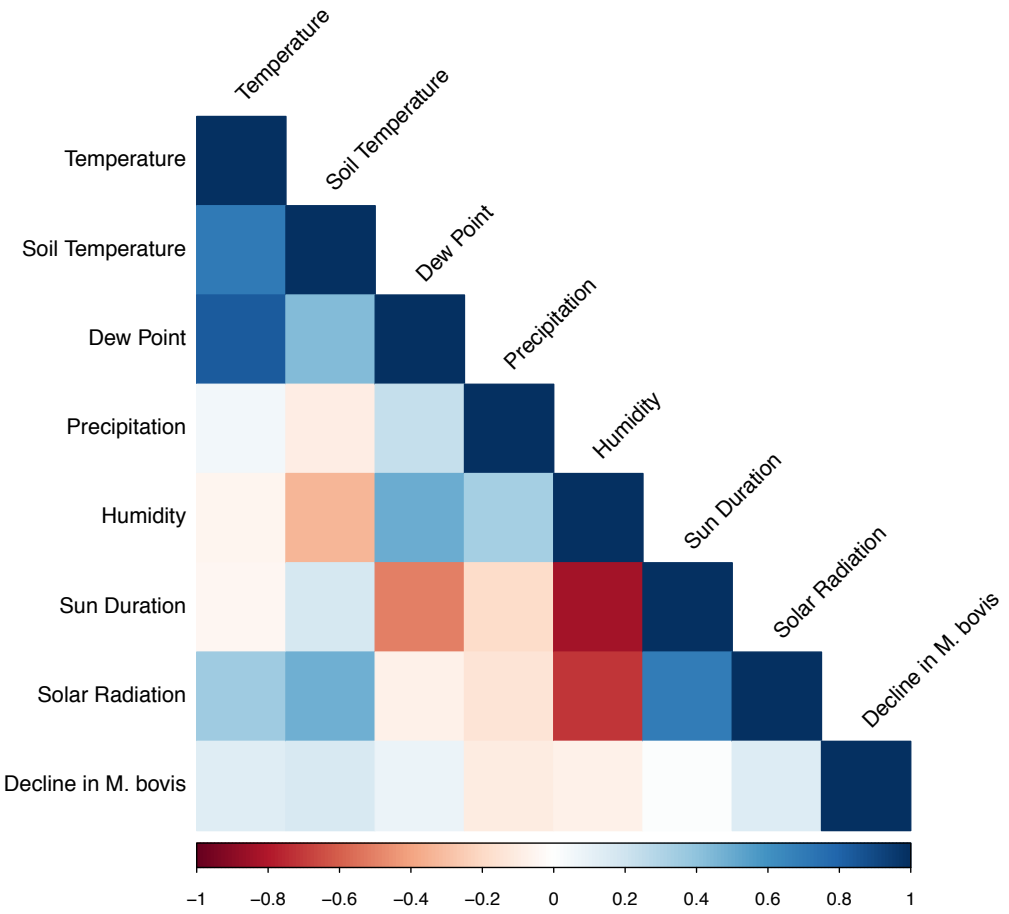

Figure 4.15: Correlogram of key meteorological variables and the percentage of M. bovis that remain detectable from the previous days' measurements.

The statistical significance of the relationships between the meteorological variables and their impact on *M. bovis* were also determined. Two variables appeared to exert a protective effect over faecal *M. bovis* as demonstrated in Figure 4.15, but neither precipitation amount ($p = 0.076$), or humidity ($p = 0.20$) were significant. Factors which related to a greater decrease in *M. bovis* GEs were soil temperature ($p = 0.005$), air temperature ($p = 0.026$), and solar radiation/UV ($p = 0.016$). After the application of Benjamini and Hochberg comparison for multiple corrections, air temperature ($p = 0.04$), soil temperature ($p = 0.01$) and solar radiation ($p = 0.03$) demonstrated a statistically significant effect on the decrease in survival time of *M. bovis* in faeces.

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4.47 The effect of direct exposure to UV radiation

Latrines occur under different environmental conditions and so half the samples were shaded to simulate being in a woodland latrine, and the other half exposed to natural light conditions simulate a latrine located on open pasture (n = 9 replicates per condition, per season). The shaded samples consistently retained higher percentage levels of *M. bovis* than those exposed to sunlight for the 30-day sampling period (Figure 4.16) however there was no statistically significant difference between sun exposure and shade and *M. bovis* decline (Mann-Whitney U = 399.5, p = 0.61). Individually, a statistically significant difference between the two conditions was only detectable in the spring (t = 2.443, df = 17, p = 0.02).

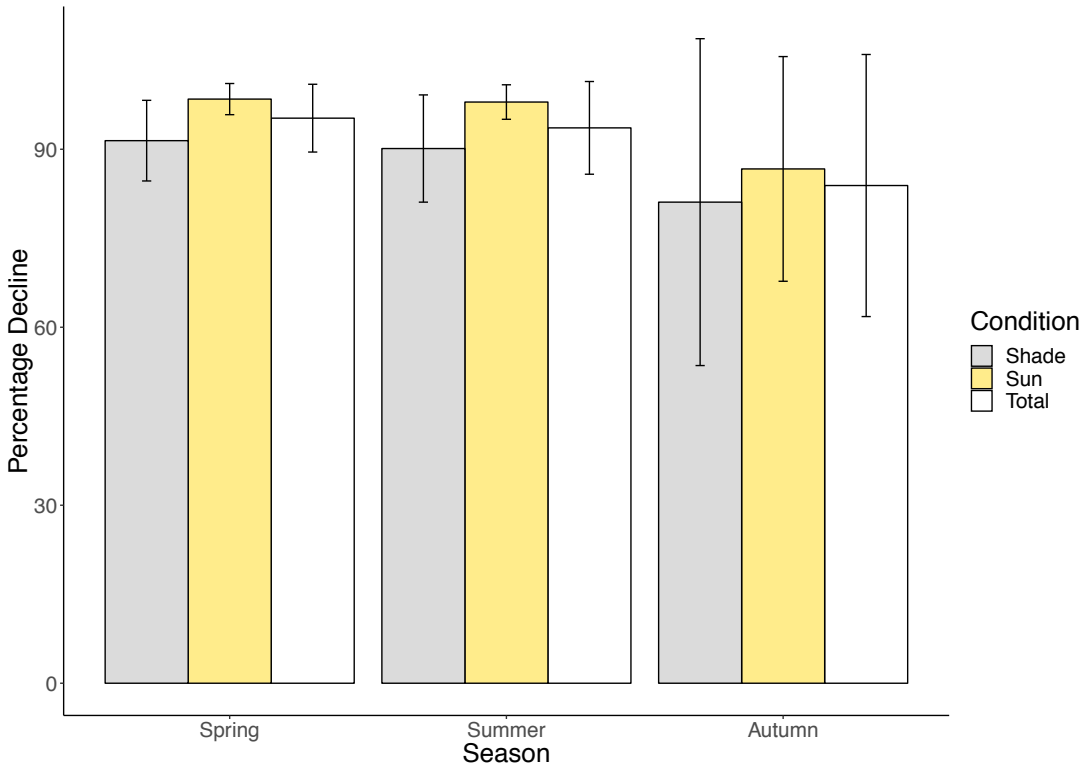

Figure 4.14: The mean percentage decline in detectable genome equivalents when exposed to direct sunlight or held under shaded conditions from day 0 (100% of their M. bovis count) to day 30 (error bars representing the standard deviation).

The declines demonstrated over the time course of this experiment show reduction of less than 2 orders of magnitude, with average values clustering around one order of magnitude. As such, a significant number of bacteria remain within the sample. The counts after 30-days of environmental exposure across the seasons are given in Table 4.3 to illustrate the high number of persisting pathogens under these conditions.

Table 4.3: The mean genome equivalents per gram of badger faeces before and after 30 days of environmental exposure under different conditions.

Season	Condition	Initial	Remaining	Loss
Spring-Summer	Sun	7.95×10^4	2.83×10^3	7.67×10^4
Spring-Summer	Shade	1.86×10^5	2.03×10^4	1.66×10^5
Summer-Autumn	Sun	1.95×10^5	3.90×10^3	1.91×10^5
Summer-Autumn	Shade	9.14×10^4	1.24×10^4	7.90×10^4
Autumn-Winter	Sun	7.08×10^4	5.54×10^3	6.52×10^4
Autumn-Winter	Shade	1.00×10^5	4.70×10^3	9.53×10^4

4.5 Discussion

4.51 Study design

The current study aimed to identify the fate of *M. bovis* in badger faeces in the most ecologically relevant study possible; with this comes limitations regarding high data point variability and a number of factors beyond the control of the study. A common technique in environmental biology is artificial inoculation which facilitates the establishment of homogenous bacterial counts and the option to increase sample numbers and sample homogeneity. However, it is also documented that artificially inoculated bacteria from cultures are detectable for longer than those naturally present in the sample (Anon., 1979, Morris et al., 1994). This may be due to abnormally high starting numbers of bacteria in combination with presence-absence based culture techniques, or the cells being in a better physiological state having been transferred from nutrient rich media rather than a GI tract.

Culture remains the method employed by the APHA for the purposes of detection of *M. bovis* in faeces but has many limitations associated with the complexities of isolating slow-growing mycobacteria from complex matrices with rich microbiomes (Chambers et al., 2017). To facilitate this method other survival studies have sterilised the starting material prior to inoculation to negate the requirement for harsh decontamination methods and hypothetically increases the yield of cultivable *M.* bovis (Corner et al., 1995, Chambers et al., 2011). However, the removal of competing microorganisms, as well as bacterial feeding microarthropods leads to atypically high levels of persistence, with *M. bovis* having been shown to persist for longer in sterile matrices, compromising the applicability of these studies (Taylor et al., 2003, Hibbing et al., 2010, Mardare et al., 2013).

A further limitation of culture is the presence of 'differentially' culturable cells that are unlikely to produce colonies under standard laboratory conditions (Hobby et al., 1954, Saito et al., 2017). Conclusions and comparisons will be drawn from work conducted on *M. tuberculosis* however it must be acknowledged that these organisms are adapted to different hosts and evolved to cope with different stresses. *M. tuberculosis* can enter a non-culturable state on solid media, which requires either dilution in suitable liquid media, the addition of Rpf (resuscitation promoting factor), or the addition of supernatant

from actively growing cells in order to return the dormant cells to a culturable state (Votyakova et al., 1994, Mukamolova et al., 2010). This was placed into the context of clinical medicine by a study of 25 untreated individuals suffering from active TB infection in the UK; the results of the study demonstrated that 80-99% of viable bacteria within 80% of the patients were not detectable by using traditional culture methods (Mukamolova et al., 2010). These non-culturable cells are highly resistant to environmental stresses, and exhibit increased antimicrobial resistance in contrast to their labile forms (Garton et al., 2008, Deb et al., 2009). The dormancy regulon (DosR) associated with this persistent state is well distributed through both pathogenic and environmental species of mycobacteria (Boon and Dick, 2002, Chen et al., 2013). Therefore, it is hypothesised that similar strategies employed by dormant *M. tuberculosis* would be evident in environmental *M.* bovis, rendering them not detectable under standard culture conditions yet the most persistent within the environment (Mukamolova et al., 2010). It is also hypothesised that current faecal culture methods vastly underestimate the population of cells, due to the cells transitioning to 'persister' states as a response to environmental exposure, and due to the current methods of decontamination which involve harsh acidic treatments that would trigger further translational switching to a persistent/non-culturable state or cell death (Corner et al., 1995, Deb et al., 2009, Saito et al., 2017). Therefore, culture is unlikely to capture the cells towards the tail-end of the time-decrease resulting in the false assumption that an area is not contaminated. For this reason, qPCR was used as the method of quantification within this study; while culture definitively demonstrates the presence of viable cells it is also likely to hugely underestimate the abundance of cells due to cell loss during sample decontamination, cell clumping and dormant/persistent cells (Corner et al., 1995, Downing et al., 2005, Drewe et al., 2010).

This study used naturally infected samples partly due to the restrictions of working with a high category pathogen. It is also important to acknowledge that using natural samples under environmental conditions is more ecologically relevant than artificial conditions. The high variability within the data is thus likely to be representative of true environmental persistence which is impacted by the interplay of a number of factors such as the excretion state of the bacteria, the moisture content and composition of the faeces, the competing faecal microbiome and the accessible organic nutrient content.

Declines in *M. bovis* were monitored daily for the first 8 days of environmental exposure rather than undertaking a 'time to negative' approach which had been the approach in previous studies. This sampling strategy was used in order to determine the shape of the decay curve for *M. bovis* under environmental conditions in order to inform hypotheses regarding the state of bacteria (labile or persistent) within naturally infected faeces in the environment. Undertaking a shorter sampling period also meant that an increased number of replicates could be performed for each time-point, in anticipation of the high variability, than would have been feasible if longer term sampling had been undertaken. These increased replicate numbers were vital for removing some of the uncertainty linked to using heterogenous environmental samples. To mitigate the otherwise short-term nature of this approach, a follow up sample was taken at day 30 to examine longer-term persistence. These 30-day values were used to investigate the effects of natural UV exposure on survival, as well as the level of contamination (in GEs) that persists a month after excretion in badger faeces.

4.52 *M. bovis* exhibits a biphasic decay rate in environmental samples *in situ*

Environmental persistence is a key element of any model developed for disease transmission and for understanding and predicting spread. The current gold-standard mathematical model for cattle bTB spread uses a first-order kinetic model for *M. bovis* decay with a half-life of 34 days (CI: 20-71 days) for estimating the role of environmental persistence in *M. bovis* transmission (Brooks-Pollock et al., 2014). However, the data generated by this study indicates this model is unsuitable for predicting *M. bovis* decline in naturally infected samples. There is a prevailing trend across the three seasons indicative of the excretion of both labile bacteria and resistant bacteria (Figure 4.17). While labile bacteria rapidly succumb to the stress of environmental exposure within the first 1-3 days, resistant bacteria persist and result in the long tail of the distribution of the hypothesised model before succumbing to a combination of environmental stresses and nutrient deprivation (Wray, 1975, Brouwer et al., 2017).

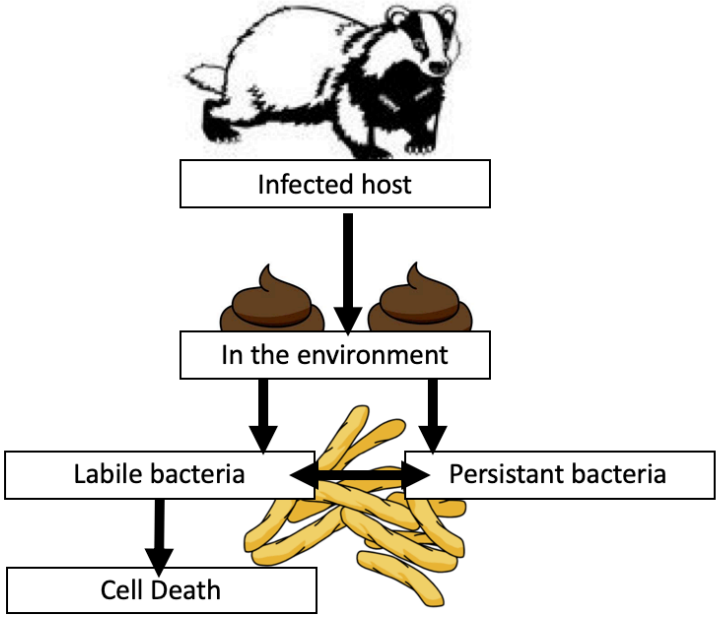

Figure 4.17: Demonstration of the proposed relationship between susceptible (labile) and persistent faecal M. bovis under environmental conditions.

The biphasic decay model has been well documented in the study of faecal indicator species but it was unknown whether it was applicable to environmental *M. bovis* (Rogers et al., 2011, Miller-Dickson et al., 2019). For example, *Escherichia coli* O157:H7 has exhibited a similar decay pattern under laboratory and environmental conditions (Easton et al., 2005, Wang et al., 2018). The percentage of O157:H7 declined to below 10% of the starting value within 2 days at 23 °C in sewage contaminated water but took another 2 days to decline to circa 2% of the starting value. This biphasic decline was mirrored in the 9 °C mesocosm, though the rates of decline were slower than those demonstrated under warmer conditions (Easton et al., 2005). In soil artificially inoculated with *Salmonella spp.* and O157:H7, held under environmental conditions, there was a multi-log reduction in all both genera over the first 2.5 days of the experiment followed by a period of slower decline (Wang et al., 2018). In a more related pathogen, *Mycobacterium avium* subspecies *paratuberculosis* isolates from cattle and sheep which were inoculated into their respective hosts' faeces have been shown to demonstrate a biphasic response to environmental exposure (Eppleston et al., 2014).

While many studies have sought to understand how long contamination is likely to persist, most of the studies have design flaws that limit their applicability to environmental conditions. The current study was the first to use naturally infected faeces to produce a survival curve for *M. bovis* in an environmental matrix and the first to use qPCR targeting RD4 for bacterial enumeration. However, parallels could be drawn with a study which monitored the survival in 4 different matrices under environmental conditions in Michigan, USA (Fine et al., 2011a). This study similarly demonstrated a decline which, when interpreted across the seasons, appeared to be biphasic but had a variety of limitations. Firstly, owing to spatial constraints and the complexity of culture there are only a small number of replicate samples (n = 4). Furthermore, the time to first sampling ranged from 2 days in the autumn/winter, to 7 days in the winter/spring and 3 days in the spring/summer, with further variation for the duration of the sampling. In relation to the current study, soil was the matrix of key interest, but would be different compositionally than to faeces, and also inoculated artificially with a laboratory culture. Additionally, the storage of the inoculated samples in pots would also have partially protected the sample from the effects of drying and again potentially compromised the applicability to natural situations. Finally, Michigan has an extremely different climate which was impossible to reconcile with British conditions. This study aimed to produce greater detail on the fate of faecal *M. bovis* in the first week post-excretion under British conditions.

The main limitation of using qPCR in this context, as addressed in Chapter 2, is the likelihood of detecting environmentally persisting extracellular DNA (eDNA). While this is a known flaw of the method, levels of eDNA from *M. bovis* BCG decreased in a linear fashion in spiked soil microcosms held at 10 ºC under laboratory conditions and was undetectable after 3 days (Young et al., 2005). It is therefore suggested that the turnover of eDNA under the harsher environmental conditions of this study would be sufficiently rapid to have not significantly influenced the results. Furthermore, while the potential for eDNA to persist and be detected is a limitation of qPCR methodology, the risks associated with underestimating the abundance of pathogens through alternative culture-based techniques is thought to be more detrimental in the context of disease control (Mukamolova et al., 2010, Adams et al., 2013). However, confirmation of the persistence hypothesis should be sought through the culture-independent isolation of cells (such as via the immunomagnetic capture method developed in Chapter 5) and latter

cell staining and visualisation for the identification of dormancy phenotypes, such as those seen in *M. tuberculosis* (Deb et al., 2009).

These data produced within the current study suggests that there is a large persistent population of bacilli remaining after one week of environmental exposure; while this is only circa 20% (1.86×10^4 cells g^{-1} (SE 7.40×10^3 cells g^{-1})) of the initial population (mean of all seasons) it takes far longer for cells presenting with the persister phenotype to succumb to the environment (Brouwer et al., 2017). Consistent with this hypothesis, after 30-days of environmental exposure, 9% (8.27×10^3 bacilli g^{-1}) of the initial population of *M. bovis* persisted (mean of all seasons). As one bacillus is considered capable of causing infection by aerosolisation these data suggest that environmental transmission can pose a significant and persistent transmission risk to naïve animals (Chausse, 1913).

4.53 Seasonal variation in bacterial decline

The initial period of rapid cell death was particularly pronounced in the spring-summer and summer-autumn periods, with circa 60% of the original *M. bovis* population being undetectable by qPCR after the first 3 days. The decline was less evident in the autumn/winter period with a slower rate of initial decline and circa half the original number of cells still detectable by the fourth day of testing. The difference in gradients between the spring/summer and summer/autumn against the autumn/winter rate of *M. bovis* decline, were statistically significant. It is suggested that while seasonally linked conditions promoted the speed of initial cell loss, that the majority of cell loss can be attributed to cells which were either poorly adapted or unable to adapt to the change in their environment (Brouwer et al., 2017).

After approximately 3 days, the *M. bovis* population entered a second period of more gradual decline, characterised by gradients of circa 30% of the initial decline phase. The gradients for spring/summer and summer/autumn declines decreased by 72.1% and 71.2% respectively, whereas the rate of the cell decline in the winter declined by 81.8%. This suggests that the decline rate in the spring and summer remained relatively high, despite the initial population of labile cells succumbing to environmental factors; this is likely to be due to the conditions being more harmful to the bacteria and will be further

explored in the section dedicated to the meteorology (Van Donsel et al., 1967, Fine et al., 2011a, Barbier et al., 2017). The autumn/winter population saw a more extreme shift towards a gentler gradient, suggesting that the meteorological parameters that characterise winter in the UK are more protective to cells which survive the initial adjustment period. This was similar to findings of Fine *et al.* in which CFUs recovered from artificially inoculated soil more rapidly declined over the winter-spring period and spring-summer period, with a slower rate of initial decay observed in the autumn-winter period (Fine et al., 2011a).

The winter model exhibited greater variation which was also evident in the research by Fine *et al.*; this could hypothetically be due to the smaller sample number within this sample or could be accountable to the stronger influence of daily meteorological parameters, as not all samples were placed within the exclosure at the same time due to sampling limitations (Fine et al., 2011a). The levels of variation within the data could also be attributed to the physical composition of the sample due to badgers' omnivorous diets which vary substantially both within seasons, between seasons and individuals. This greatly impacts faecal consistency and therefore *M. bovis* survival (Robertson et al., 2015, Rose et al., 2015, Barbier et al., 2017).

4.54 The relationship between meteorology and *M. bovis* decline

Very few studies exist regarding the survival of *M. bovis* in an environmental matrix; the majority of the existing literature investigates the seasonal effects, with conclusions drawn from the effect of shading the samples from UV radiation. With the seasons being particularly 'atypical' in 2018, and with the large fluctuations in conditions being typical of the UK's climate, it highlighted the importance of considering local weather patterns when assessing timescales of persistence on contaminated land. It is also important to acknowledge that predicted survival periods are extrapolated from a short period of time, and that while cells may be excreted in one set of conditions, that the period of predicted decline may continue into a separate set of conditions (e.g. the transition of a slower decline in winter to a swifter decline in spring).

In order to explore the effect of meteorological parameters on bacterial decline multivariate models were constructed. This project did not permit the isolation of

individual meteorological parameters, as these are not independent of each other, though it is possible to speculate which variables were likely to be the most influential, both on each other and on *M. bovis* survival. Comparisons will be drawn between these results and the existing literature conducted either under laboratory conditions or in other locations.

Factors that correlated with a decrease in environmental *M. bovis* within this study, given in decreasing order of significance, were soil temperature, solar radiation and air temperature. Despite solar radiation being statistically the second strongest determinant of *M. bovis* decline, it is impossible to rule out factors that correlate with strong solar radiation as being the key driver. In the spring-summer period, characterised by the highest mean levels of solar radiation and the highest mean levels of total sunshine duration, the provision of shade resulted in a statistically significant level of protection. However, in the summer-autumn and autumn-winter periods shading did not have a statistically significant effect on *M. bovis* survival despite prolonging its persistence. These findings were in keeping with those of several studies that found slightly longer survival rates in shaded conditions, approaching significance in times of greater UV radiation (Duffield and Young, 1985, Tanner and Michel, 1999, Fine et al., 2011a). The lack of a strongly characterised response to UV radiation is counterintuitive, as irradiation is a common method of reducing bacterial counts (Riley et al., 1976, Peccia and Hernandez, 2004). It is hypothesised that the limited effect of UV on survival is due to both the density of faeces reducing the sample's permeability to light, as well as the crust that forms on the outer layer of the faeces functioning as a protective barrier, helping to maintain the internal environment at higher moisture levels and less extreme temperatures (Haynes and Williams, 1993). It is therefore hypothesised that solar radiation is not the critical element, but it instead a factor that correlates strongly with high solar radiation.

Previous studies have concluded that sunny but moist conditions increased *M. bovis* survival, and that only in the absence of moisture was sunlight a significant factor in cell-death (Stenhouse Williams and Hoy, 1930, Maddock, 1933, Tanner and Michel, 1999). The presence of moisture is likely to slow the heating of the sample, and therefore is suggestive of internal sample temperature playing a more significant role (Tanner and Michel 1999). Within this study, soil temperature had the most significant relationship

with *M. bovis* decline. This is likely to be due to soil and faeces being more similar in consistency than air, with the internal part of the sample requiring the conduction of heat in order for the air temperature to play a significant effect. The average daily soil temperature was consistently warmer than that of the air temperature (with air temperature being 20.5%, 15.8% and 8% cooler in the spring, summer and autumn respectively), as well as exhibiting lower amounts of variation (see Appendix, figures 16 and 17). This warmer and more consistent environment could explain why soil temperature had a more significant relationship with bacterial decline than that of air temperature and should therefore be considered a better predictor of natural environmental decontamination.

In terms of causal factors relating to the decline in *M. bovis*, it is likely to be a complex interaction of the meteorological factors and the consistency of the sample. However, this study indicates that the key factors pertaining to the loss of *M. bovis* from faeces are likely to be UV and air temperature, but that neither would be sufficient without interaction with other factors. It is also highly likely that factors relating to the moisture content of the sample (e.g. precipitation, humidity) are important co-factors (Haynes and Williams, 1993, Tanner and Michel, 1999).

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4.55 Are faecally persisting *M. bovis* infectious?

The presence of *M. bovis* bacilli within the environment would be of little concern unless thought to be infectious. Current knowledge of the state of *M. bovis* in naturally infected, environmental samples is limited, with few studies having sought to address this question. Stenhouse Williams and Hoy indicated infectiousness but the results are limited by the likely presence of NTM which could present clinically in a similar manner in guinea-pigs but were not understood in the 1930s (Aronson and Whitney, 1930, Stenhouse Williams and Hoy, 1930, Feldman, 1936, Ordway et al., 2008, Silva-Gomes et al., 2015). However, more recent research investigated the survival and subsequent infectiousness of three members of the MTC inoculated into sterilised soil and maintained in opaque pots at room temperature for 12 months (Ghodbane et al., 2014). Survival was assessed through viable count on solid agar without the use of decontamination methods due to the sterility of the matrix. *M. bovis* was culturable after 12 months yielding 150 CFU g^{-1} however, when a 1 x 10^5 CFU suspension of the isolated

M. bovis bacilli were inoculated intraperitoneally into five mice they failed to develop lesions typical of mycobacterial infection. However, further *post mortem* analysis cultured $4.96 \times 10^4 \pm 1.21 \times 10^4$ CFU mL^{-1} *M. bovis* from the spleen and $2.43 \times 10^3 \pm 1.38 \times 10^3$ CFU mL^{-1} from the lungs. Of greater interest is the development of infection from ingestion or aerosolisation of bacilli from infected material as these are the likely transmission pathways (Chausse, 1913, Phillips et al., 2003). Unfortunately, the study focussed on the experimental feeding of mice with material contaminated with *M. tuberculosis*, though this did demonstrate that a member of the MTC was capable of causing tuberculous pathology in 3/5 mice and was cultured from 5/5 mice after 12 months outside a host.

When the two studies mentioned above are taken in combination, they present a valuable argument for the infectiousness of *M. bovis* in naturally contaminated substrates. While the survival aspect of the Ghodbane *et al.* study is limited by the artificial conditions under which the samples were kept, it does demonstrate that a member of the MTC is capable of remaining infectious after a long period outside of a host. Meanwhile, the 1930's study demonstrates that environmental survival in faeces is probable but was hampered by the inability to differentiate between members of the mycobacteria at that point in history and by using guinea pig models as a proxy for infectiousness. While cattle have been observed to avoid grazing close to badger latrines, incidences were recorded during the study of lower ranking cattle and curious cattle grazing near to or sniffing faeces (Benham and Broom, 1991). This study suggests that even after thirty days of environmental exposure that up to 2×10^4 *M. bovis* persisted in highly infected samples and therefore poses a realistic risk to farm biosecurity (Chausse, 1913).

4.56 Impact

From the literature review, this appears to be the first study of its kind due to the use of naturally infected badger faeces held under environmental conditions. This study focussed on quantification rather than presence-absence allowing the exploration of factors on a finer scale than previously achieved and therefore the interaction between the samples and meteorology to be understood. Furthermore, the use of daily subsampling to elucidate survival levels over the first days of exposure demonstrated the

biphasic decline of *M. bovis* cells and suggested the formation and/or presence of persistent cells.

The first implication is the interpretation of this work in the context of biosecurity. Cattle are generally turned out onto pasture in early May, when the forage has achieved sufficient growth in the early spring so as to not be negatively impacted later in the year by the grazing of cattle. The results could also be (with caution and caveats) extrapolated to cattle faeces, and the possible contamination of both pasture and sheds with faecally derived *M. bovis*. This work demonstrated that faeces presents with a hospitable environment for *M. bovis*, with 2.90×10^3 bacteria g^{-1} remaining even after a month of direct exposure in June (British summertime). Therefore, it is suggested that badger latrines should be either fenced off from cattle contact or have the faeces continually and safely removed in order to reduce the risk of badger-to-cattle transmission.

The second implication is that of possible remnant contamination post-badger cull, and the likelihood of naïve badgers being infected upon relocation to the sett under natural territorial expansion. This study highlights that if infected latrines persist (which badgers use to identify individuals within that territory), as well as contaminated sett soil, that there is a considerable risk of the new (potentially naïve) badger population being infected and establishing a new reservoir on that farm. The infectious dose of *M. bovis* is approximately 1 CFU by aerosolisation and 1000 CFU by ingestion; with the samples within this study containing sufficiently high numbers so as to establish infection post 1 month of exposure to sunlight (Chausse, 1913). It is therefore suggested that badgers should either be excluded from the area until there is a strong likelihood of the environment no longer containing bacilli, that potential locations of high-risk should be decontaminated (either by the removal and incineration of potentially infectious material, or environmental decontamination with disinfectants on Defra's approved disinfectant list) and that the area should be tested to ensure that decontamination has been effective prior to allowing animals (whether cattle or wildlife) to enter the HRA.

4.6 Conclusion

This study confirms that cells excreted by badgers exhibit a biphasic decay pattern within the environment, and though numbers significantly decrease, can still present a significant and persistent environmental biohazard. Furthermore, the results of the above research suggest that the interaction between the British climate and badger faeces are conducive to the long-term survival of *M. bovis* at levels capable of establishing infection.

While making any assessment of the level of biohazard, the surveyor must account for the recent weather conditions, in particular that of soil temperature. These factors will exhibit a greater effect on the surface of the faeces, and it is likely that the bacilli towards the edges of the faecal samples will succumb to these environmental factors, while the bacilli in the inner aspect will be greater protected by the crust forming and maintaining and more consistent and moist internal environment. In accordance with the recommendations made by Maddocks and Stenhouse-Williams & Hoy in their research from the 1930s, it is hypothesised that a good approximation for the removal of infectivity of an area is when the infected organic matter disappears (Stenhouse Williams and Hoy, 1930, Maddock, 1933). This would be an easy measure for farmers and/or surveyors to implement as a part of ongoing biosecurity checks, by ensuring the degradation of organic matter in latrines on pasture prior to allowing cattle access.

Chapter 5:

Optimisation of an immunomagnetic capture based method for the isolation of *M. bovis* from badger faeces.

5.1 Abstract

The state of environmental *M. bovis*, and not merely the absolute number, is vital to our understanding of the likelihood of contaminated material presenting as infectious but is impossible to reliably mimic within laboratory conditions. An optimised immunomagnetic capture method resulted in the capture of 0.3% of *M. bovis* BCG from spiked badger faeces, as determined by viable count, and circa 4.5% by qPCR. This method can have important downstream applications for detecting the presence of persistent cells within the environment and increasing the understanding of conditions that invoke the dormancy phenotype. In terms of infection control, due to the hypothesised non-culturability of persistent cells under standard laboratory conditions, this would permit the isolation of bacilli from complex sample types that currently reduce the range of tests that can be conducted. Furthermore, current disinfection regimes focus on the application of disinfectants tested by viable count and are likely to either not be effective on dormant and stress-tolerant phenotypes or for these cells not to be captured by culture and therefore appear to have higher efficacy. The culture-free isolation method would permit greater insight into the state of environmental *M. bovis* and greater clarity regarding the efficacy of current regimes and testing methods.

5.2 Introduction

5.21 Environmental detection

While there are many methods of bacterial detection, environmental studies typically use culture and qPCR despite little correspondence between the two (Pathak et al., 2012, Oliver et al., 2016). Culture demonstrates the viability of isolated cells but in the context of environmental mycobacteria is limited by the harsh decontamination methods required for removing contaminating and faster growing microorganisms (Figure 5.1) reducing levels of detectable *M. bovis* (Corner et al., 1995). Furthermore, during attempts to isolate *M. bovis* BCG from spiked faecal samples by standard culture-based-methodologies faecal particles appeared to shield microorganisms from the effects of decontamination or produced low viable counts for the known number within the spike (Figure 5.2). Culture is also hampered by the hydrophobicity of mycobacteria and their tendency to form aggregates that reduce the sensitivity of CFU based enumeration (Alsteens et al., 2008, Mazumder et al., 2010). This is evident on solid media when harsher decontamination methods are employed through the aggregation of CFUs (Figure 5.2) and in pure culture by microscopy for visualisation of bacilli (Figure 5.3). The sensitivity of culture dependent assays is further compromised by the presence of VBNC cells either due to environmental conditions or the decontamination methods (Meyers et al., 1998, Shleeva et al., 2002). While, qPCR increases assay sensitivity, due to detecting individual cells within cellular aggregations and VBNC cells, it is likely to provide an overestimation as DNA can persist for brief periods outside of the cell (Young et al., 2005, Sweeney et al., 2007, Travis et al., 2011).

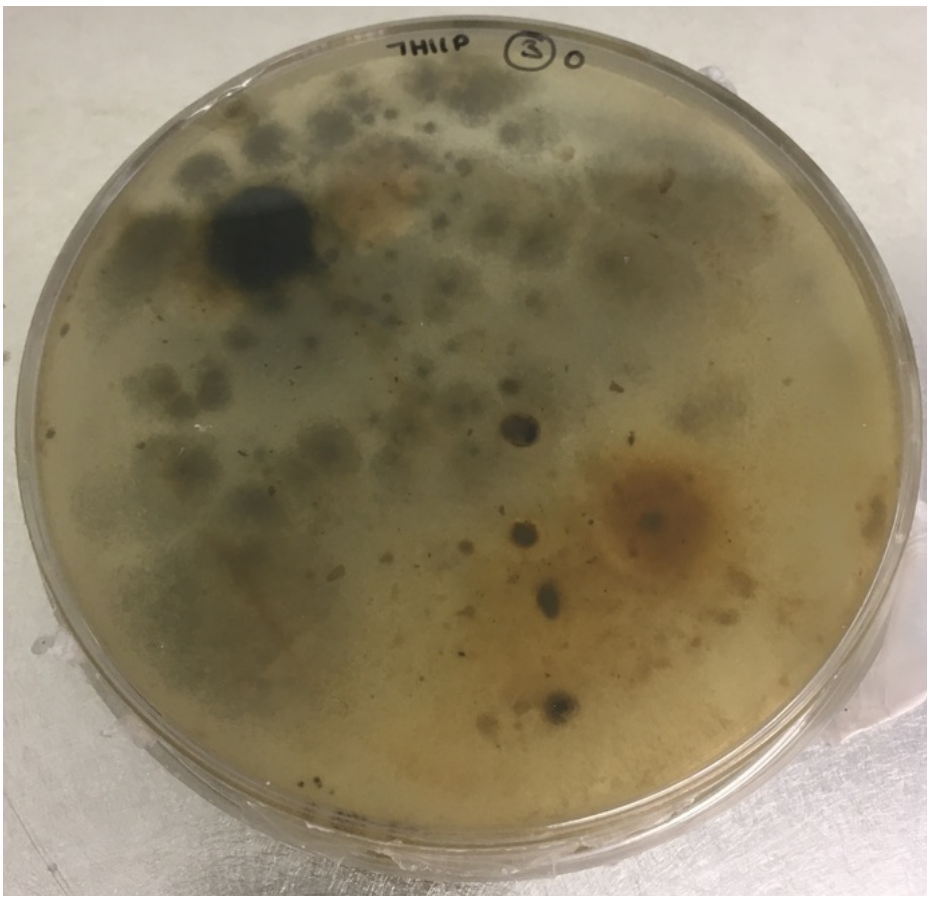

Figure 5.1: Demonstration a 'mild' faecal decontamination method for culturing M. bovis BCG Pasteur from badger faeces. Produced from spiked faeces decontaminated with 2.9% sodium citrate, 1% NaOH and 0.5% NALC; plated on Middlebrook 7H11 + PANTA antibiotics. Incubated for 7 days at 37 °C.

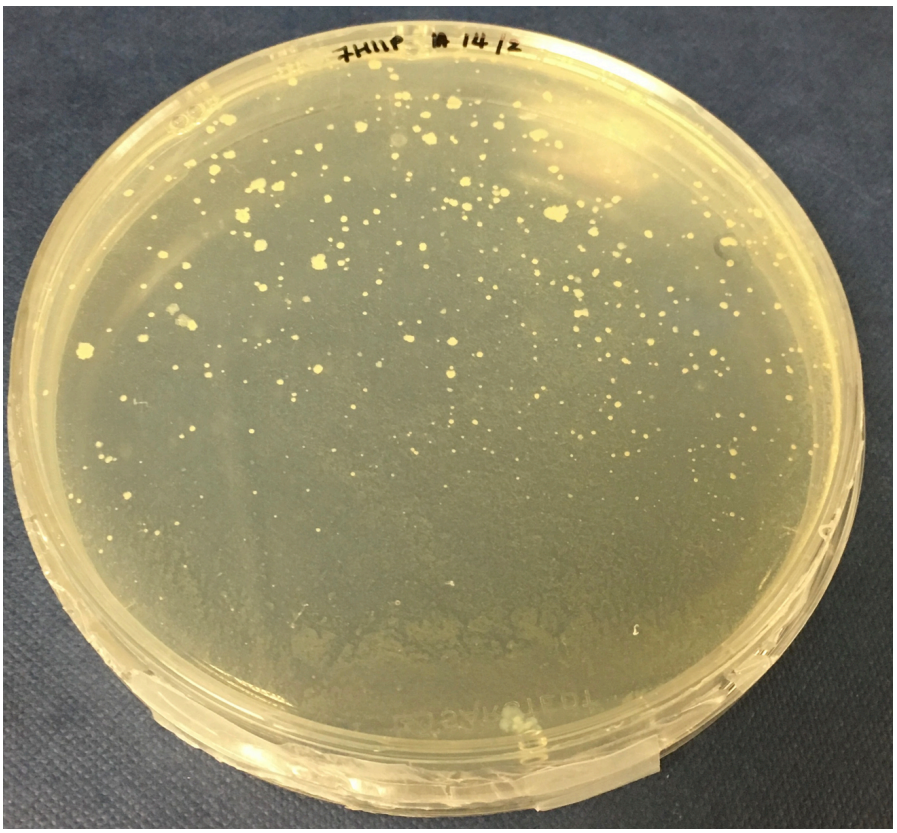

Figure 5.2: An undiluted viable count plate from badger faeces spiked with 2 x 10^8 bacilli and decontaminated with 0.85% saline overnight followed by 5% oxalic acid for 30 mins. Incubated on Middlebrook 7H11 + PANTA for 3 weeks at 37°C.

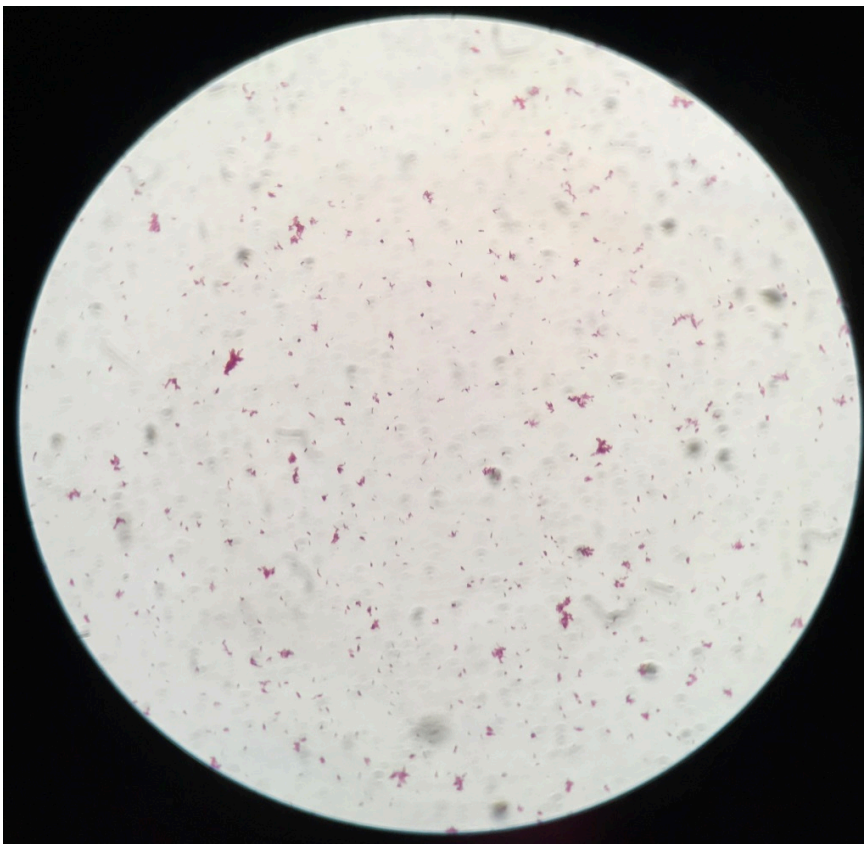

Figure 5.3: Demonstration of M. bovis BCG in culture forming cellular clumps under a x40 objective.

5.22 Immunomagnetic Capture

Immunomagnetic capture (IMC) is a method most frequently referred to in literature associated with the food industry for the isolation of contaminating microorganisms from complex sample types (Austin and Pagotto, 2003, Chakraborty et al., 2011, Madden, 2016). Beads of superparamagnetic iron oxide (Fe_3O_4) and polystyrene are coated in antibodies or peptides that are capable of reacting with suitable primary or secondary antibodies for the target cells (Austin and Pagotto, 2003). The cells are then immobilised against the internal side of the tube using a magnet and the sample matrix is removed by a variable number of wash steps (Patel, 1995, Bennett et al., 1996). Once the matrix has been removed, the beads bound to the target cells can be eluted and used for downstream applications such as culture (Austin and Pagotto, 2003). The major drawback of the method is that it is expensive to do on a small-scale basis and time consuming. However, it eliminates the requirement for strong decontamination methods, potentially increasing the sensitivity of the assay in contrast to traditional decontamination-based-culture (Corner et al., 1995). The method has been successfully used for the detection of *Bacillus anthracis, Listeria monocytogenes, Escherichia coli* O157:H7 and *Salmonella spp.* (Skjerve et al., 1990, Skjerve and Olsvik, 1991, Kobayashi et al., 2004, Shields et al., 2012).

5.23 Optimisation

Optimisation of environmental IMC presents a significant challenge due to the method having a large number of components and the complex interactions between the target cells, non-target cells and the sample matrix. Areas that require optimisation include bead size, antibody selection, direct or indirect capture methods, incubation time, and methods of final quantification. Applying the method to environmental samples increases its complexity due to the potential for the selected antibody to react with a common faecal bacterial species. Furthermore, faeces harbours a high number of bacteria per gram (circa 9.2×10^{11}) and therefore all faecal material must be removed as any residual faeces could pose a significant threat of contamination (Sender et al., 2016). Increasing the number of wash steps will reduce the amount of residual sample matrix; however additional wash steps could hypothetically reduce the sensitivity of the assay due to increasing the potential for target-cell loss.

There are also a number of factors related to working with mycobacteria that are likely to limit the applicability of the method. Firstly, the highly hydrophobic cell wall will result in the cells attaching to organic particulate matter and being discarded within the wash steps (Alsteens et al., 2008) The tendency of the bacilli to form aggregates also results in high variability due to the non-homogenous distribution throughout the stages of concentration and quantification (Meyers et al., 1998, Beste et al., 2005). Finally, *M. bovis* is a slow growing organism and thus culture plates are particularly susceptible to the growth of contaminating organisms that can mask the presence of *M. bovis* colonies (Portaels, 1988, Allen, 1991, Corner et al., 1995).

5.24 Advantages of immunomagnetic capture

Despite the limitations of the method and multiple boundaries to optimisation, IMC has a number of potential applications that make it worthy of investigation. The principle area of interest is the possibility to understand the state of *M. bovis* within the environment. Current methods of quantification (viable count and qPCR) will only provide information on the presence of the organism. Direct isolation without culture could provide scope for investigations into metabolic activity or permit the application of viability stains such as propidium iodide for fluorescence microscopy. Furthermore, this could provide scope for infectiousness studies to establish whether bacilli directly isolated from the environment are capable of forming infection in a host.

The possibility of conducting analyses regarding the state of *M. bovis in situ* would also provide greater clarity on which detection methods are the most suitable. Once the target cells are isolated and concentrated, it is possible to stain the eluate with nile red which will stain lipid inclusion bodies within the organism (Greenspan et al., 1985, Garton et al., 2008). Lipid bodies within mycobacteria are visible by fluorescent microscopy under the x100 objective and are indicative of the organisms residing within a dormant state (Garton et al., 2008). This is an important consideration in an environment which routinely uses culture for the determination of sterility, as these dormant bacilli will not be culturable under standard conditions (Mukamolova et al., 2010). Furthermore, dormant mycobacteria are better able to tolerate stress and have demonstrated increased resistance to antibiotics (Garton et al., 2008, Daniel et al., 2011, Rodríguez et al., 2014). It is hypothesised that this increased tolerance will expand to the use of Defra

disinfectants which are tested firstly on fast growing *M. fortuitum* and secondly with culture-based methods (Anon., 2016). The use of an unverified model species combined with culture-based methods which are unlikely to detect stress-adapted cells could have large implications on our current understanding or appropriate biosecurity strategies.

5.25 Antibody selection

Immunomagnetic capture is not a novel approach to *M. bovis* detection. Sweeney *et al*. published a method in 2006 for the isolation of *M. bovis* using a primary antibody to adhere to the *M. bovis* cells and secondary antibody coated to the beads in order to react with and capture the primary antibody. However, the study used an antibody which is no longer commercially available and thus other antibodies must be identified. Antibody clonality was considered during method development as polyclonal antibodies (pAbs) offer increased sensitivity in comparison to monoclonal antibodies (mAbs) as pAbs permitting the binding of multiple epitopes of an antigen (Ivell et al., 2014, Cox et al., 2019). For example, when pAbs and mAbs derived from the same goat were analysed via western blots, the pAbs demonstrated increased assay sensitivity (Ascoli and Aggeler, 2018). Sensitivity is an important consideration in the context of samples that are likely to contain low levels of target bacilli within a complex matrix (Skjerve and Olsvik, 1991). However, when increasing sample sensitivity, there is a trade off in the specificity of the assay (Parikh et al., 2008). Environmental samples have the potential to contain a diverse range of mycobacterial genus members, therefore additional confirmatory tests should be used in order to confirm the likely identity of isolated bacteria (Young, 2003).

Two antibodies were selected based on their reactivity with antigens expressed by *M. bovis* BCG Pasteur for the optimisation stages, and in *M. bovis* wildtype for the targeted application. The first antibody was a mouse monoclonal IgG1 antibody raised against Ag85 (Fisher Scientific); Ag85 is a mycolyltransferase which binds to fibronectin and is a major secretary protein in active, pathogenic *Mycobacteria spp.* (Wiker and Harboe, 1992, Kuo et al., 2012). Ag85 is a complex of three related gene products and despite being principally described as a secretory component of the *M. bovis* proteome, Ag85 will also be retained within the cell wall making it a suitable target for the purposes of this assay (Abou-Zeid et al., 1988, Vettiger, 2014). The second antibody was a rabbit

IgG polyclonal raised against the whole cell proteins of *M. bovis* (Bioss Inc, Stratech Scientific Limited). Mouse raised antibodies exhibit low reactivity with Dynabeads® Protein A, while rabbit antibodies exhibit high reactivity. Therefore, a rabbit, anti-mouse IgG (H+L) secondary antibody was selected (Jackson ImmunoResearch, Stratech Scientific Limited) to determine whether the capture rate of the mouse monoclonal would be increased by the addition of a secondary antibody.

5.26 Quantifying capture success

Quantitative-PCR and viable plate counts will be conducted on the culture used for spiking the faecal samples and on the recaptured cells with the addition of colony PCR to determine the efficacy of the capture method against these two measures, as well as determining the plate count discrepancy.

5.3 Methods

5.31 Growth *M. bovis* BCG Pasteur

Cultures of 25 mL were established in Middlebrook 7H9 media supplemented with 10% ADC and 0.05% Tween 20. Briefly, a mid-exponential phase of *M. bovis* BCG Pasteur was taken (circa OD_{600} 0.6-0.9), the OD_{600} read on a spectrophotometer and a sufficient volume of this starter culture added to the new flask so as to achieve an OD_{600} of 0.05 (Mukamolova et al., 2010). The new culture was sealed, swirled by hand and incubated at 37 ˚C, 120 rpm until the culture reached mid-exponential phase. A blank of the supplemented media was incubated alongside so as to ensure turbidity was not due to contamination.

5.32 Sample and spike preparation

The experiment was performed in triplicate with a negative control (no spike) and a positive control (seed, but no faeces). Briefly, 0.5 g weights of known-to-be-negative badger faeces were diluted with sterile 2% BSA to create a slurry (final volume of 2 mL) and 200 µL of mid exponential culture was added to each positive sample (or an additional 200 µL of PBS for the negative control) and vortexed for 5 seconds. A positive control consisting of 200 µL BCG culture was also blocked and included. The samples were blocked overnight at 4 ˚C to prevent non-specific binding of antibodies.

5.33 Antibody preparation

Upon receipt of the antibodies they were aliquoted into 10 µg portions and frozen at -20 °C until use to increase the shelf life and to prevent degradation associated with successive freeze-thaw cycles. Three antibody combinations were trialled as outlined in the introduction including a mouse monoclonal (‘mouse-direct’), a mouse-monoclonal attached to the magnetic beads via a rabbit polyclonal (‘mouse-indirect’) and a rabbit polyclonal (‘rabbit-direct’).

5.34 Capture of *M. bovis*

For each sample, 20 µL of Dynabeads® Protein A (2.8µm) were washed twice in 100 µL of PBS + 0.05% Tween 20 (PBS-T) and resuspended in 50 µL of PBS-T. For each wash step, a magnetic rack (Dynal Biotech) was used to capture the beads prior to the removal of the liquid/waste fraction. Either 10 µg of mouse monoclonal, 10 µg of rabbit polyclonal, or 10 µg of rabbit-anti-mouse polyclonal antibody were added to 20 µL of the washed beads and pipetted up and down three times following incubation in the dark at room temperature for 1.5 h. For the indirect (dual antibody) method the mouse monoclonal antibody was then added to the rabbit-anti-mouse-Dynabeads; all combinations had a total incubation of 3 h. BCG culture (250 µL) was added to three separate microcentrifuge tubes per antibody-combination. To this, 20 µL of labelled beads were added and incubated for 1 h at room temperature with regular mixing. The magnetic beads were then trapped, the supernatant removed, and the beads eluted in 250 µL of PBS-T before repeating the prior step. Finally, the beads were suspended in 125 µL of PBS-T.

5.35 Eluate investigations

From the resultant beads-BCG mix, 80 µL was plated on to separate modified Middlebrook 7H11 plates and incubated for 4 weeks at 37 °C; 25 µL was used for qPCR and a 5 µL microscopy smear prepared for Ziehl-Neelson staining.

5.36 Ziehl-Neelson staining

Captured cells (5 µL) were spread onto a microscopy slide and allowed to air dry. The slide was placed on a heat block set to 80 °C for 3 mins to fix the captured cells to the slide. Subsequently, the slide was flooded with carbol-fuschin and remained on the heat block until fumes were visible before being removed and left to stand for 5 mins. The slide was washed with di-H_2O before being flooded with acid-alcohol for 1 min, rewashed and counter-stained with methylene blue (1.5% w/v) for 1 min and rinsed with water. The slide was allowed to air dry, a coverslip mounted and then visualised on the 40x objective on a Zeiss light microscope for AF bacilli retaining the carbol-fuschin stain.

5.37 DNA extraction

Total community DNA was extracted from the produced pellet using the FastDNA™ Spin Kit for Soil (MPbio) as per the manufacturer's instructions, with minor modifications. In brief, 0.1 g of faeces, 978 µL of sodium phosphate buffer (Na_2HPO_4) and 122 µL of MT buffer were added to Lysing Matrix E tubes, vortexed and ribolysed at 6000 wibbles min^{-1} for 2 rounds of 40 s. The tubes were centrifuged at 13,000 x g for 12 mins and the supernatant transferred to microcentrifuge tubes containing 250 µL PPS, inverted by hand 10 times and incubated at room temperature for 10 mins. The tubes were again centrifuged for 5 mins at 13,000 x g and the supernatant added to 7 mL universals containing 1 mL of binding matrix. The universals were inverted by hand for 2 mins, settled for 5 mins before 400 µL of the top fraction was discarded. The remaining mixture was resuspended and repeat aliquots of 650 µL transferred to a tube containing a spin filter and centrifuged for 3 mins at 13,000 x g and the flow-through discarded. Subsequently, the residue was eluted in 500 µL of SEWS-M and incubated for 5 mins at room temperature, centrifuged twice at 13,000 x g for 5 mins and the flow-through discarded. Finally, the spin basket was transferred to a new catch tube and air dried for 5 mins prior to gentle elution in 100 µL of DES. The tubes were incubated at 60 ˚C for 5 mins before being transferred to the centrifuge and spun at 13,000 g for 3 mins (twice). The spin baskets were discarded and the DNA frozen at -15 ˚C ± 2 ˚C until use.

5.38 qPCR reaction

M. bovis BCG DNA was quantified using qPCR assays targeting the RD4 deletion region, unique in *M. bovis.* Samples were tested using an ABI 7500 Fast qPCR machine (ABI) with negative and positive controls. A panel of standards from 10^6 to 10^{-1} µL $^{-1}$ were included for the production of a standard curve. The qPCR reaction mix included 10 µL of either standard or total community DNA, 900 nM of RD4-forward primer $^{5'}$-TGTGAATTCATACAAGCCGTAGTCG-$^{3'}$, 900 nM of RD4-reverse primer $^{5'}$-CCCGTAGCGTTACTGAGAAATTGC-$^{3'}$, 250 nM probe AGCGCAACACTCTTGGAGTGGCCTAC-TMR, 1 mg/mL of BSA, 12.5 µL of Environmental Mastermix 2.0 (ABI), and made up to 25 µL with sterile, DNA-free water (Pontiroli et al., 2011). The conditions for the reaction were as follows; 50 ˚C for 2 mins, 95 ˚C for 10 mins, 40 cycles of 95 ˚C for 15 s and 58 ˚C for 1 min.

5.39 Colony confirmation by PCR

Suspected *M. bovis* BCG colonies were picked from plates with a pipette tip and homogenised in 50 μL STW. The liquified colony was heated to 95 °C for 2 mins on a heat block and 10 μL used as the PCR template DNA and amplified with the above primers, using PCR Mastermix 2x (Thermo Scientific™). The PCR product was visualised by gel electrophoresis (2.5% agarose gel) against a 100 bp ladder (Invitrogen).

5.40 Data analysis

Data was recorded using workbooks in the laboratory and transferred to Excel 2018 on the same day. R version 3.4.1 was used for analysis and graphical representation.

5.4 Results

The method was initially trialled with *M. bovis* BCG culture for the identification of suitable antibodies. In the early stages of optimisation, it was noted that there were high levels of variation between replicates. Attempts were made in order to establish the source(s) of this variation and hypotheses will be suggested. The optimisation procedures were as follows:

1. Determine the most definitive method of quantification.
2. Tests on pure culture to identify the antibody combination with the highest capture rate.
3. Compare percentage recovery in faeces to culture.
4. Further steps to optimise by increased washing stages and higher faecal dilutions.

5.41 Trials on pure culture

Microscopy of the eluate demonstrated clumps of captured cells (Figure 5.4) which would result in variation between replicates during the aliquoting stage, with further variation introduced due to a clump of cells forming a single colony on agar. To remove this limitation in quantification, qPCR was used rather than viable counts to increase the sensitivity of detection (Figure 5.4). While the non-homogenous aliquoting would still affect the qPCR results, it would eliminate the effect of a clump of cells being identified as a single cell.

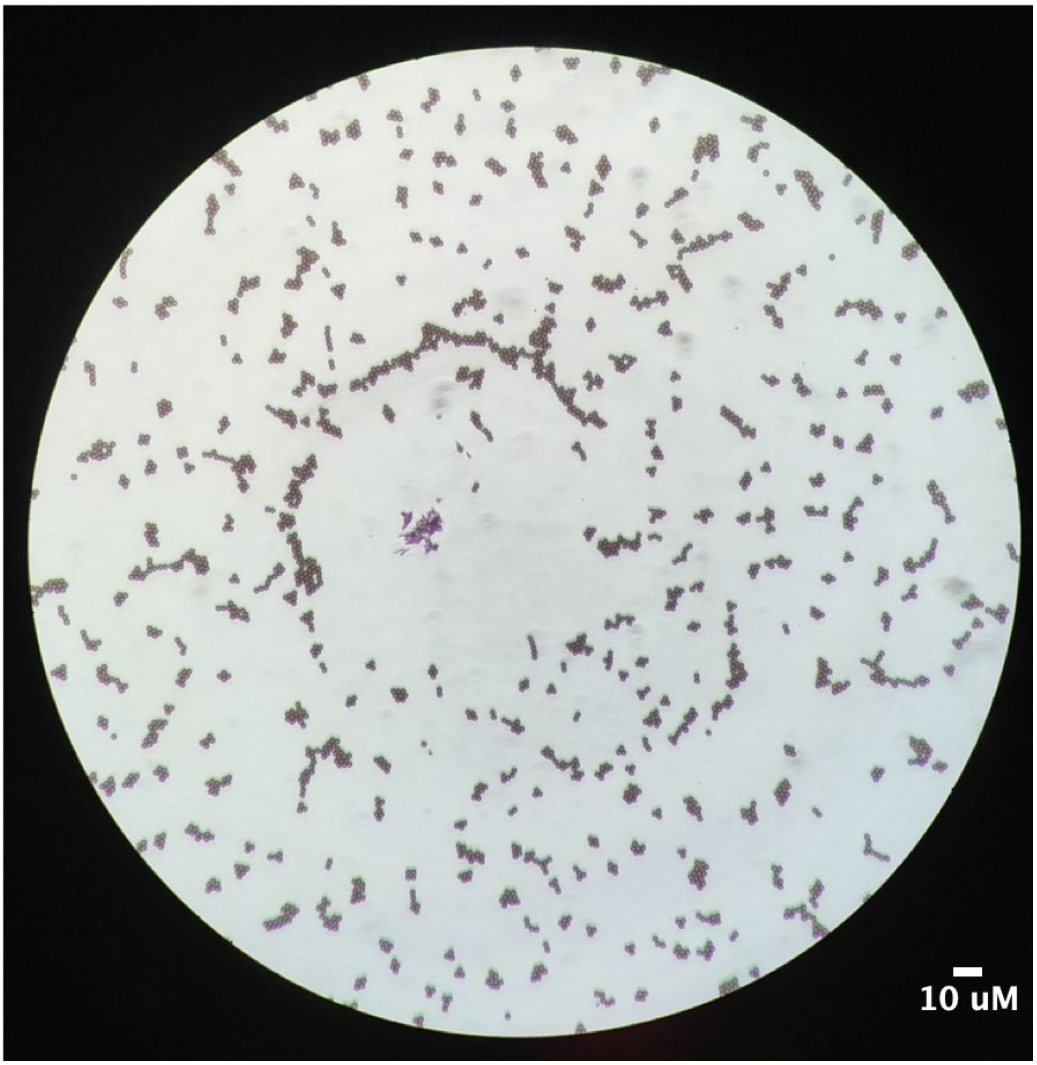

Figure 5.4: Cells captured with magnetic beads and a rabbit polyclonal antibody from a pure culture of Mycobacterium bovis BCG, eluted in PBS-T. Visualised on the x40 objective.

Initial work was conducted with the mouse-direct method but due to the limited capture capacity an alternative antibody was sought and a rabbit antibody for direct capture used. However, the rabbit-direct method did not demonstrate an improvement in capture efficacy and was largely comparable to the mouse-mAb used initially.

To determine whether the capture rate of the mouse-mAb method was limited by the lower affinity for mouse raised antibodies for Protein A on the selected Dynabeads®, a secondary rabbit-anti-mouse pAb was added to increase the adherence of the mouse mAb to the beads (referred to as the 'mouse indirect' method). The capture results in Table 5.1 demonstrate the success of each of the three methods across 3 technical replicates per antibody combination in pure culture. These results were determined by qPCR and show the increased number of captured cells with the inclusion of the secondary antibody in the mouse-mAb method.

Table 5.1: Percentage of captured cells from a culture of M. bovis BCG (performed in triplicate) as determined by the recovered number of genome equivalents.

Antibody Combination	**Mean capture (N (%))**	**Range of capture (N (%))**
Rabbit polyclonal (rabbit – direct)	1.46×10^7 (42)	$2.30 \times 10^6 – 2.64 \times 10^7$ (4- 52)
Mouse monoclonal (mouse-direct)	1.44×10^7 (45)	$7.47 \times 10^6 – 2.95 \times 10^7$ (16- 55)
Rabbit polyclonal 2ndry– Mouse monoclonal (mouse indirect)	2.17×10^7 (67)	$1.00 \times 10^7 – 3.98 \times 10^7$ (22- 78)

Subsequently, a single culture of *M. bovis* BCG was divided into 12 aliquots. Three aliquots of approximately 4.8 x 10^7 cells were tested via the above IMC method using the three antibody combinations, with latter DNA extracted in triplicate for each of the resultant eluates for qPCR quantification. The results, and variability, are demonstrated in Figure 5.5. The two primary antibodies exhibited similar abilities to bind and capture BCG to the magnetic beads (W = 33, p = 0.815). However, the inclusion of the intermediate rabbit-anti-mouse antibody significantly increased the capture rate of the mouse mAb (W = 63, p = 0.05; Figure 5.5). After the financial implications and practicality implications were taken into account the indirect method was brought forward for optimisation within faecal samples.

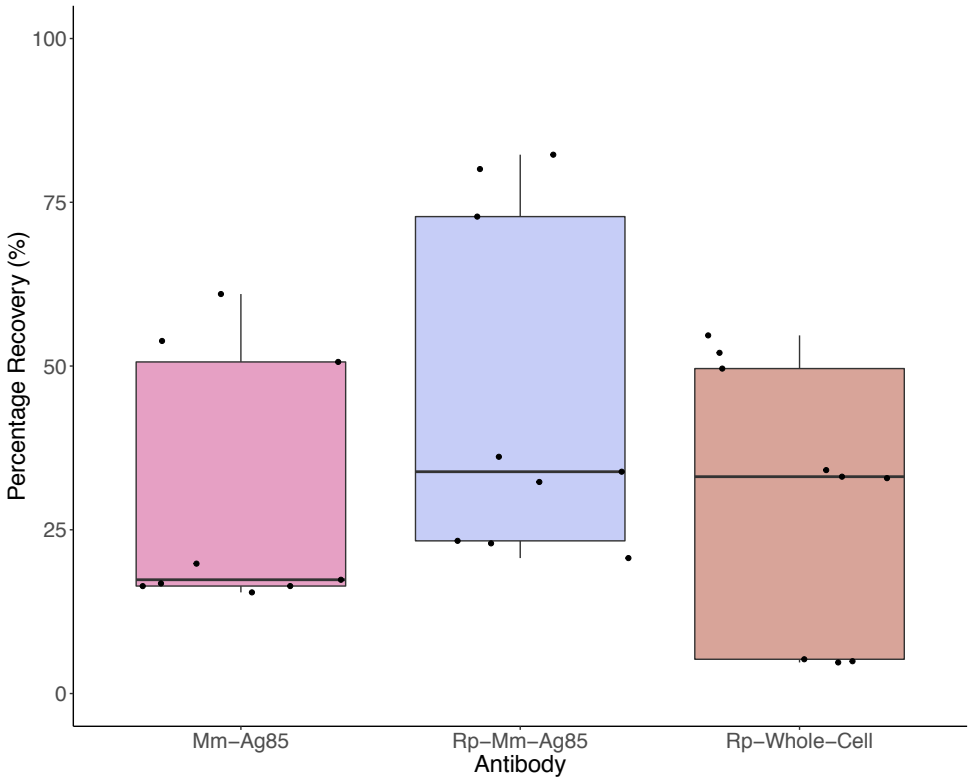

Figure 5.5: The percentage recovery of BCG from culture by antibody combination across 3 biological replicates as quantified by triplicate DNA extractions and qPCR. Mm-Ag85 represents the mouse-direct (median 18%); Rp-Whole-cell represents the rabbit-direct method (median 18%); Rp-Mm-Ag85 represents the mouse-indirect method (median 34%).

5.42 Trials on spiked badger faeces

When the indirect capture method was applied to spiked faecal samples, there was a large decrease in the number of cells recoverable compared to the number captured from culture. The number of recovered cells reduced from a mean of 67% for the indirect-mouse method in culture to an average of 1% in spiked faeces (n = 3) as determined by qPCR. The sample was diluted to 5 mL to test the hypothesis that sample fluidity was a limiting factor on bacilli recovery (Figure 5.6). Reducing the turbidity of the sample increased the recovery of bacilli to 4.5% ($t = -3.99$, $df = 4.0$, $p = 0.02$, $n = 3$). However high variance in the percentage of recovered cells remained an issue despite the decrease in relative variance in higher sample volumes (1 mL slurry $\bar{x} = 1.0\%$, variance = 1.08%; 5 mL slurry $\bar{x} = 4.5\%$, variance = 1.17%).

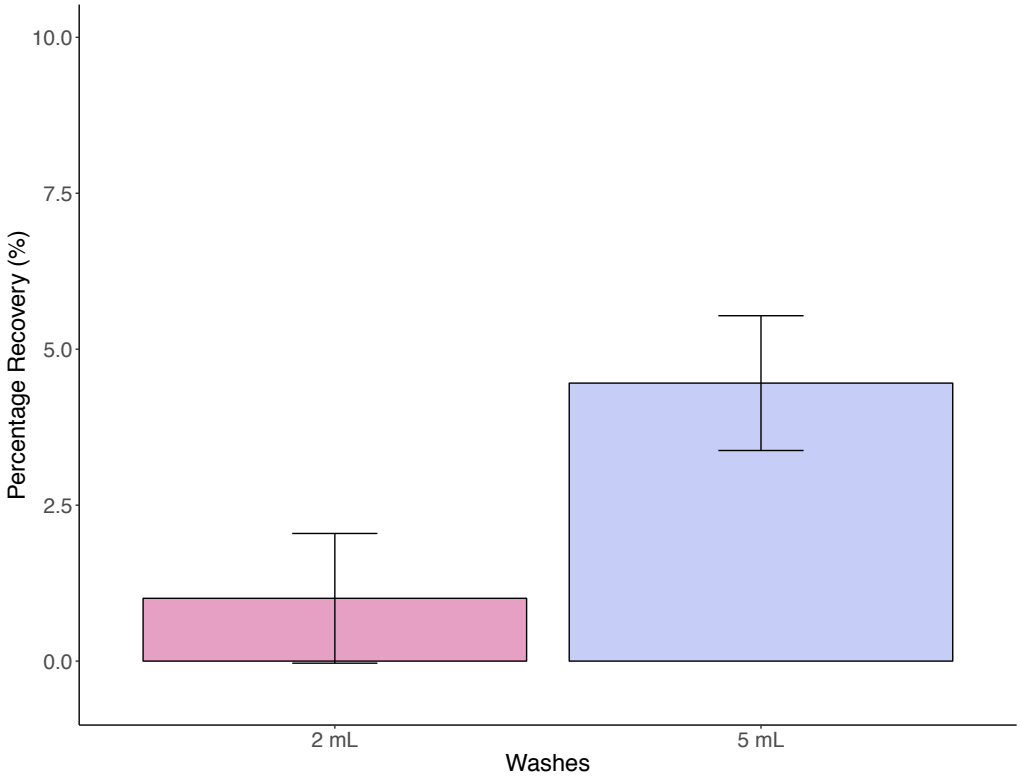

Figure 5.6: The effect of decreasing the samples' viscosity on percentage recovery rate, as determined by qPCR. Badger faecal samples spiked with 4.0 x 10^9 bacilli.

Even with increased sample liquidity, when a culture of *M. bovis* BCG was tested in triplicate, and triplicate faecal samples spiked with the same culture and tested in parallel, the recovery rates were not comparable (recovery from culture rate: $\bar{x} = 42.8\%$, $\sigma = 21.9\%$, $n = 4$; recovery from faeces rate: $\bar{x} = 3.2\%$, $\sigma = 1.4\%$, $n = 4$). It was deemed impractical to undertake testing on higher faecal dilutions due to the additional time it would take in order to capture target cells from a larger volume. A significant difference remained between the two matrices despite the increased liquidity of the faeces ($W = 49$, $p < 0.001$) and it is was deemed unlikely that the capture rate in faeces would approach the success seen in culture (Figure 5.7).

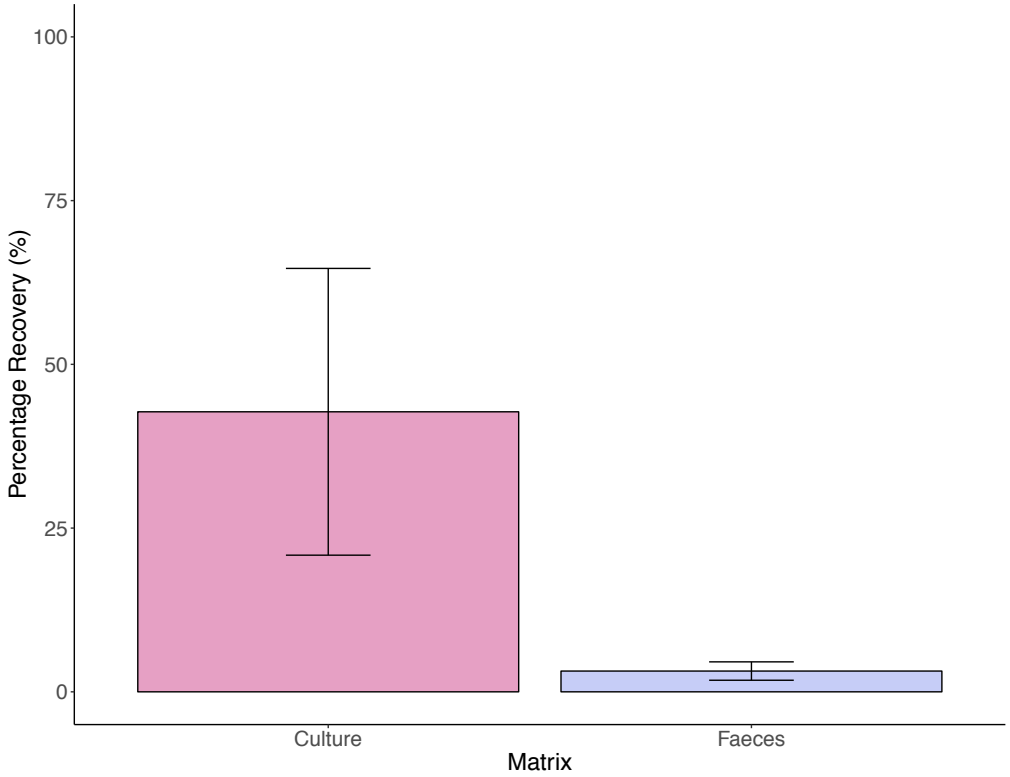

Figure 5.7: The difference in cell recovery from liquid culture, and spiked faecal samples.

To explore the effect that cell and bead aggregation had on the number of detected bacteria, two methods of quantification were tested in parallel on the eluate from four seeded badger faecal samples (Figure 5.8). It was found that viable counts detected a significantly lower number of cells than qPCR ($t = -3.733$, $df = 6$, $p = 0.01$).

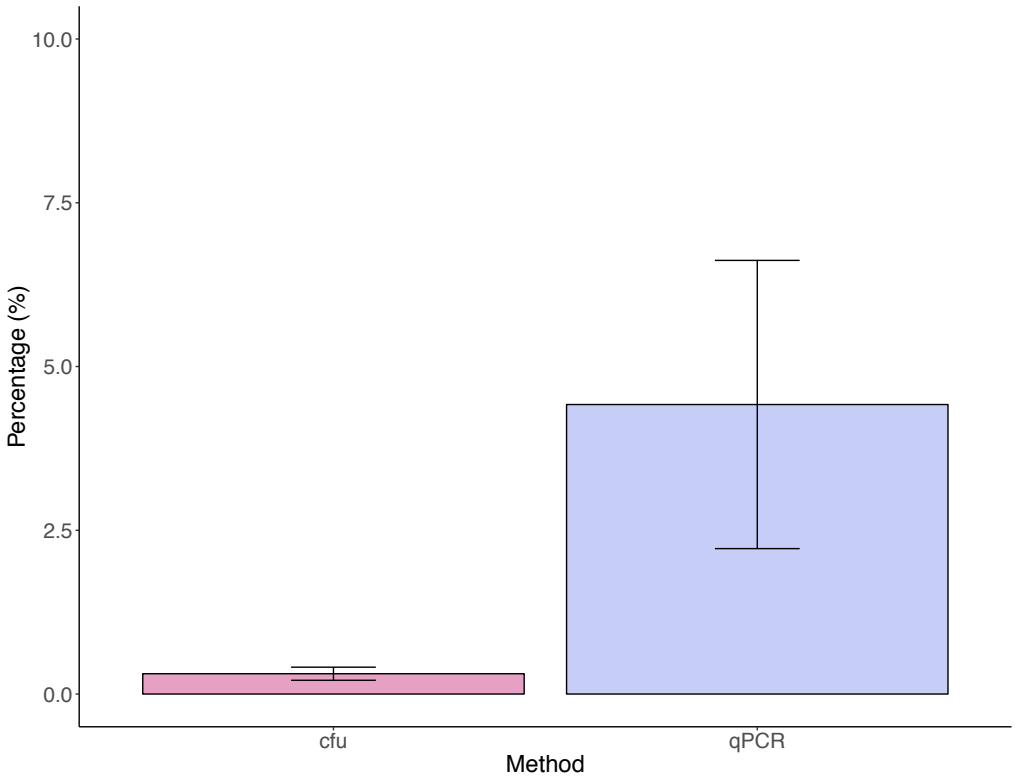

Figure 5.8: Difference in quantification of captured M. bovis *BCG from spiked faecal samples as determined by CFU and qPCR ($\bar{x} = 0.31$, $\sigma = 0.10$, $n = 4$) and qPCR ($\bar{x} = 4.42$, $\sigma = 2.2$, $n = 4$)).*

To attempt to increase the recovery of the beads, the methodology was altered so that the supernatant produced from the sample was subjected to sequential magnetic captures (Figure 5.9).

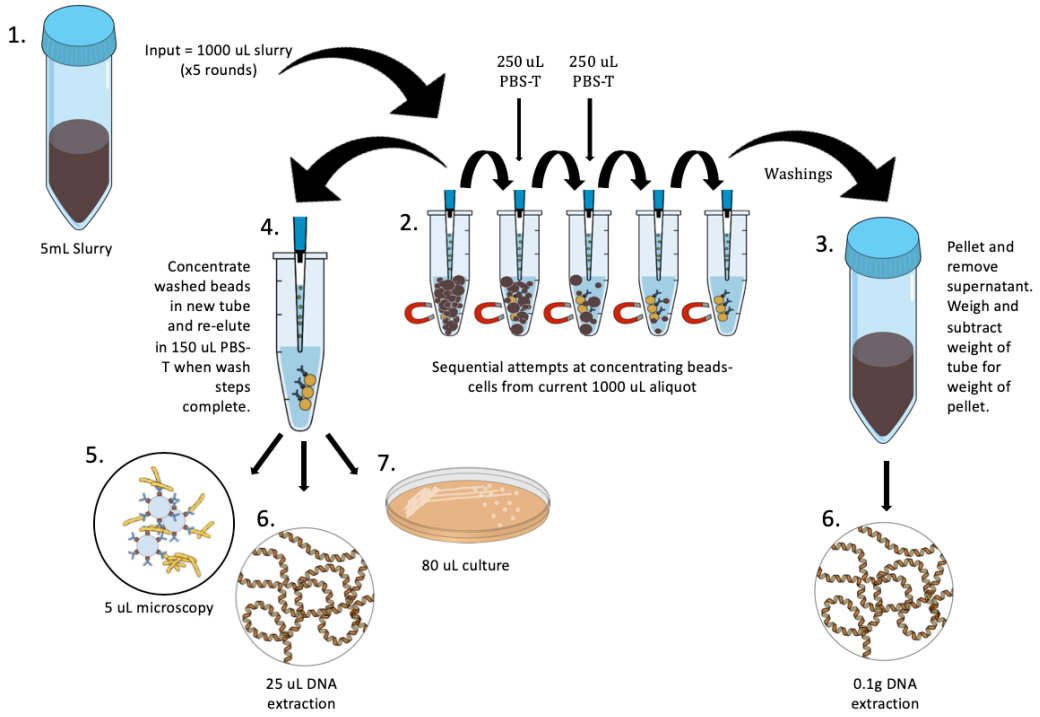

Figure 5.9: ***Flow diagram representing the capture of magnetic beads from faeces:***

1. the original faecal slurry, 2. the transferring of 1000 uL aliquots at a time with two further dilution steps and sequential isolation attempts within the magnetic rack, 3. the collection of cell washings for later pelleting and DNA quantification for non-captured cells, 4. the transferring of washed beads to a new tube to be concentrated via one further round of capture and elution in 150 uL of PBS-T, 5. the use of the eluate for microscopy, 6. DNA extraction for qPCR quantification in the eluate and pelleted washings and 7. the culture of the washed beads-cells on Middlebrook 7H11+ PANTA as described in the methods section. Image created using the Mind The Graph platform available at www.mindthegraph.com.

To test the impact of sequential captures on capture rate and variance, one faecal sample was split into two subsamples, spiked with liquid BCG culture and homogenised. The samples were both subjected to the IMC methodology and the final eluate plated onto three modified Middlebrook 7H11 + PANTA plates for quantification of captured cells by viable count. Sequential captures increased the capture rate of the method due to the additional recovery of magnetic-bead-bound-cells from the sample wash, but also demonstrated the intrinsic variability of the method (Figure 5.10). Variation is likely to stem from the sample processing stage and the culture stage, with significant variation demonstratable between the results from the two subsamples ($t = -4.94$, $df = 3.93$, $p = 0.008$), as well as relatively large standard deviations within the replicates performed for each sample (A: $\bar{x} = 1.0 \times 10^7$ (1.63%, $\sigma = 0.82$, se = 0.47); B: $\bar{x} = 3.2 \times 10^7$ (5.18%, $\sigma = 0.94$, se = 0.54)).

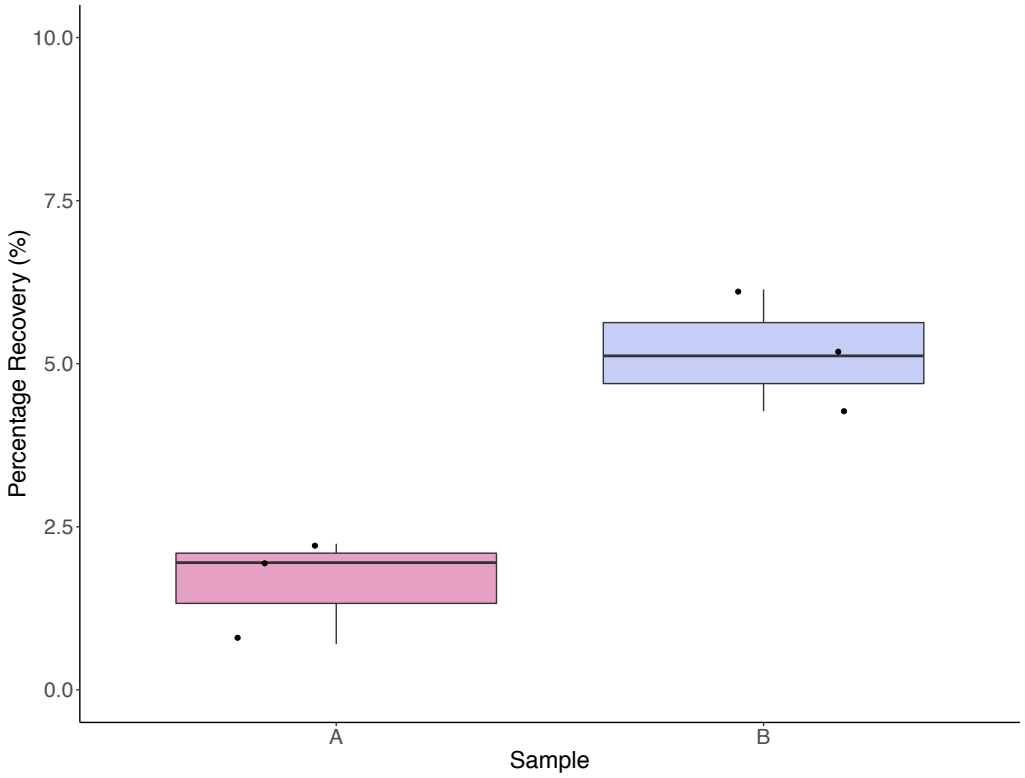

Figure 5.10: Within and between sample variation between duplicate faecal samples spiked with the same bacterial starting culture (A and B) at 6.2 x 10^8 genome equivalents as determined by qPCR.

It was hypothesised that a number of cells would either not be captured or would be discarded within the wash steps due to the slowness of the magnetic particles' movement through the slurry, adherence to faecal particles and their subsequent removal, or due to detachment during transfer of the supernatant. To identify how many cells were lost within the first four washes of the sample-beads (before relatively faecal-matter free beads are collected in a single tube for remaining wash-steps in step 4, Figure 5.9) the removed slurry was transferred to a 50 mL falcon tube and centrifuged at 3000 x g for 20 mins. The supernatant was discarded, the pellet homogenised, weighed, and 0.1g used for DNA extraction. Post quantification, the recovered DNA was multiplied to the total weight of the pellet formed from the washings and compared to the number of cells captured, as also determined by qPCR (Figure 5.11). The results demonstrated that despite the optimisation steps that had been undertaken that a significant number of cells were lost within the wash stages.

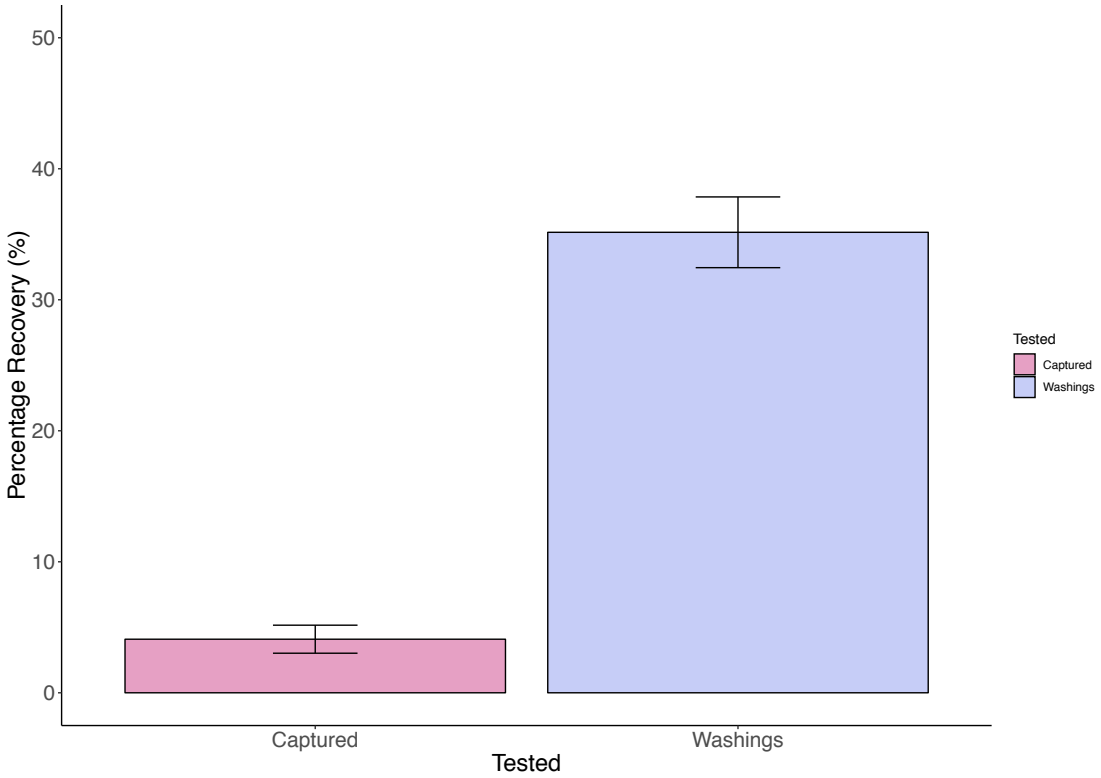

Figure 5.11: PCR quantification of the number of cells lost in the initial wash steps, in comparison to those which are captured. Faeces spiked with 3.9 x 10^9 bacteria

Finally, it needed to be ascertained whether as well as eliminating the harsh decontamination procedures necessary for isolation of environmental *M. bovis*, it would be possible to remove the PANTA antibiotic supplement from culture media. It was found that plating samples on 7H11 plates without PANTA resulted in considerable contamination after 24 h incubation at 37 °C that obscured the final mycobacterial CFU reading (Figure 5.12). It was therefore concluded that PANTA remained a necessary component of the culture medium.

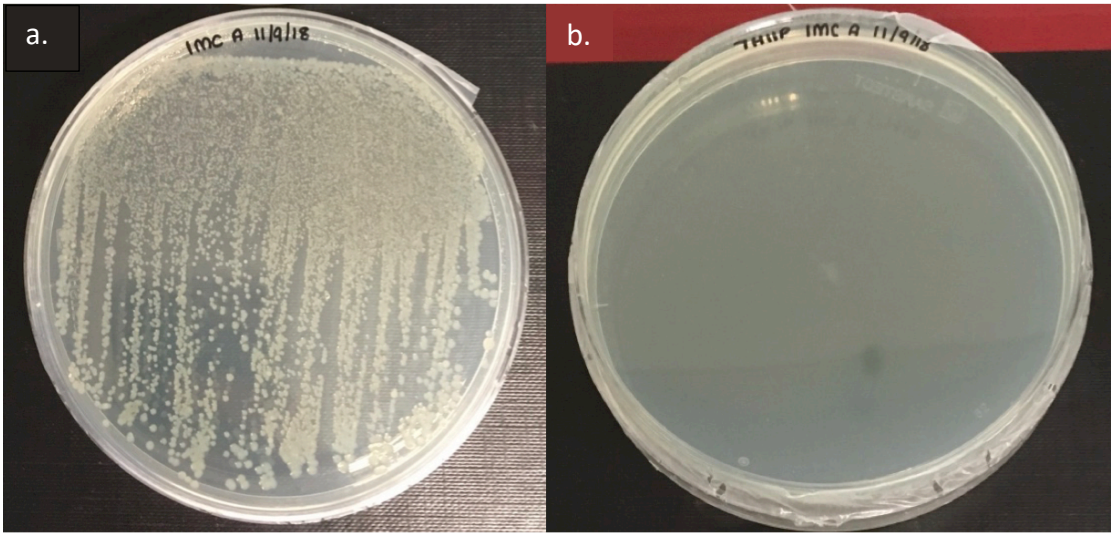

Figure 5.12: Plated IMC eluted beads from faeces without (a) and with (b) the addition of PANTA antibiotics to 7H11 after 24 hours of incubation at 37 °C.

5.5 Discussion

5.51 Methodology

The antibodies used in the two direct methods are thought to have similar antigen binding capacities but exhibit different levels of reactivity with protein A. The Dynabeads® manufacturer stated that rabbit-raised antibodies possessed a stronger affinity for binding protein A. This was confirmed by the development of the mouse-indirect method in which a secondary rabbit-anti-mouse antibody increased the adherence of the primary antibody to the beads and therefore the subsequent capture rate of the bacilli by the mouse mAb.

Though the capture of cells increased, the numbers remained low and a large level of variation persisted within the method making it unsuitable for undertaking quantification or presence-absence assessments. The first source of variation is due to the heterogenous nature of the sample; faeces is a complex matrix which naturally varies depending on the individual, the diet, and the level of hydration (Robertson et al., 2015, Rose et al., 2015). Sample consistency will firstly have an effect on the ability of the magnetic beads to encounter and capture the target cells and secondly on the migration of beads towards the magnet. If the beads cannot reach the magnet to be trapped against the side of the tube, then cells adhered to those beads will be lost during the wash. Furthermore, the extreme hydrophobicity of mycobacteria is likely to result in adherence to the high level of suspended solids; consequently bacilli bound to faecal particles are lost during the wash steps due to being insufficiently buoyant so as to migrate to be trapped by the magnet (Bendinger et al., 1993, Alsteens et al., 2008). Once captured, the hydrophobicity further increases the margin for error by the formation of aggregation of bacilli and beads rendering the cell distribution through the eluate inhomogeneous and unquantifiable by viable count.

The selection of pAbs was due to a desire to increase the sensitivity of the assay, however this also introduced additional potential for error by decreasing the assays specificity. Furthermore, the use of pAbs introduces increased variation within the assay as pAbs demonstrate higher variability between batches in comparison to mAbs (Ascoli and Aggeler, 2018).

Finally, this method is also susceptible to operator error, with reductions in capture attributable to accidental detachment of the beads from the side of the tube during the wash steps and variation in time spent capturing the magnetic particles due to laboratory restriction. The combination of all of the above factors makes it unlikely to obtain similar cell numbers during repeated aliquoting of the mixture - as demonstrated through both qPCR and viable counts. It was deemed unsuitable for the purposes of cellular quantification, though other downstream applications would be feasible.

5.52 Capture success

This study primarily focussed on capture success as quantified by qPCR to remove some of the variation from the method. By this measure, the recovery of cells was circa 4.5% within the current study; it was sought to compare this value to that previously obtained by the laboratory – 82.24% (Sweeney et al., 2006). However the previous study calculated capture success by semi-quantitative PCR against the viable count of the spike, it is therefore likely to be an overestimation of method's capabilities (Pathak et al., 2012). The only way of drawing comparison was therefore through the more variable estimation of recovery by viable plate count. While the current method resulted in a low mean recovery rate of 0.3% from spiked faeces, this was an improvement on the previous study which cited a 0.1% recovery from the same matrix (Sweeney et al., 2006).

There were multiple differences in method design that are likely to have contributed to the increased recovery of cells in comparison to the Sweeney *et al*. method. Firstly, as the antibody in the previous study was no longer available an alternative antibody was purchased; this would have impacted on antigen-antibody reactions and potentially have been more reactive than the previously used antibody, though this cannot be proved. Secondly, the wash steps were undescribed and assuming different approaches were taken, this could alter patterns of cellular detachment from the beads or bead detachment from the magnet. Furthermore, the use of the repeat capture method used within the current study (Figure 5.9) is likely to have increased the amount of time available for the beads to be retrieved from the sample and therefore the capture rate. After the capture component of the method, variation in the use of the eluate could also have added variation to the results. An increase in CFU detection could be partially explained by the use of 7H11 media within the current study which shows greater levels of *M. bovis*

growth than the 7H10 media used in the previous study (Procop, 2017). The only other study known at the time of writing does not have a specified percentage recovery rate but instead stated the limit of detection 50% (LOD_{50}) as being 2.8 x 10^5 cells/mL faecal homogenate and therefore also suggests limited application for faecal detection in naturally contaminated faecal samples (Stewart et al., 2017).

5.43 Application

For routine application to environmental samples it was deemed that there was a low return for a large time and economic input. Despite the culture sensitivity from faecal samples as a single sample type having yet to be specified, the cultivation of multiple clinical samples in parallel (i.e. faeces, urine, tracheal aspirate and oesophageal aspirate and wound swabs) have an average sensitivity of 8.0% . It is hypothesised that with faeces containing large numbers of contaminating organisms and high levels of organic substances, that the sensitivity of *M. bovis* detection by culture from faeces is likely to be lower than this average. While the IMC method eliminates the requirement to use harsh decontaminating chemicals it is unlikely to have made a sufficient improvement on the culture methods currently available, though this cannot be directly compared.

However, with additional development there is scope for utilising the method for the isolation of single cells which could be used in further experimental set ups. *M. bovis* is hypothesised to be in a dormant state when in the environment due to a combination of the stresses associated with excretion and those associated with environmental persistence (Rifat et al., 2009, Hibbing et al., 2010, Bradley et al., 2019). For the culture of organisms from environmental matrices, they must be placed into a nutrient rich environment and it is likely that the addition of these nutrients would function so as to alter the physiological and metabolic state of a proportion of the bacteria, rendering them no longer representative of the cells' states within the environment within hours (Sweeney et al., 2006, Cangelosi et al., 2010). While mRNA methods have advanced for determining the state of cells within environmental samples, only small sample sizes can be processed due to high concentrations of inhibitors which lowers then methods' sensitivities (Wang et al., 2012). Furthermore, the isolation of high purity RNA in sufficient quantities so as to detect the transcriptomic profile of dormant environmental mycobacteria has also been questioned (Stephan et al., 2004, Ignatov et al., 2015). The

most cited methods for both environmental and mycobacterial studies are based on phenol-chloroform or TRIzol® extraction, neither of which were permitted due to the use of phenol in the currently unsuitable class 1 medical safety cabinets (Reck et al., 2015). This study provides an alternative method of isolating and concentrating bacteria from larger sample volumes while also preserving their *in situ* metabolic state as far as possible. This could prove useful for transcriptomic, proteomic, and infectiousness studies for high yielding samples in facilities that support the application of these methods for containment level 3 pathogens.

It was not feasible to undertake studies examining the efficacy of disinfectants on *M. bovis* inoculated into farm substrate within the remaining time available to this research project. The aim of this proposed work was to address my concerns regarding the use of *Mycobacterium fortuitum* as a model organism for determining the efficacy of disinfectants for the use of farm decontamination post-breakdown (Anon., 2016). It is thought that testing regimes would benefit from not only the inclusion of a more suitable organism (i.e. *M. bovis* BCG) but also from testing which approaches the conditions under which it will be used within the farm environment. It is hypothesised that under field conditions it is likely that VBNC cells will arise from treated populations of *M. bovis* and furthermore that the aggregation of cells to particulate matter will demonstrate a protective effect as demonstrated in the attempt to culture from faecal material (Figure 5.1). These factors combined will result in lower efficacy than that demonstrated through *in vitro* testing using standard culture techniques as these are known to underestimate the population of viable cells due to the non-culturability of dormant/stressed cell phenotypes (Mukamolova et al., 2010).

Finally, IMC could permit the isolation and staining of captured cells by the aforementioned nile red method. Nile red dye accumulates within the lipid inclusion bodies of mycobacteria and these LBs are highly correlated with the expression of the persister phenotype in *M. tuberculosis* and *M. smegmatis* (Garton et al., 2008). This would provide a rapid and easy to assess method of determining whether these faecal bacilli are expressing the persister phenotype evident within clinical mycobacteriology and discussed above. The identification of nonculturable persister bacteria would have important implications for methods of assessing environmental contamination by *M. bovis*; culture is the current method employed by the APHA which is likely to not detect

these persistent cells as they are associated with the VBNC state (Garton et al., 2008, Mukamolova et al., 2010). Furthermore, the persistent phenotype of mycobacteria demonstrates increased tolerance to antibiotics and other stresses; it is likely that these cells have not been considered in the testing protocols for current Defra approved disinfectants and protocols targeting *M. bovis* (Barer and Garton, 2010, Rodríguez et al., 2014, Maurya et al., 2018).

5.54 Suggested future optimisation

For future application, further optimisation is required to improve efficacy though it is hypothesised that the method will continue to encounter pathogen and matrix specific complications. The first limitation of the method is the viscosity and turbidity of the sample that hampers cellular recovery (Skjerve and Olsvik, 1991). This could be mitigated by further diluting the sample to reduce the turbidity, but this would also increase the amount of processing time required unless a customised rack capable of handling larger sample volumes was created. Alternatively, the turbidity could be reduced by filtering of the sample for larger size particles prior to capture, though analysis will have to be conducted to ensure that sufficiently more beads/cell are recaptured due to the removal of these large particles than are lost through filtration (Bendinger et al., 1993, Stewart et al., 2017).

The second key limitation is the aggregation of beads and captured cells resulting in highly variable results. It is suggested that the increased viable count in the current study compared to the Sweeney *et al.* method is in part attributable to the use of a non-ionic detergent within the elution step. Further optimisation could take the form of increasing the concentration of the detergent while ensuring that it has little effect of cell growth, or the use of other detergents and methods. For example, when polyoxyethylene sorbitan trioleate (Tween 85) was used in combination with sodium chloride (NaCl) and sodium bicarbonate ($NaHCO_3$) a significantly greater number of *E.coli* O157:H7 cells were recovered from the surface of lettuce leaves than were recovered by the use of Tween 20 and 60 (Hassan and Frank, 2003). Aggregations within the eluate can be rapidly qualitatively assessed by standard staining and microscopy techniques, however it must be ensured that the detergent does not reduce the culturability of the organism.

Finally, alternative antibodies could be trialled, but this is likely to add additional costs to the experiment as the lower priced commercially available antibodies were used within this study. However, a dual antibody system using the cost-effective antibody used here in combination with another could potentially lower the increased cost and may also increase the capture rate found within this study by including a second target antigen (Stewart et al., 2017).

5.6 Conclusion

This study has demonstrated the feasibility of direct isolation of *M. bovis* from badger faeces (or other environmental sample types) while maintaining the metabolic and physiological state expressed within the environment. However, large variation persisted which was independent of the capture method employed (direct or indirect). Variation was reduced by the increased level of sample dilution; however further dilution was not feasible with the current methodology, equipment and facilities.

IMC has potential, but with the high cost of antibodies, low capture rate, and high variation between replicates, it was not suitable for application to the detection and survival elements of this project. Regardless, the method has potential future usage in the study of the metabolic and physiological state of the organism *in situ* which could better inform the approaches to quantifying the cells in the environment if lipid body analysis suggests the presence of VBNC cells. Further investigations would determine whether the state in which *M. bovis* resides in the environment is capable of forming infection in species of interest. If *M. bovis* from naturally infected faecal samples proves to be infectious, then further questions can be asked regarding the infectious dose of cells, and how this relates to the organism's persistence and the disease epidemiology.

Chapter 6

Farm level impacts of bovine tuberculosis and research impact

6.1 Introduction

The majority of work on bTB rationalises investment in research due to the economic impact of the disease, with the annual taxpayer bill frequently being stated as approaching the £100 million mark. With these large figures it is easy to overlook the other costs associated, such as those paid directly by the farmer as OTF-W farms are associated with a decreased annual net profit (Turner, 2008). Research conducted to examine the longer-term effects of bTB identified two key implications; firstly, the more commonly discussed business-economic side, and secondly was the impact on human mental health (Table 6.1).

Table 6.1: Factors related to bTB breakdowns that result in longer term impacts: summary of findings (Turner, 2008)

Area and nature of impact	**Farm type**					
	Dairy farms			**Beef farms**		
	'Lightly affected'	'Long period under restriction'	'Large number of cattle taken'	'Lightly affected'	'Long period under restriction'	'Large number of cattle taken'
Farm business economics (strategic decision-making)	+	++	+++	+	++	+++
Human mental health (psychiatric morbidity)	++	+++	++++	+	+++	++

Note: Plus signs (+) indicate a positive effect as a factor associated with longer term impacts, the more (+) the stronger the association.

6.2 Economics

Firstly, to address the farm-level economics, there are further reaching impacts than simply the finances associated with the loss of cattle numbers. Before a positive test is read, the stress of bTB testing decreases milk production in otherwise healthy cattle which results in a loss of profits, furthermore suckler cows put on less weight due to stress (FCN, 2009). There are also additional economic considerations associated with increased workloads (particularly in when ongoing surveillance is mandatory), inability to continue with the normal workloads of the farm and increased requirement for the presence of a veterinarian or lay-tester (Turner, 2008, FCN, 2009). Once under restriction, costs increase due to the requirement for additional housing in order to segregate the animals (when possible), but also due to the inability to buy or sell cattle. The ban on cattle movements is particularly felt by businesses which rely on the movement of beef cattle for 'finishing', as these cattle require specialist high-energy diets and then may not be able to be sold when ready resulting in a doubly negative effect on profits. Furthermore, having to maintain cattle on the farm that may otherwise have been sold creates a backlog in the planned work of the farm as well as additional requirements for food, housing and veterinary costs that may or may not be met.

When a bovine is tested positive by the SICCT and subsequently slaughtered, the farmer will receive compensation from the government for the cows estimated value. In 31% of cases, compensation was directed towards the replacement of slaughtered cattle, however the payment did not cover the cost of replacement in 67% of cases. This is particularly important in England where standard cattle values are used which may significantly underestimate the value of a bovine, and particularly in the case of organic raised cattle or cattle from prized lineages. This has been mitigated slightly in Wales as cattle are valued by auctioneers prior to slaughter, however this still will only account for the price of the cow, and not the loss of earnings associated with the test-and-slaughter regime (FCN, 2009, Godfrey, 2018).

6.3 Health

The summer of 2019 saw a surge in news reports highlighting the problem of mental health in the farming industry, with 81% of farmers surveyed under 40 believing the mental health is the largest hidden problem for farmers (FarmingUK, 2019). This has been supplemented by reports from the Health and Safety Executive (HSE) who released figures stating that the overall illness rate for agricultural workers is 46% higher than the industry average, and that stress, depression and anxiety are significant causes of illness. A report conducted by the Farmers Crisis Network (FCN) was issued and Table 6.2 summarises the responses of 68 farmers who were asked how bTB impacts their wellbeing with only 46% reporting to be calm or coping with the issue of bTB, and 20% of farmers reporting they were either panicked or devastated by a breakdown (FCN, 2009).

Table 6.2: Table summarising the responses of farmers regarding the degrees of stress caused by bTB (n=68), 30% of interviewees were currently free from bTB restriction regulations (FCN, 2009).

	Number
Feeling pressure but coping	26
Stressed or depressed	29
Stress leading to physical illness	5
Don't want to carry on	3
Calm	5

Many farmers interviewed as a part of studies into bTB and mental health have cited the uncertainty around bTB as having a significant impact on their mental health. As they cannot predict the number of cattle that will be taken, or the duration of a breakdown it is difficult to mitigate potential cashflow problems (Turner, 2008). Furthermore, there is often a significant amount of frustration due to the disagreement between government organisations and scientific groups regarding the source of infections leading to disbelief that the problem will be resolved. The government has been unable to convince farmers the recommended biosecurity measures for the eradication of bTB will help, with 95%

of farmers interviews stating that they did not believe that they would contribute and 24% believing that testing was increasing the number of reactors (FCN, 2009).

There are further reaching social effects associated with bTB (Table 6.3) such as the deterioration of relationships with family or neighbours either due to the stress of the work or due to conflicting interests (e.g. badger conservation, biosecurity implementation) which will have further impacts on mental health. Table 6.3 demonstrates the wide range of health-related effects that are identified within the farming community as a consequence of, or are exacerbated by, bTB breakdowns. These feelings of helplessness and stress are a result of what is seen as political inaction, and the perceived lack of will to address the concerns raised by the farming community (Turner, 2008, FCN, 2009, Godfrey, 2018).

Table 6.3: Adverse effects on human health and well-being related to bTB (Turner, 2008).

Health effects	Stress
	Very severe physical problems such as heart trouble, stroke and suicide
	No time off or holidays
Social effects	Marital breakdown
	Deterioration of relationships with neighbours
	Lack of participation in community events
	Increased dependence on family members for help leads to extra family hardship
Other human effects	Loss of confidence in the farming industry and therefore no interest in succession from family members
	Loss of confidence in the industry
	Lack of confidence in authority on policy making
	Effects on the environment and landscapes
	Change in attitudes to wildlife

6.4 Impact of the current study: addressing the concerns of farmers

Understanding the disease epidemiology and how strategies can be implemented for the prevention of bTB transmission both within and between herds is something which many farmers feel has been an overlooked component of the current approach. While farmers are told what they should do, the evidence for implementing biosecurity strategies is often not provided (FCN, 2009). This is likely to be the underlying cause for the limited uptake of biosecurity strategies until after the farm has suffered from a breakdown (Turner, 2008, Godfrey, 2018). It is suggested that an important component of strategic updates would be to directly address the concerns of farmers; this could be achieved by either providing the results of research or by funding research to justify both the benefits and the financial outgoings associated with implementing new measures. In accordance with the statements above, addressing the concerns of farmers was considered to be an important element of any work undertaken as a part of this thesis.

The primary objective was the optimisation of viability-qPCR to address the concerns regarding the use of PCR for testing potentially contaminated materials providing the ability to rapidly differentiate between viable and non-viable *M. bovis*. The second, complimentary method was the optimisation of IMC (Chapter 5) to increase our understanding of the suitability of culture for routine purposes. While culture has the benefit of isolating viable and replicating organisms in PCR positive samples, it is likely to significantly underestimate the number of cells present and gives no insight into the state in which the cells persist within the environment. This could have further reaching applications for testing the suitability of the current farm decontamination legislation post bTB breakdown as current disinfectants are tested on *M. fortuitum* culture under standard laboratory conditions (Anon., 2016). Under field conditions it is likely that VBNC cells will arise from treated populations of *M. bovis* and that particulate matter will demonstrate a protective effect over the bacilli (as demonstrated in the attempt to culture from faecal material in Chapter 5) resulting in lower efficacy than that demonstrated *in vitro*. Furthermore, if it is demonstrated that the environmental *M. bovis* is present within the environment in the stress-tolerant persister phenotype, it is likely that these cells would be unculturable under standard conditions and therefore go undetected (Garton et al., 2008, Zhang et al., 2012). For farmers who comply with available legislation and have introduced strategies for bTB control yet remain chronically under restriction questions are raised regarding the suitability of the current

strategies. For example, 59% of farms interviewed were having their cattle tested every two months with every test taking an average of 48 hrs labour; the introduction of novel methods and application of knowledge from clinical studies to veterinary and environmental microbiology may provide answers to the question of why it is so difficult to remove infection from some farms (FCN, 2009).

For some farms who wish to increase measures for protection against wildlife harbouring bTB, the implementation of biosecurity strategies can be too costly across large swathes of land. The South-West of England which is also the HRA of England also contains the highest density of badgers and therefore total segregation of cattle from badgers may not be plausible (Judge et al., 2017). Many factors associated with farming encourage the foraging of badgers e.g. the presence of pasture, growth of maize and wheat, and food stores intended for cattle and may be difficult to deter without unfeasibly large alterations to practice (Benham and Broom, 1989, Winkler and Mathews, 2015, Woodroffe et al., 2016). This was the case on the chronically infected dairy farm on which this study was conducted and undertaking this work demonstrated a hotspot of infection and therefore the ability for the farmer to directly alter his practices in relation to this spatial information. Furthermore, the identification of a singular social group with high levels of excretion provided the farmer with peace of mind after his decision not to participate in a wildlife cull on his farm as it was likely that infection would spread and stabilise in other (perhaps wider) areas of the farm (Woodroffe et al., 2006, Jenkins, 2007, Jenkins et al., 2008). It is hoped that in the future, additional surveying and sample testing such as that which was undertaken in Chapter 3 will be available to farmers in order for them to have greater information relating to the state of wildlife infection before committing to any costly biosecurity measures.

A concern surrounding the implementation of a badger cull on the study farm is the knowledge of the ability of *M. bovis* to persist for long periods under British environmental conditions without a host (Chapter 4) (Maddock, 1933, Stenhouse Williams and Hoy, 1930, Anon., 1979). Environmental persistence presents the opportunity for the infection of colonising badger populations by persisting organisms within the soil, setts or latrines of the previous occupants (as highlighted in Chapter 4). This is particularly relevant as culling occurs in the September-November period, resulting in the bacilli excreted prior to culling being under the conditions most-

conducive to their survival, as demonstrated by Chapter 4. The result of persistent bacteria within the environment is the potential infection of naïve colonising individuals in the spring when there is an increase in badger activity and movement prior to the main mating season (Anon., 2015a). This environmental contamination, and the recently destabilised badger population could result in the spread of disease to uninfected areas of the farm or neighbours' farms through perturbation and altered territories farm (Woodroffe et al., 2006, Jenkins, 2007, Jenkins et al., 2008) .

Badger culls are of high cost to farmers, both due to the financial burden placed on the farmers as they must bear the cost of four years of active culling, but also due to the disturbance to proceedings on the farm and potential legal ramifications if guidelines are misinterpreted. In an already struggling industry, in which significant working hours and finances are given over to participating in bTB testing regimes, there needs to be greater clarification of the potential cost-benefit analysis for farmers when they are contemplating engaging within a cull. It is also likely that the uptake of culling, and the pressure placed on neighbouring farmers to cooperate with culling, can detract from efforts and finances that can be channelled towards implementing and encouraging the uptake of biosecurity strategies (Godfrey, 2018). It is thought that the utilisation of a non-invasive monitoring process for the establishment of hotspots of infection could prove to be a cost-effective method of screening farms for their suitability for undertaking culls or to target areas for improved biosecurity relating to preventing cattle-badger contact.

6.5 Statement from the farmer

The farmer and landowner, Mr. Nigel Finch, provided this statement regarding the situation on his farm and the work that we have undertaken:

Woodlands Farm (Chedworth) Ltd 10/8/19 bTB Response

"Our observations following three and a half years observing the work and reports presented to us annually by Sian Powell from the Warwick University Bovine T.B. Research Team.

The sampling programme and area covered were extremely thorough covering 218 ha including 50 ha of dispersed and undulating woodland. Our first encounter with bTB was in 1930 when as producer retailers we felt obliged to have the cows bTB tested for the first time. We tested clear from that time until 1950. In the late 1960's we had four Badger setts and roughly 50 fallow deer. Since 1950 we have only tested clear for two short periods. observation cameras in and around the buildings showed no sign of Badgers at all. All male calves are finished for beef inside their entire life with only one failure (no visible lesions) probably an immune reaction.

Initial observation by Miss Sian Powell had identified some four Badger Setts built into our Cotswold stone banks, largely in the wooded areas, and 150 deer. At the end of the first year we were astounded to learn of the estimated total number of badgers and the fact that one sett had been identified as infected. The following two and a half years confirmed the same basic level, with odd samples showing adjacent to the infected sett. Deer numbers had continued to rise in excess of 250. Earlier Deer culls, some 45 animals annually, had shown several bTB infections, but none in the last two years. Our 12, 18-month cattle most recent post mortem results at slaughter have shown only "Head lesions" and several "no visible lesions".

Question! as "The tuberculin skin test is more sensitive than post mortem it may detect the immune response to bovine TB bacterium". Are we killing immune animals? Also, the head is now always destroyed, and the carcass enters the food chain.

Finally, Sept 2019, our herd replacement numbers have been severely reduced leaving us milking only 60 cows instead of 100, we do not feel it wise to import replacements.

As a result, we have concluded that the sensible approach to the problem with all the enormous evidence we have, would be to eliminate the infected sett entirely and prevent re habitation, however this is impossible without breaking the Law.

Consequently, we are in an impossible situation, the cattle failures have continued with all those cattle in close proximity to the infected sett, none from the clear areas. Therefore, this summer we have not allowed cattle to graze anywhere near the infected sett, and by so doing have had to accept the 6ha loss of crop, in the hope of reducing the risk, only time will tell if worthwhile.

Our awareness and knowledge have benefitted immensely from the research, and we are now much more aware of the risks involved. Our problem is being unable to eliminate the infection problem and by so doing rebuild our cattle numbers to profitability and remove the everlasting disastrous situation facing both cattle and healthy Badgers. The only other way is to give up cattle altogether with the dire consequence that would have on U.K. Soil, Landscape, Human diet and Wildlife.

6.6 Conclusion

The work presented within this thesis has provided a molecular based method by which cells' viability can be assessed within environmental samples, with its primary function thought to be for examining environments in which cells may be persisting in a VBNC state. This work has been supplemented by the development of an IMC method to assess their survival strategies through microscopy as well as their infectiousness capabilities without the requirement for culture-based isolation techniques. These methods can provide us with scope for improving our understanding of environmental transmission, as well as testing and modifying our approaches towards the decontamination of environmentally residing cells rather than testing on laboratory adapted populations of cells.

The fieldwork elements of this research have demonstrated that there are levels of persistence both within badger social groups and within latrines in their local environment. The notion of disease clustering, and the detection of disease clustering through non-invasive methods, could improve the understanding of disease maintenance attributable to the principal environmental reservoir. Within the current framework, this research could be used for farmers to implement testing to identify potential contamination on their holdings or to inform the farmer as to whether a cull would be beneficial to their disease eradication strategy. Furthermore, the ability to track levels of contamination within the environment gives a quantifiable result by which to measure the success of culls in controlling the environmental reservoir.

An increased understanding of how *M. bovis* is maintained and transmitted within and between badger social groups could be used to update policy and control methods. For example, if disease clustering is found to not be a local phenomenon, culls of social groups excreting *M. bovis* in their faeces could provide a more long-term and more effective strategy. This would require supplementary updates to long standing policy surrounding the protection of badger setts, as it is suggested that the setts associated with infected social groups, and their latrines, are decontaminated or destroyed to prevent the potential transmission to newly colonising animals.

It is hoped that the combination of the information presented within this thesis will be useful to not only the farmer with whom the work was conducted, but to other farmers experiencing chronic bTB breakdowns and are presented with 'the badger problem'. Furthermore, it is hoped that the techniques optimised within this thesis have scope for future application and enhancing not only our knowledge of *M. bovis* but also to facilitate the improvement of current control strategies.

7.0 References

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8.0 Appendices

8.1 Appendix A

Table A1: Co-ordinates of the main points identified on the farm

Name	Type	Lat	Long
Big Tree Ext	Satellite	51.8146476	-1.975183
Extension	Main	51.8149554	-1.9750363
Crater	Satellite	51.8151915	-1.9756336
Wood road strip	Satellite	51.8133319	-1.9511825
Pinswell wood RHS	Satellite	51.8140069	-1.9540578
Pinswell field	Satellite	51.8137972	-1.9532961
Pinswell wood LHS	Main	51.8139962	-1.9554311
Spring source	Satellite	51.8172572	-1.9740224
Fallen trees	Satellite	51.818008	-1.9742262
Nettle	Satellite	51.8179841	-1.9748914
Sycamores	Main	51.818707	-1.9761467
Valley	Satellite	51.8180485	-1.9762633
Quarry	Satellite	51.8109204	-1.9541095
Far Fields	Satellite	51.8202511	-1.9799768
Road bends	Satellite	51.813522	-1.9546458
In tree	Satellite	51.8145913	-1.9683948
Dec-pine-spring	Satellite	51.8175354	-1.9737043
Quarry down	Satellite	51.8105173	-1.9544555
Qurry Pentagon	Satellite	51.809075	-1.9545735
Quarry far	Satellite	51.8080083	-1.9545301
Powerline field	Satellite	51.8091993	-1.9578494
X-woods	Satellite	51.8143785	-1.9751038
X-woods2	Satellite	51.8132443	-1.9738056
Quarry main	Satellite	51.8083523	-1.9545802
Under birch	Satellite	51.8209943	-1.9736492
Fallen Tree	Latrine	51.8147556	-1.9754469
Tree-mound	Latrine	51.8147561	-1.9756937

Top-right	Latrine	51.8145213	-1.9747226
Inbetween	Latrine	51.8149516	-1.9752484
Mid-top	Latrine	51.8151196	-1.9748782
Cow-wood-edge	Latrine	51.814893	-1.9688058
Wood pile	Latrine	51.8143884	-1.9682591
Crater	Latrine	51.8153306	-1.9760983
Wrongun	Latrine	51.8145846	-1.9764274
Sheep Fence Line	Latrine	51.8143795	-1.971084
Long Drop	Latrine	51.8150133	-1.9751392
Old oak to birch	Latrine	51.8184346	-1.9746342
Nettle Latrine	Latrine	51.8182908	-1.9750895
Sycamore to birch	Latrine	51.8184241	-1.9757832
SW of big wood	Latrine	51.8170181	-1.9762767
Tractor trail	Latrine	51.8152329	-1.9711402
Roadside-strip	Latrine	51.8132148	-1.9510485
Sycamores Long Drop	Latrine	51.8188927	-1.9759321
Field slope	Latrine	51.813857	-1.9533583
Pinswell wood 1	Latrine	51.8140078	-1.9553905
Pinswell wood point	Latrine	51.8146017	-1.9556163
Sycamore-fields	Latrine	51.8155077	-1.9525837
Fieldline	Latrine	51.8165086	-1.9688785
Farfieldline	Latrine	51.8205804	-1.9798821
Powerline	Latrine	51.8114633	-1.954488
Quarry Wood	Latrine	51.8082315	-1.954407
New Quarry-wood	Latrine	51.8082659	-1.9548113
Quarry Hawthorn	Latrine	51.8081121	-1.9543426
Scrub North	Latrine	51.8180399	-1.9512804
Scrub South	Latrine	51.8177746	-1.9513126
Top wood Y	Latrine	51.8204077	-1.9570805
Pathside top wood	Latrine	51.820779	-1.9573058
Three Corners	Latrine	51.8161008	-1.9707728

8.2 Appendix B

Meteorological conditions

In the following subsection, the results will focus on the weather experienced over the sampling seasons, and how these potentially influenced the decline in *M. bovis*.

The daily mean temperature is demonstrated in Figure B1 below, with autumn averaging 6.9 °C (σ = 3.4 °C, n=1057) spring at 14.0 °C (σ = 3.9 °C, n=1081) and summer at 14.9 °C (σ = 3.6 °C, n=1033).

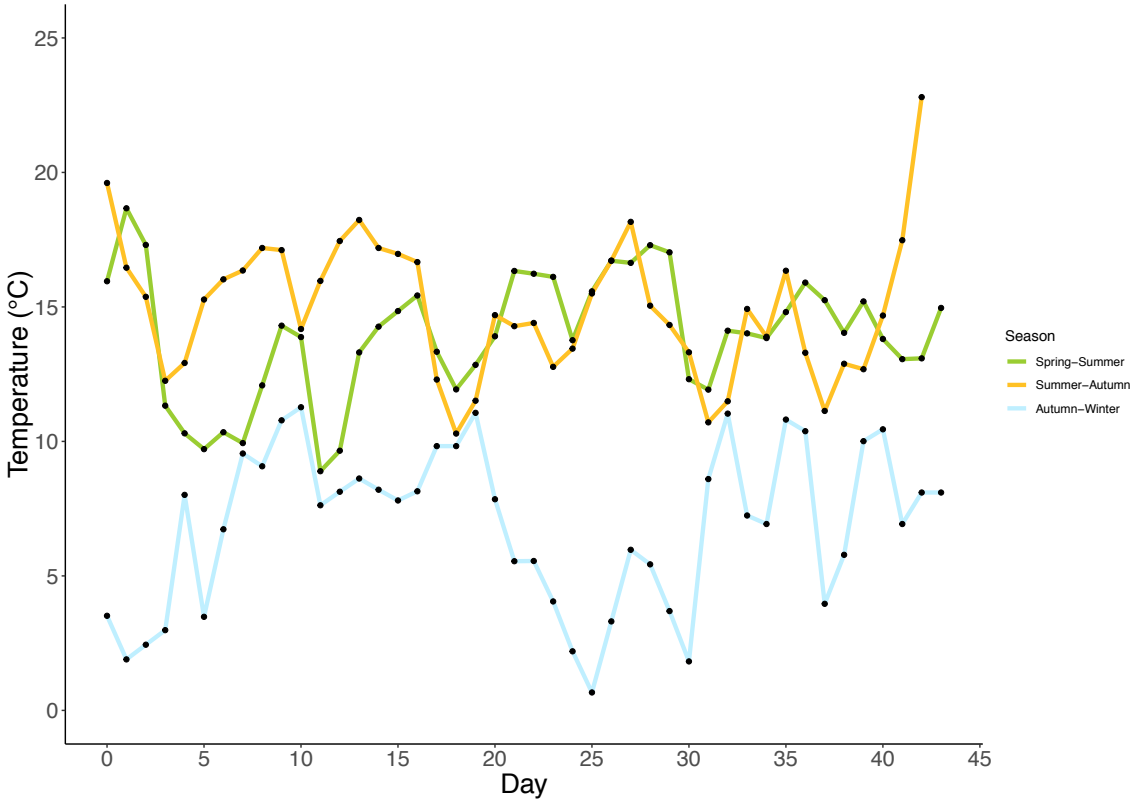

Figure B1: Daily mean temperature over the sampling periods.

Soil temperature analysis was measured at a depth of 10 cm as the local weather station does not acquire values for depths of 5 cm which would have been the preferred depth. The daily mean soil temperature is demonstrated in Figure B2 below, with autumn averaging 7.5 °C (σ = 1.7 °C, n=1057), spring at 17.6 °C (σ = 2.6 °C, n=1081) and summer at 17.7 °C (σ = 2.8 °C, n=1033).

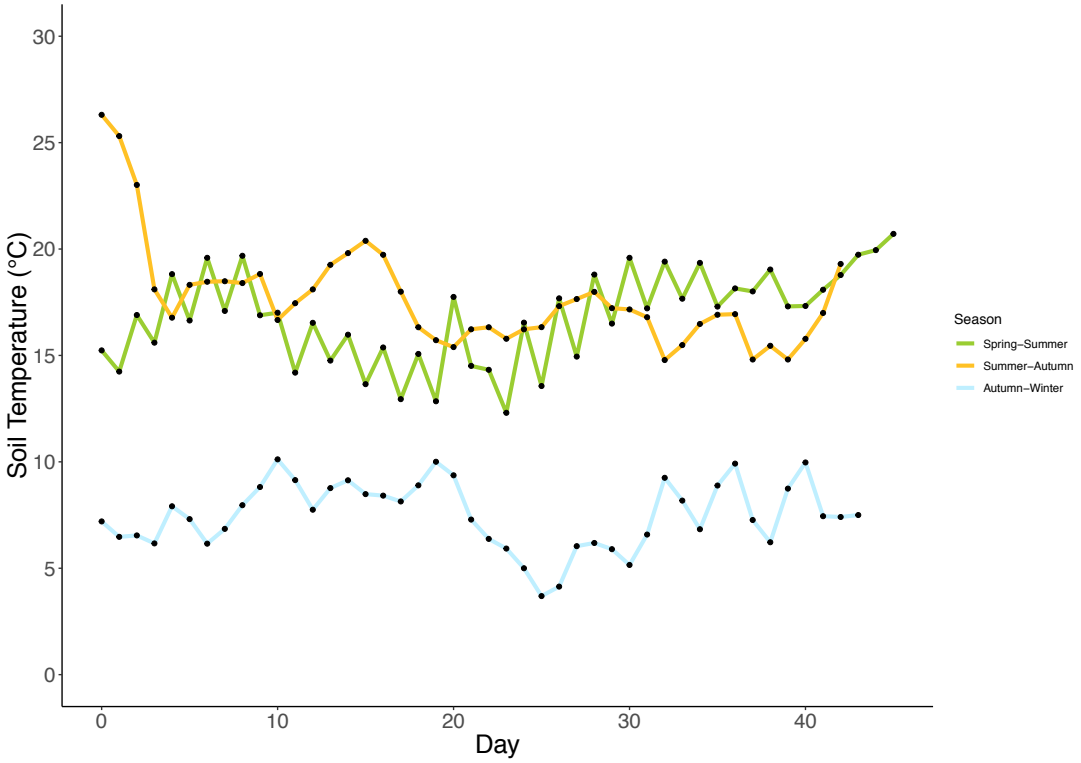

Figure B2: Mean daily soil temperature over the sampling periods.

The mean daily relative humidity was calculated from the hourly data measurements, with spring having a mean relative humidity of 77.9 % (σ = 16.3%), summer of 80.1% (σ = 14.6%), and autumn of 91.8% (σ = 8.3%) (Figure B3).

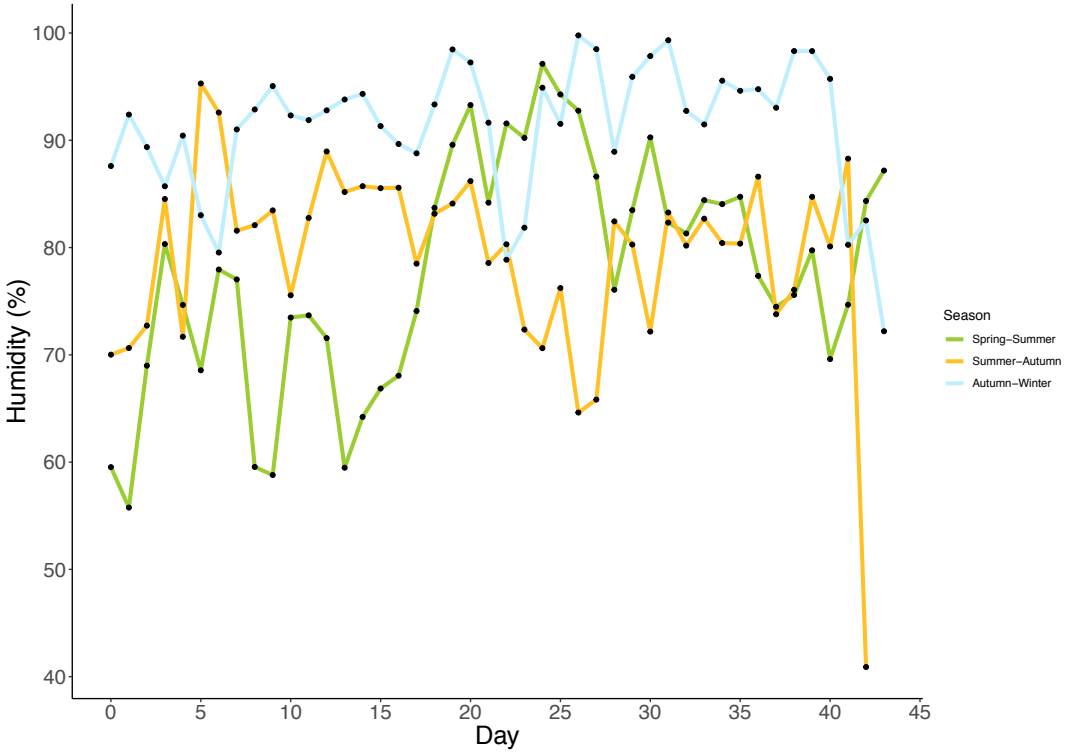

Figure B3: Daily mean humidity over the sampling periods.

The mean sum of daily rainfall was calculated from the rainfall data (Figure B4). The average daily rainfall in spring was 4.2mm (σ = 9.5mm), summer was 3.2 mm (σ = 4.3mm), autumn was 6.1mm (σ = 6.9mm).

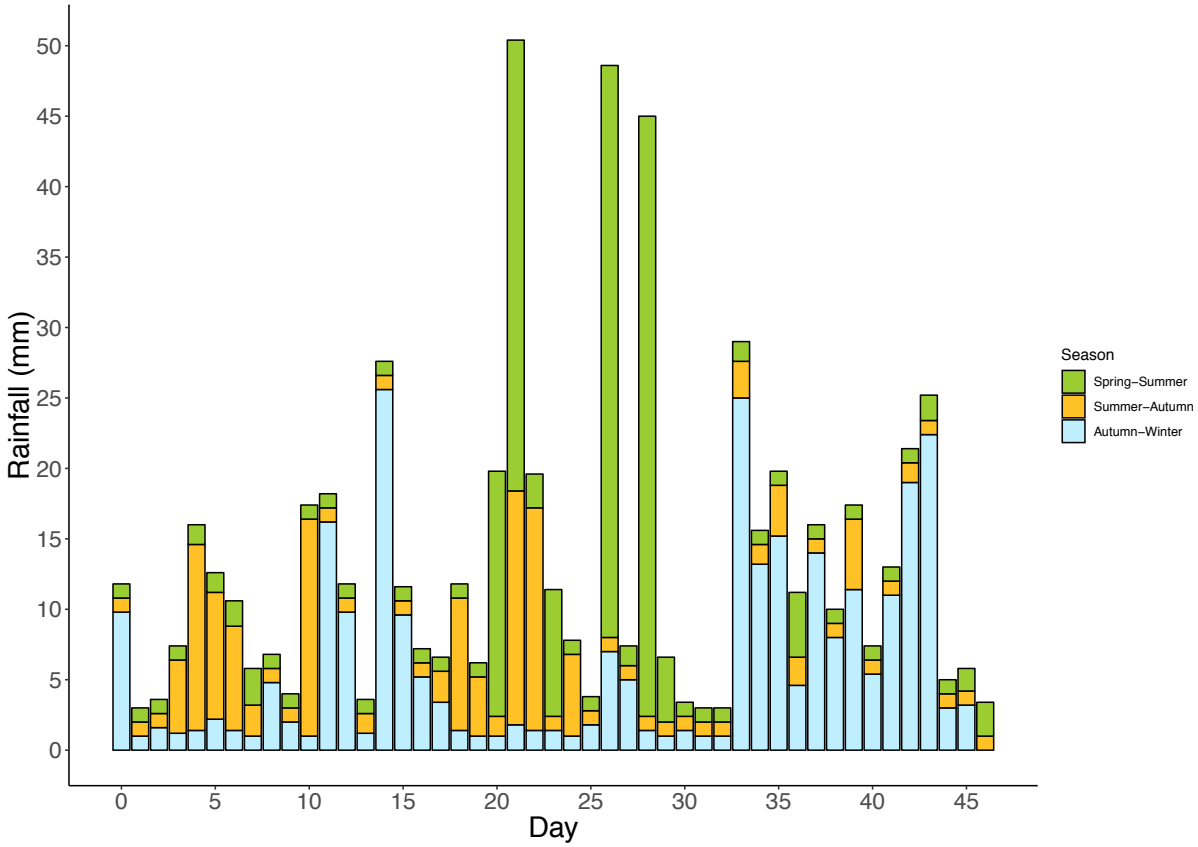

Figure B4: Sum of daily rainfall over the sampling periods.

The daily mean dew point is demonstrated in Figure B5 below, with autumn averaging 5.6 °C (σ = 3.65°C, n=1057), spring at 9.8 °C (σ = 3.4 °C, n=1081) and summer at 11.1 °C (σ = 3.0 °C, n=1033).

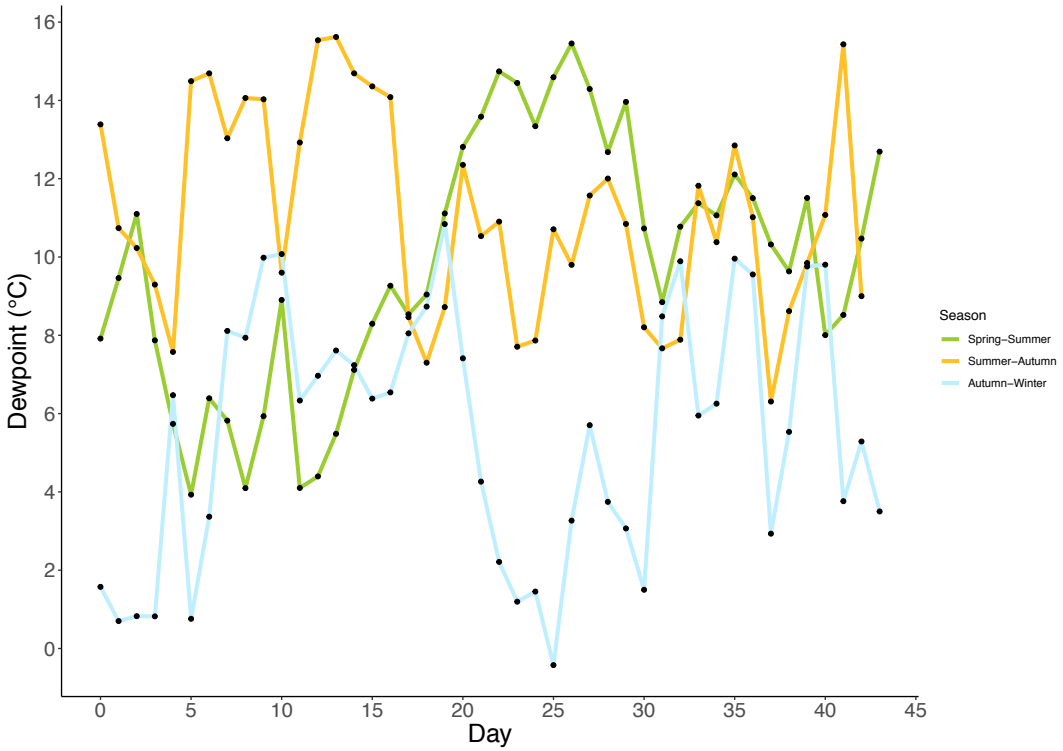

Figure B5: Mean daily dew point over the sampling period.

The duration of sunshine (in minutes) to which the samples were exposed was also measured for every hour of the 24-hour period (Figure B6). The samples were exposed to a greater average amount of sunlight in the spring with a mean sunshine duration of 452.8 minutes (σ = 292.0), in the summer they were exposed to an average of 259.6 minutes (σ =206.6), and in the autumn of 150.8 minutes (σ = 139.2). Hourly sunshine duration values are calculated from the MMS global radiation minute values by a formula which determines whether each minute classifies as 'sunshine'. Overall, no difference was attributable to mean sunshine duration over the 24-hour period.

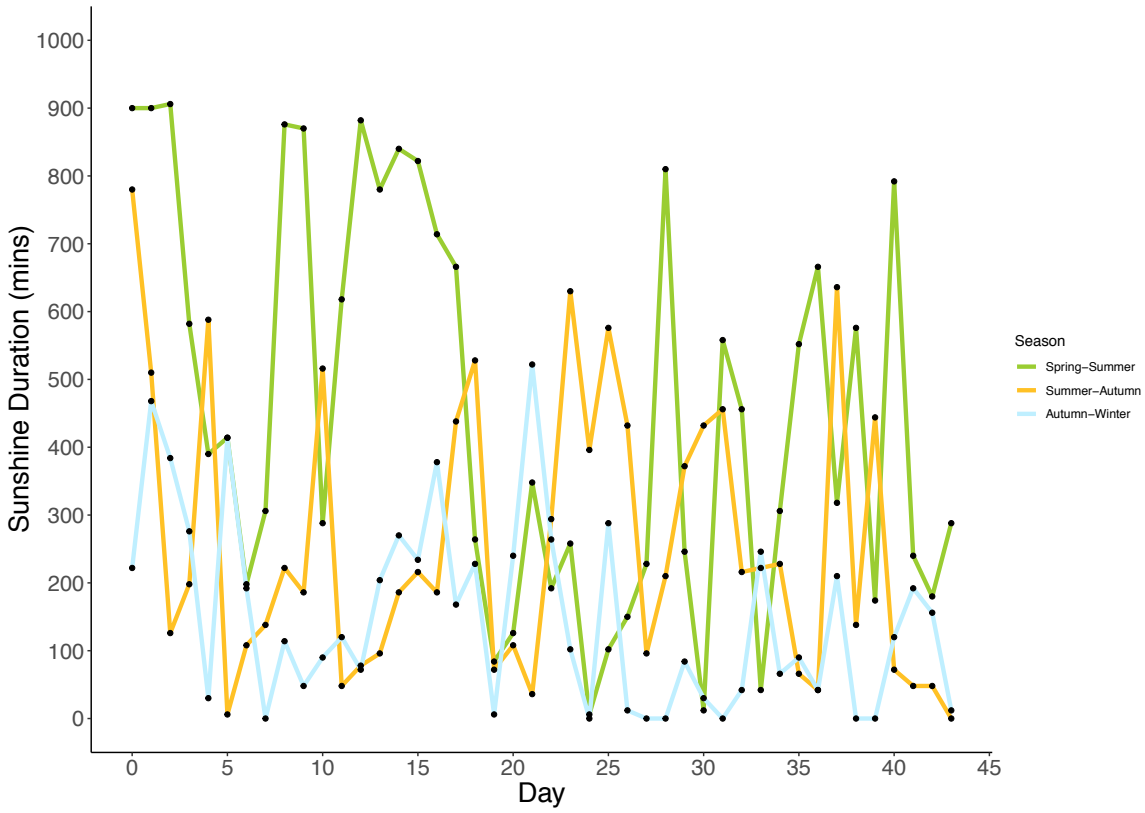

Figure B6: Sum of daily sunshine duration over the sampling periods.

The level of solar radiation was extracted to determine whether this was an important factor when it came to the decline in *M. bovis* genome equivalents. The mean values were as follows: for spring it was 2.6 kWh/m^2 (σ = 2.9 kWh/m^2); for summer it was 1.7 kWh/m^2 (σ = 2.1 kWh/m^2) and for autumn it was 0.5 kWh/m^2 (σ = 0.8 kWh/m^2). The figure below illustrates the maximum levels of UV that the samples were exposed to within that 24-hour period.

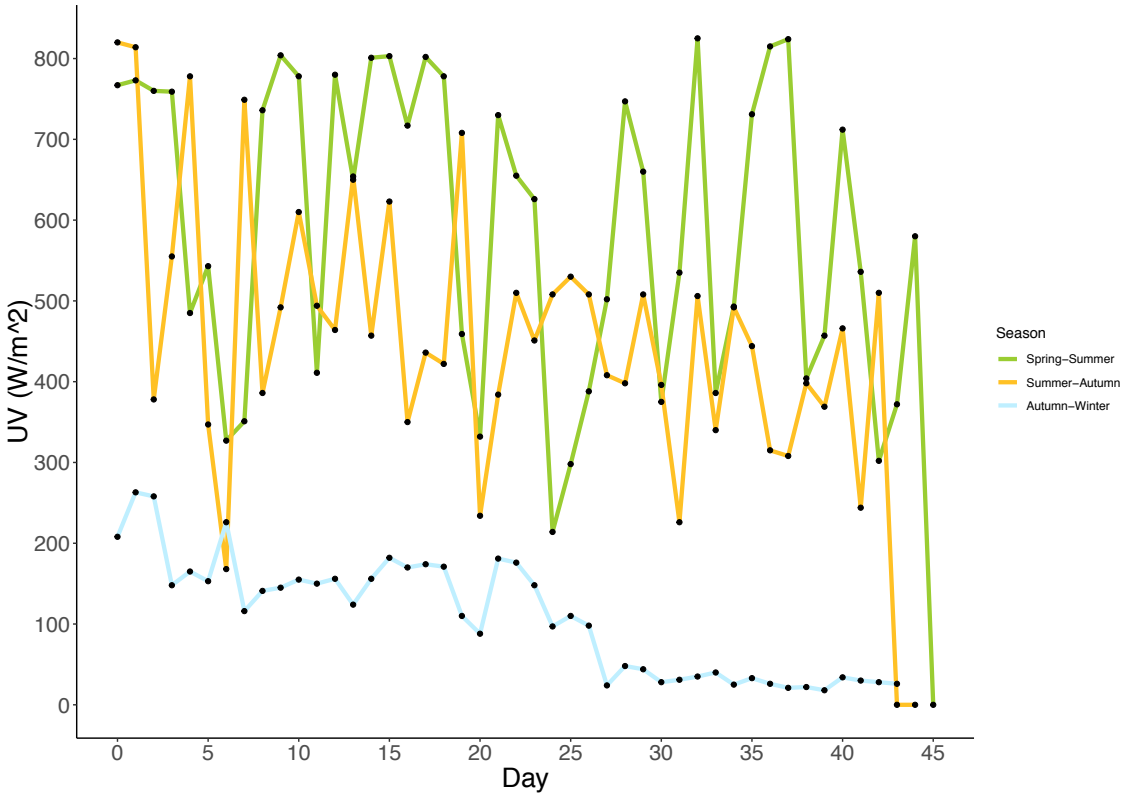

Figure B7: Maximum daily UV radiation over the sampling period.

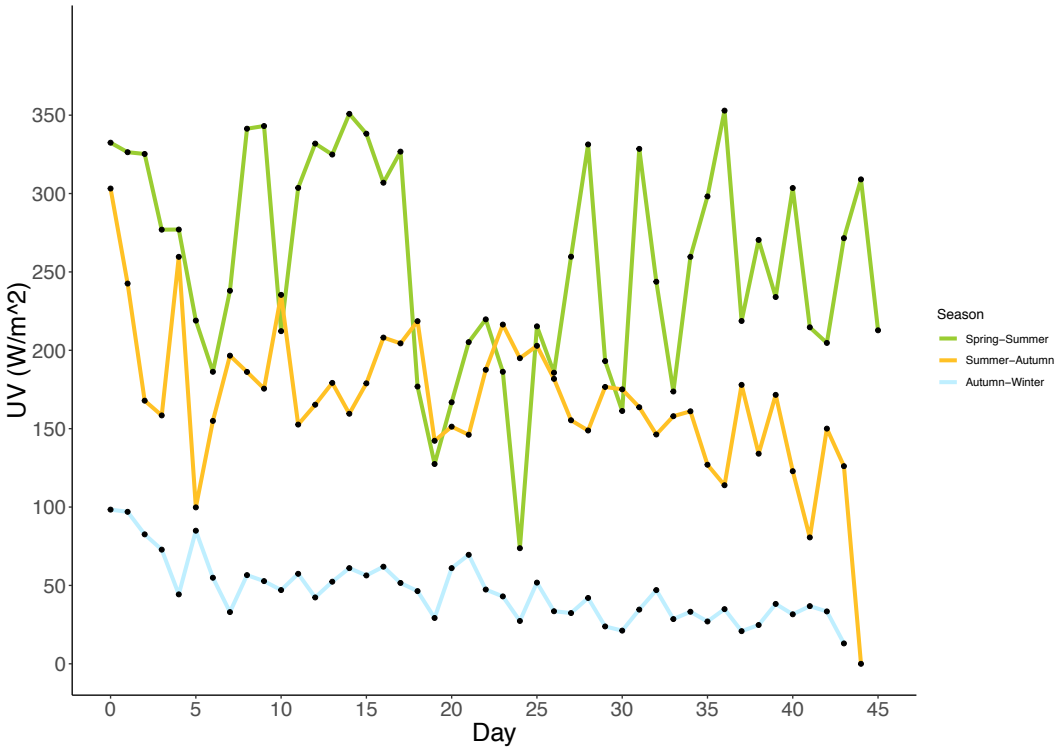

Figure B8: Mean daily UV radiation over the sampling period.