

BETTER WATER QUALITY

INDICATORS FOR

UNDERSTANDING MICROBIAL

HEALTH RISKS

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Abstract

The aims of the research were to evaluate existing microbial water quality indicators, and refine and/or develop alternative, improved indicators for determining the source of faecal contamination in urban and rural surface waters. There has been concern that because *E. coli* is capable of long term persistence in the environment in temperate climates that it is no longer a valid frontline tool for water quality monitoring. This research explored urban (untreated human sewage) and rural (cow faeces) impacts on water quality, and investigated relationships between faecal source tracking (FST) markers, faecal ageing determinants, microbial indicators and pathogens. The variables measured were FST markers (quantitative Polymerase Chain Reaction (qPCR), and faecal steroids), the faecal ageing ratio of atypical colonies/total coliforms (AC/TC), and the indicator microorganism, *Escherichia coli*. In the urban river study, additional determinants were indicator microorganisms, *Clostridium perfringens* and F-RNA phage; potential pathogens belonging to the genera of *Campylobacter*, *Giardia* and *Cryptosporidium*, and the FST marker, fluorescent whitening agents (FWA).

In the urban study, a river had been impacted by major discharges of untreated human sewage. Variables were monitored in the river water and underlying sediment at three locations both during discharge, and up to eighteen months post-discharge. Relationships between *E. coli* and potential pathogens in water demonstrated that *E. coli* was a reliable indicator of public health risk. As a signal of a recent human faecal input, F-RNA phage were identified as suitable, cost-effective indicators to be measured in conjunction with *E. coli*. In contrast, the ubiquitous *C. perfringens* was observed to accumulate in sediments, confounding its ability as an indicator in water. PCR markers and faecal steroids in water were similar and even superior to *E. coli* as predictors of protozoan pathogen presence, and hence indicative of human health risk. The faecal ageing ratio, AC/TC in water, was significantly, negatively correlated with increasing pathogen detection. *Campylobacter* had the weakest associations with all microbial and FST indicators. It was observed, however, that where elevated *E. coli* levels were detected in water, identification of the HumM3 PCR marker in conjunction with F-RNA phage and a low AC/TC ratio <1.5 was indicative of fresh pollution and an associated health risk from *Campylobacter*. River sediments appeared to be a reservoir for steroids and FWA, *Cryptosporidium* and *Giardia* but not *Campylobacter* or F-RNA phage. FST PCR markers were not assayed in the sediments. There was no relationship observed between chemical FST markers in sediments and the overlying water, and few correlations between chemical FST markers and target microorganisms in sediment.

In the rural study, the decomposition of cowpats was investigated to determine the mobilisation rates of water quality determinants when irrigated and non-irrigated cowpats were

subjected to simulated flood and rainfall runoff events. It was observed that decomposing cowpats harboured concentrations of *E. coli*, which were available for mobilisation after flood and rainfall events for at least five and a half months post-deposition under flood conditions, and for at least two and a half months after lighter rainfall. Persistent levels of total coliforms in ageing cowpats showed that AC/TC ratios would indicate fresh sources of faecal contamination in a waterway after flood conditions up to four months post-deposition. An amplicon-based metagenomic study of the ageing cowpat investigated shifts in microbial populations as the cowpat decomposed. Major bacterial community shifts were observed over 161 days in the mobilised fraction from decomposing cowpats. Dominant bacteria that inhabited the cow rumen and fresh faeces, such as a *Ruminococcus* species, were displaced by bacterial groups that could be utilised as potential PCR targets of aged bovine faecal sources. Faecal steroid ratios were observed to be reliable and stable FST markers during the ageing process. The PCR marker ratio of BacR/TotalBac (ruminant (BacR)/Total Bacteroidetes) has potential as an indicator of 100% contribution from fresh bovine sources.

Recommendations for water managers are outlined for the cost-effective application of FST tools based on findings from this current research. The differential fate and transport of microbial and FST markers noted in this research supported the use of multiple lines of evidence through application of a cohort of indicators for tracking the source(s) of faecal contamination and indicating the associated public health risk. In the urban river study, strong to moderate correlations between PCR and steroid markers suggested they could be used individually or combined for greater confidence in the result. Some of the FST host-associated PCR markers (HumM3 and CowM2) were shown to be useful indicators of recent faecal inputs to a waterbody. The lack of correlation between chemical FST markers and microorganisms in sediment suggested that chemical markers in sediment were indicative of historical faecal sources, and restricted their predictive value for health risks. Due to the persistence of potential pathogens, re-suspension of sediment has the potential to increase risk to human health for those who participate in recreational and work activities in the river environment. It is suggested that where runoff from non-flood conditions may confound water quality monitoring, application of the Bacteroidales host-associated PCR markers would be preferable to the more persistent *E. coli*. In addition, AC/TC testing should only be performed during baseflow conditions. The sequence information generated from the cowpat metagenomic study could be used for development of a metagenomic FST library of bacteria. Mobilisation rates of FST markers from cowpat runoff determined in this rural study can contribute to models designed to apportion contamination from agricultural sources.

Abbreviations

α -diversity	Alpha diversity
AC/TC	Atypical Colonies/Total Coliforms
β -diversity	Beta diversity
BS	Boatsheds
CBD	Central Business District
CDC	Communicable Disease Centre
CFU	Colony forming units
Cp	Cycle threshold (in qPCR)
CV	Coefficient of variation
ddPCR	Droplet Digital PCR
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
dw	Dry weight
EDCs	Endocrine-disrupting chemicals
EMA	Ethidium monoazide
EPA	Environmental Protection Agency
ESR	Institute of Environmental Science and Research Ltd.
FC	Faecal coliforms
FIB	Faecal indicator bacteria
FITC	Fluorescein isothiocyanate
FST	Faecal source tracking
FWA	Fluorescent whitening agents
<i>g</i>	Gravitational force
GC	Gene copies
GCMS	Gas Chromatography Mass Spectrometer
GI	Gastrointestinal illness
GR	Global radiation
HPLC	High Pressure Liquid Chromotography
HUS	Haemolytic uraemic syndrome
IRR	Irrigated (cowpat supernatant)
IUPAC	International Union of Pure and Applied Chemistry
KR	Kerrs Reach
LOD	Limit of detection
LOQ	Limit of quantification
m-endo agar	Modified endo agar
MPN	Most probable number
mtDNA	Mitochondrial DNA
MST	Microbial source tracking
m/z	Mass to charge ratio
NGS	Next generation sequencing
NIR	Non-irrigated (cowpat supernatant)
NIWA	National Institute of Water and Atmospheric Research
NTU	Nephelometric Turbidity Unit

NZ	New Zealand
OT	Owles Terrace
OTU	Operational taxonomic units
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing 0.1% of Tween 20
PCR	Polymerase chain reaction
PCA	Principle component analysis
PCoA	Principle co-ordinate analysis
PFU	Plaque forming unit
PMA	Propidium monoazide
QIIME	Quantitative insights into microbial ecology
qPCR	Quantitative polymerase chain reaction
r^2	Coefficient of determination
RDP	Ribosomal Database Project
RNA	Ribonucleic acid
RWQC	Recreational water quality criteria
r_s	Spearman Ranks correlation
RT-PCR	Reverse-transcriptase polymerase chain reaction
SA:V	Surface area to volume ratio
SD	Standard deviation
SIM	Selected ion monitoring
S/N	Signal to noise ratio
sp.	Species
TC	Total coliforms
T_{90}	Time taken for a one log reduction in microbial concentration
UniFrac	Unweighted unique fraction
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
ww	Wet weight

Units used in this thesis

kg	kilograms	km	kilometres
g	grams	cm	centimetre
mg	milligrams	mm	millimetres
μg	micrograms	s	second
ng	nanograms	min	minute
L	litres	h	hour
mL	millilitres	$^{\circ}\text{C}$	degrees Celsius
μL	microliters	mS	milliSiemens
MJ	mejjoules		

Steroid abbreviations

Cop	coprostanol	24-E-epicop	24-ethylepicoprostanol
Cholestan	cholestanol	24-Mcholesterol	24-methylcholesterol
24-Echolestan	24-ethylcholestanol	Stig	Stigmasterol
Epicop	epicoprostanol	Chol	cholesterol
24-Echolesterol	24-ethylcholesterol	24-Ecop	24-ethylcoprostanol

1 Chapter One

Introduction

1.1 Faecal pollution and its effects

1.1.1 What is the nature of faecal pollution?

The intestinal tracts of mammals and birds contain microorganisms belonging to the protozoa, viruses, fungi and bacteria (Arumugam et al., 2011; Halaihel et al., 2010; Hundesa et al., 2006; Ott et al., 2008; Pallen, 2011; Zhou et al., 2004). It has been suggested that the naturally present microbial community in humans, termed the human microbiome, is tenfold more numerous than the number of cells in the human host. The bacterial community of the large bowel, for example, is the most abundant community inhabiting humans, having 10^{12} bacterial cells per gram of the intestinal contents (Pallen, 2011). Most of these bacteria are an inherent part of the microbiome and do not cause disease in the individual host (they are termed commensal). They are recognised as an important asset for healthy functioning (Falk et al., 1998), including helping to maintain the integrity of the mucosal membranes that line the intestine, and supporting the immune system by preventing infection by disease-causing organisms (pathogens). The most numerous group of bacteria in the human intestine, the Bacteroidales, produce enzymes that breakdown complex polysaccharides into simple sugars for absorption by the host (Pallen, 2011). In addition, intestinal microbes can synthesise nutrients such as biotin, folic acid and vitamin K, supplementing the dietary intake of the host.

The solid waste excreted by an animal from the digestive tract is termed faeces and includes a significant portion of the microbial population, which inhabited the intestine. If the host is infected with a pathogenic microorganism, then this species will also be excreted in faeces. For example, an infection by a virus causing gastroenteritis, such as rotavirus or norovirus, can result in 10^{10} viral infectious units per gram of faeces, with excretion occurring for periods of one to four weeks after an infection (Leclerc et al., 2002).

1.1.2 Faecal pollution in water and its effects on human health and ecosystems

The input of faeces into a waterbody can be through direct deposition from animals including birds, or a discharge of sewage or wastewater directly into the water. Indirect inputs can occur as overland run-off from faecal deposits on land, particularly during rainfall events (Shehane et al., 2005) and links between heavy rainfall and waterborne illness have been identified (Curriero et al., 2001). Waterborne illnesses are those transmitted through the consumption of water, where

water acts as the vector of pathogens (Leclerc et al., 2002). Ingestion of water includes its inadvertent consumption during recreational and work activities conducted in or on water.

Table 1 outlines the microbial pathogens that have been identified as the more common causal agents in waterborne outbreaks. Waterborne clinical disorders can be categorised as either infection by the microorganisms leading to asymptomatic infection; or disease where infection is accompanied by symptoms of illness such as vomiting and diarrhoea (Leclerc et al., 2002). Animal hosts who are infected without symptoms are, however, potential carriers providing a host for replication and acting as disseminators of disease. Therefore, it is appropriate to refer to

Table 1: Examples of microorganisms identified as the primary cause of waterborne disease outbreaks in freshwater (Baldursson and Karanis, 2011; Heymann, 2008; Hlavsa et al., 2014; Janda and Abbott, 2010; Leclerc et al., 2002; Neogi et al., 2014; Schets et al., 2011; Staff and Association, 2006)

	Microorganism	Symptoms	Secondary symptoms
Bacteria faecally-derived	Pathogenic <i>E. coli</i> (e.g. <i>E. coli</i> O157:H7)	Gastroenteritis	haemolytic uremic syndrome
	<i>Campylobacter jejuni</i> , <i>C. coli</i>	Gastroenteritis	Guillian Barré
	<i>Salmonella</i> strains	Gastroenteritis	
	<i>Shigella</i> sp.	Gastroenteritis	
	<i>Vibrio</i> sp. (e.g. <i>V. cholerae</i>)	Gastroenteritis	Ear infection, wound infection
	<i>Yersinia enterocolitica</i>	Gastroenteritis	
Bacteria identified in urine of animals	<i>Leptospira</i> sp.	Wide range of symptoms including fever, headache, and muscle pain	Respiratory illness, meningitis, encephalitis, kidney and liver disease
Bacteria that are natural inhabitants of aquatic environments	<i>Legionella pneumophila</i>	Respiratory illness	
	<i>Aeromonas</i> sp.	Gastroenteritis, wound infections	Respiratory illness
	<i>Mycobacterium</i> sp.	Respiratory illness	
	<i>Arcobacter butzleri</i>	Gastroenteritis	
	<i>Pseudomonas aeruginosa</i> <i>Listeria</i> sp.	Ear infections Gastroenteritis, septicemia, meningitis, spontaneous abortion	Respiratory illness
Protozoa	<i>Giardia</i> sp.	Gastroenteritis	
	<i>Cryptosporidium</i> sp.	Gastroenteritis	
	<i>Entamoeba histolytica</i>	Gastroenteritis	
	<i>Cyclospora cayetanensis</i>	Gastroenteritis	
	<i>Naegleria fowleri</i>	Meningoencephalitis	
Viruses	Hepatitis A	Gastroenteritis	
	Adenovirus	Gastroenteritis, conjunctivitis	Respiratory illness
	Norwalk	Gastroenteritis	
	Rotavirus	Gastroenteritis	

waterborne infections as those which include infection only, and infection with symptoms of illness. An outbreak of waterborne illness is defined by the Communicable Disease Centre (CDC) as the occurrence of a similar illness in at least two people whose cases are linked by epidemiological evidence of exposure to recreational or drinking water (Leclerc et al., 2002).

There are differences in the number of microorganisms required to initiate an infection. Bacteria such as *Salmonella* require approximately 10^4 organisms to cause infection in humans, however pathogenic *E. coli* strains have lower infectious doses with an estimation of half of a population becoming infected (ID_{50}) after ingesting 750 *E. coli* (McBride et al., 2012). In comparison, <100 infectious organisms can cause disease for both viruses and protozoa (Leclerc et al., 2002; McBride et al., 2012). Many microbes will form clumps or clusters of organisms, which increases the variability of transmission, in that one person can ingest a large number of pathogens whereas others ingest few to none (Gale, 1996).

Examples of waterborne outbreaks

It is commonly acknowledged that cases and outbreaks of waterborne infection are vastly underreported as they are often not recognised as an outbreak (Poullis et al., 2005). The United States Environmental Protection Agency (USEPA) estimated the numbers of acute gastrointestinal illnesses (GI) attributed to community drinking water systems as approximately 8.5% of total GI, which equated to approximately 16.4 million cases per year (Weintraub and Wright, 2008).

There have been multiple instances of faecally contaminated water causing episodes of waterborne illness from drinking water supplies and/or recreational water contact. Some of the worst examples include the Walkerton incident in Ontario, Canada, where more than 2,000 people succumbed to illness due to *E. coli* O157:H7 and *Campylobacter* contamination of drinking water (O'Connor, 2002). This incident resulted in seven deaths from complications associated with *E. coli* O157:H7 infection such as haemolytic uraemic syndrome (HUS). The largest waterborne outbreak in a developed country occurred in Milwaukee, Michigan, USA in 1993 when approximately 400,000 people were affected and 600 clinical specimens of *Cryptosporidium* from individual cases were confirmed (MacKenzie et al., 1994). An outbreak attributed to recreational activity occurred at a waterpark in South Korea where groundwater was identified as the probable transmission route for norovirus, which infected a school party visiting the recreational complex (Koh et al., 2011). In France, in 2000, a local community was affected by faecal contamination of groundwater used for drinking water, and multiple pathogens

(*Campylobacter coli*, norovirus and rotavirus) were detected in patient stools, including as co-infections (Gallay et al., 2006).

In New Zealand (NZ), there was a large waterborne outbreak of gastrointestinal illness at a skifield, where 218 people were affected by symptoms ascribed to norovirus (Bartholomew et al., 2014). An outbreak of campylobacteriosis in a small rural township of Darfield, NZ, occurred in 2012, where 29 cases were confirmed as due to *C. coli* or *C. jejuni* and one case due to *Giardia*, while a further 138 cases were defined as probable cases of gastroenteritis. This episode was linked to the failure of the township's drinking water supply after a period of heavy rainfall (Bartholomew et al., 2014). Contamination was suspected to result from unprotected bore well heads in paddocks where sheep grazed, or from pasture runoff into the river from which the well drew source water. Faecal specimens from local sheep were identified as carrying subtypes of *Campylobacter* that were closely related to those identified in clinical specimens.

Impacts of faecal waste applied to land

In NZ and internationally, one practice for biological treatment of agricultural faecal waste is application of animal effluent onto land (Baker and Hawke, 2007; Sobsey et al., 2006; Wright, 2012). Effluent application has beneficial effects for waste disposal and nutrient recovery by reducing the need for other fertilisers, and decreasing direct discharge of waste and water to rivers and lakes (Wang et al., 2004). However, there have been concerns about the detrimental effects because animal effluent may contain pathogens, heavy metals and low levels of endocrine-disrupting chemicals (EDCs) such as oestrogen (Sobsey et al., 2006; Wang et al., 2004). EDCs alter hormone function by mimicking or interfering with the biosynthesis of the organism's own hormones, and as such have been implicated in disruption of the reproductive cycle of wildlife and humans (Bai et al., 2013; Dickerson and Gore, 2007; Handelsman et al., 2002; Jobling et al., 1998; Schug et al., 2011) including the feminisation of fish species (Jobling et al., 2009; Johnson et al., 2006; Matthiessen et al., 2006; Wei et al., 2011).

It is important that the proper procedures relating to residence time of agricultural faecal waste in stabilisation ponds is followed before dispersal on to land (Wang et al., 2004). Incorrect timing and rates of liquid effluent application on to land can lead to waterlogging of soils resulting in runoff of nutrients and pathogens to shallow groundwater aquifers which can directly impact drinking water sources (Baker and Hawke, 2007). High levels of nitrate in drinking water has caused the "blue baby syndrome" where ingested nitrate is converted to nitrite in the body, which combines with haemoglobin in the blood to form methaemoglobin

(Majumdar, 2003). The methaemoglobin has a reduced capacity to carry oxygen, leading to oxygen starvation and the blue colouration of skin and lips, hence the syndrome's name.

The rise of pathogen resistance to antimicrobial agents has been an area of increasing concern to public health officials, and exacerbated by the limited discovery of new antimicrobials to combat infectious disease (Xin et al., 2015). In many countries, in addition to human medicine, antimicrobials are used in agricultural practices to promote the growth of livestock, and to prevent and treat disease in animals and poultry. There is concern about the dissemination of antibiotic resistant bacteria, particularly, the prevalence of clinically relevant bacteria resistant to multiple antibiotics (Cameron-Veas et al., 2015). Studies have identified a high level of resistance to the antibiotics tetracycline and ampicillin by *E. coli* isolated from sediment and water samples impacted by urban runoff and agricultural practices (Ibekwe et al., 2011), and from the faeces of farm and feral animals (Nhung et al., 2015). These two antibiotics are commonly used in farm husbandry practices and are important in clinical settings.

Ecological effects from faecal inputs

Eutrophication is a natural ageing process in waterbodies whereby the ecosystem becomes enriched with nutrients over time. This process, however, can be accelerated by inputs from sources of faecal pollution (Ryding and Thornton, 1999; Thornton et al., 2013). The ecological effects of eutrophication can result in the establishment of nuisance aquatic species such as algae and aquatic plants (Dorgham, 2013; Ryding and Thornton, 1999). The high levels of nitrogen and phosphorus sources found in faeces and wastewater facilitate their excessive growth. The die-off of the algae and plants can then result in large masses of decaying organic matter with attendant episodes of oxygen depletion (Khan and Mohammed, 2013). Other detrimental effects from algae include the production of algal toxins, for example, microcystin, which can cause mortality in terrestrial and marine animals (Ryding and Thornton, 1999).

Human waste and agricultural sewage inputs can influence the microinvertebrates such as protozoa, nematodes and some insect larvae that inhabit aquatic ecosystems like wetlands, lakes and streams (Neogi et al., 2014). Microinvertebrates can have a negative influence on the numbers of bacteria in an ecosystem by the forces of predation, with known high grazing rates for protozoa of up to 2000 bacteria ingested per hour (Macek et al., 1997). However, some bacteria, including pathogens, have developed mechanisms to evade predation (Sun et al., 2013). Evidence now suggests that protozoa such as amoeba can act as vectors of various bacteria including the pathogens *Legionella*, *Campylobacter* and *E. coli* (Buse and Ashbolt, 2011; Greub and Raoult, 2004; Ji et al., 2014; Thomas, 2013). These bacteria survive and grow within the

microinvertebrate by evading the host immune system (Neogi et al., 2014). This causes concern because the protozoa and their intracellular bacterial companions are resistant to the doses used in chlorination of drinking water sources (Codony et al., 2012; King et al., 1988). In addition, ingestion of microinvertebrates by avian species can provide a transmission route for dissemination of pathogens (Neogi et al., 2014). Nematodes and stream-dwelling macroinvertebrates have been observed to feed on and produce viable and infective forms of the oocysts of *Cryptosporidium* and *Giardia*, acting as vectors of these disease-causing organisms (Anderson et al., 2003; Huamanchay et al., 2004; Reboledo-Fernandez et al., 2014).

Anthropogenic inputs can also stimulate the growth of microorganisms that are natural inhabitants of waterways but have the potential to be opportunistic pathogens featuring in waterborne infections/outbreaks. *Aerobacter butzleri* is a bacterium known to survive in aquatic environments, but is also isolated from the faeces of agricultural animals and humans (Oth et al., 2005). Other potential pathogens include *Vibrio* spp. and *Pseudomonas aeruginosa* (Hlavsa et al., 2014; Schets et al., 2011) (Table 1). *Vibrio* produce the enzyme chitinase and are able to colonise microinvertebrates (Chiavelli et al., 2001), degrading their chitinous exoskeleton discarded during moulting. Degradation of the chitin releases a rich source of nutrition in the benthic environment of water bodies, playing an important role in the recycling of nutrients, fuelling population increases at all trophic levels (Neogi et al., 2014). In addition, biofilm formation by bacteria on chitinous microinvertebrates provides a unique niche for replication to levels, which if copepods are ingested in drinking water could result in infection from colonising species of *Vibrio* and *Aeromonas* (Lipp et al., 2002). Jahid et al. (2006) has observed that intracellular storage of phosphorus can be beneficial to *Vibrio cholerae* as it is essential for activation of the stress response sigma factors, which regulate bacterial survival in aquatic environments.

The changes in nutrient status of an ecosystem produce many consequences that lead to an imbalance in the regulation of natural populations (Callisto et al., 2013). A study of wetlands receiving inputs from human wastewater plants observed a significantly increased prevalence of bacteria pathogenic to waterfowl, including a higher incidence of avian botulism due to *Clostridium botulinum* (Anza et al., 2014), which has also been associated with floating mats of the nuisance green alga *Cladophora* in the Great Lakes (Lan Chun et al., 2015). All of these factors have the potential to degrade natural environments and increase the pathogenicity of the inhabitants of aquatic systems.

1.2 Microbial faecal indicators

The role of the indicator in water quality assessment is to identify the potential presence of other substances that could be a risk to human health. In the case of faecal contamination of water, the indicator is a substance that is strongly associated with faeces, and therefore, indicates risk of diseases spread by the faecal-oral route (Leclerc et al., 2001; Standridge, 2008). Factors that determine the ideal microbial indicator include (Standridge, 2008; USEPA, 2015):

- Identification in high concentration in faeces
- No multiplication outside of the host, and therefore, not present in the environment
- Die-off in the environment is slower compared with that of pathogens
- Safe to work with in the laboratory
- Cost-effective analysis with quick turnaround time

An indicator of faecal contamination can be chemical or microbial but it is required to be associated with the faeces or wastewater derived from the targeted species to ensure detection when faecal contamination is present. There should be a strong and significant correlation between the presence of pathogens and the indicator of choice. Human pathogens may be present when faeces are detected, but they are often present in much lower concentrations compared to the indicators (Standridge, 2008). In addition, there are many different types of pathogens associated with faecal pollution, making it expensive and time-consuming to try to identify all pathogen candidates in a sample, hence the need for cost-effective indicators (Harwood et al., 2014). Determining the health risk associated with a water body when there is uncertainty about the types of pathogens circulating in a community makes it imperative to rely on indicators to identify a faecal event (Wu et al., 2011).

1.2.1 Indicator use in drinking and recreational water assessment

The reduction in waterborne disease over the last 100 years in developed countries such as NZ has been assisted by the detection of culturable faecal indicator bacteria (FIB) in water as sentinels of faecal contamination. In fresh, untreated sewage, the USEPA has affirmed that *E. coli* and enterococci are good indicators of potential risk to human health from pathogenic bacteria and protozoa (USEPA, 1996). Once sewage discharge occurs into receiving waters, however, a range of physical and environmental factors may, over time, alter the relationship between these indicator bacteria and the pathogens of concern (Kinzelman et al., 2004; Sinclair et al., 2012; Sobsey, 1989).

Recreational water quality criteria (RWQC) are based on scientific conclusions from the relationship between concentrations of FIB and rates of illness, and on criteria which determine

the acceptable risk of illness for those who participate in recreational activities. In epidemiological studies of recreational waterways, the coliform bacterium, *E. coli*, has shown a strong correlation with the rates of gastrointestinal illness (GI) associated with freshwater bathers, whereas enterococci are recognised as better predictors of GI illness in marine waters (Booth and Brion, 2004; Strachan et al., 2012; Wade et al., 2003). This correlation led to the incorporation of specified levels of *E. coli* in freshwater, and enterococci in marine water quality guidelines (USEPA, 1996). Recently, the USEPA has designated that the criteria for either enterococci or *E. coli* levels can be used for the assessment of freshwater but only enterococci for marine waters (USEPA, 2012).

The *New Zealand Microbiological Water Quality Guidelines for Marine and Freshwater Recreational Areas* (Ministry for the Environment, 2003) state that fresh water containing ≤ 260 *E. coli* per 100 mL (Alert Level) is acceptable for primary recreational activities which result in full immersion such as swimming, but that concentrations higher than 550 *E. coli* per 100 mL are not acceptable for any recreational contact (Action Level). In marine waters, the levels of enterococci for the Alert level are ≤ 140 enterococci/100 mL and for the Action level are ≤ 280 enterococci/100 mL. In drinking water, identification of faecal contamination is based on the detection of *E. coli* and levels of *E. coli* are required to be less than 1 colony forming unit (CFU) or most probable number (MPN) per 100 mL (Ministry of Health, 2013).

The alert and action levels for *E. coli* in freshwater in NZ have been developed with the purpose of keeping the risk of illness below 2% per 1000 healthy adult swimmers (McBride et al., 2002; Ministry for the Environment, 2003; Till et al., 2008). A study of NZ freshwater recreational sites concluded that *Campylobacter* and human adenoviruses were the pathogens most likely to cause human waterborne illness (McBride et al., 2002; Till et al., 2008). A correlation between *E. coli* and *Campylobacter* levels was identified, and it was proposed that approximately 5% of the *Campylobacter* infections in NZ were attributable to recreational water contact (Till et al., 2008). The other pathogens examined (*Salmonella*, F-RNA bacteriophage, somatic coliphage, enteroviruses, adenoviruses, *Cryptosporidium* oocysts and *Giardia* cysts), however, could not be related to *E. coli* concentrations in fresh water.

1.2.2 Limitations of current faecal indicator bacteria

The role of faecal microbes such as *E. coli*, as indicators of water quality has been publicly questioned due to research revealing the growth and/or persistence of FIB in the environment (McLellan et al., 2001; Solo-Gabriele et al., 2000). The debate has evolved in the developed world as the source of faecal microbes in water has moved from high levels of contamination

related to sewage/wastewater inputs (point sources), to lower levels of diffuse pollution (non-point sources) from a range of human and non-human sources (Tyrrel and Quinton, 2003). Point sources of faecal contamination such as wastewater from municipal treatment plants and slaughterhouses are more easily identified compared with diffuse pollution and generally result in very high levels of microbial indicators. Non-point sources of diffuse pollution include leaking sewer pipes (Guérineau et al., 2014) or septic tanks (Keegan et al., 2014), wildlife sources and runoff from agricultural land including land application of manure (Frey et al., 2015; Oun et al., 2014). This diffuse pollution may be reflected in lower but persistent concentrations of FIB, which are difficult to trace.

The USEPA believes that RWQC are protective of human health irrespective of the source of the faecal contamination. However, section 6 of USEPA, 2012, now describes site specific protocols for determining health hazards based on faecal sources particular to a location because not all animal species have been reported as having the same health hazard attributed to their faecal inputs. For example, Soller et al. (2010) has suggested that faecal inputs from birds have a lower public health risk compared with either human or agricultural sources. This lower risk from birds is attributed to the lower level of pathogen carriage by bird species.

Faecal sources from diverse animal types are associated with different health risks

Human faecal contamination presents the greatest risk for infectious disease, followed closely by livestock faecal contamination from cattle and dairy cows (Schoen et al., 2011; Soller et al., 2010). The human health risks from recreational water impacted by pollution from either human, gull, chicken, pig or cattle faeces has been investigated using quantitative microbial risk assessment (QMRA) (Soller et al., 2010). In water that contained the same level of faecal indicator from each source there was a potentially lower risk of illness when the water was impacted by chicken, gull and pig faecal material, than either human or cattle faeces. This reduced risk was attributed to the lower carriage of pathogenic microbes by these other animal species. Faecal contamination from beef cattle and dairy cows is considered to be a similar risk as human inputs due to significant carriage of pathogens such as *E. coli* O157:H7, *Salmonella*, *Campylobacter*, *Cryptosporidium* and *Giardia* (Callaway et al., 2005; Castro-Hermida et al., 2007; Cookson et al., 2006; Grinberg et al., 2005; Moriarty et al., 2008). In general, animal viruses are not considered to be infectious to humans (zoonotic) because it is believed that there are strong barriers to prevent viruses crossing between animal species (Cavirani, 2008; Kallio-Kokko et al., 2005).

In a 2014 paper, Soller et al. extended their initial QMRA work. The starting point was that 35 enterococci/100 ml provided an acceptable level of risk, and was based on the source of those enterococci being human faeces or sewage (that is, point sources) (USEPA, 2012). The risk of illness was defined as 36 GI per 1000 swimmers. Using QMRA modelling they estimated the level of enterococci that would provide an equivalent level of protection if those enterococci were from non-human sources. Their analysis suggested that if the enterococci are entirely from chicken, pig or gull sources, the equivalent level of enterococci that would provide the same protection, ranged from threefold to 50 times higher. This analysis illustrates the importance of determining the faecal source attributed to elevated FIB levels in water so that locations with the greatest potential health risk can be prioritised for remediation efforts.

Another key finding from the Soller et al. (2014) study was that where there are mixed sources of contamination identified, the risk is dependent on the most potent source of faecal contamination. The risk of illness decreased slowly as the contribution from human sources reduced from 100%, so that by 30% human source attribution to FIB levels, the predicted risk of infection had lowered by 50% compared with the risk if all detected FIB were solely derived from human sources. Thereafter, the risk declined more rapidly, so that at $\leq 20\%$ human contribution to the mixed faecal source, the predicted risk was five times lower compared with a pure human source. These predictions were based on the faecal source being from recent faecal events and did not account for the differential die-off between FIB and pathogens. The fact that the most potent faecal source (human or cattle, (Soller et al., 2010)) was the driver of predicted risk is of particular relevance to rural areas where ruminant agricultural sources are detected often in conjunction with avian sources. Therefore, unless the ruminant signal accounts for less than 30% of the mixed contamination, then the health risk could be 50 to 100% of the risk associated with a solely ruminant faecal source.

Environmental sources of FIB

Concerns have been raised about the potential for putative environmental sources of faecal microbes to confound water management practices for identifying faecal pollution (Anderson et al., 2005; Byappanahalli et al., 2003a; Ferguson et al., 2016; Whitman et al., 2006). When faecal coliforms (FC) were first proposed as a method of assessing water quality, the paradigm was that they were only able to survive and replicate in the homeostatic intestinal environment of their animal/bird host (Geldreich, 1966). Survival and persistence in the environment external to an intestinal habitat was believed to be short-lived.

Replication of FC such as *E. coli* in aquatic environments was considered highly improbable where ambient temperatures ranged from 4 to 25°C and nutrient status was in continual flux. Early work on carriage of FIB in the intestinal tract of freshwater fish provided strong evidence that the FIB population in fish was not stable but that carriage and replication of microbes was affected by pollution status of the water body (Geldreich and Clarke, 1966). Fish species exposed to water containing FIB (10⁴ CFU/100 ml) intermittently carried low levels of FC (range <2 to 22 MPN/g) from Day 7 to 16. Higher carriage of FC in fish occurred in waters with summer temperature ranges of 16-20°C compared with FC below the detection limit during winter (1-10°C). Furthermore, when these fish, contaminated with total coliforms, were placed in a tank of unpolluted potable water, <2 MPN/100 mL of FC were detected in the water up to 9 days after fish had been residing in the tank. In contrast, the same tank water had variable levels of total coliforms (10³ to 10⁴ MPN/100 mL) over the same period.

Studies have shown that even in the absence of recent faecal inputs, the faecal indicators *E. coli* and enterococci can occur in soil, sediment, vegetation and algal mats in waterbodies as part of the natural microflora (Berthe et al., 2013; Byappanahalli and Fujioka, 2004; Byappanahalli et al., 2003b; Whitman et al., 2005). Initial reports of survival of intestinal microbes in the environment were limited to tropical areas where higher temperatures were suggested as aiding their survival (Jimenez et al., 1989). Further work established the same trend for persistence of indicator bacteria in subtropical environments (Anderson et al., 2005; Badgley et al., 2010a; Badgley et al., 2010b; Byappanahalli et al., 2012a; Byappanahalli et al., 2012b; Desmarais et al., 2002; Solo-Gabriele et al., 2000). This work has been extended to the identification of FIB in temperate environmental reservoirs (Badgley et al., 2011; Byappanahalli et al., 2003a; Byappanahalli et al., 2006a; Byappanahalli et al., 2006b; Ishii et al., 2006; McLellan, 2004; Whitman and Nevers, 2003; Whitman et al., 2003). Some of these studies also provide evidence of *E. coli*'s potential to actively grow in soil environments and algal mats across the climate spectrum of tropical to temperate. In a study of the survival of *E. coli* strains in water microcosm experiments, Berthe et al. (2013) noted that, in general, *E. coli* derived from water impacted by recent faecal events had reduced persistence in water compared with those strains derived from waters containing low levels of faecal contamination and FIB, suggesting those latter *E. coli* strains had adapted to the aquatic environment. It is now recognised that after defecation from the host, FIB may persist in reservoirs such as beach sand, soil, river sediment, algal mats and terrestrial plants (Heaney et al., 2014; Nevers et al., 2014).

Further confounding the use of FIB is recent research identifying putative environmental strains of *E. coli* and enterococci as “naturalised” inhabitants of such environmental reservoirs as

soil, sand and sediment (Cohan and Kopac, 2011; Leimbach et al., 2013; Luo et al., 2011; Weigand et al., 2014). These FIB strains are phenotypically and taxonomically indistinguishable to the enteric FIB. Whole genome sequencing of these “naturalised” *E. coli* and enterococci strains has, however, suggested that whilst they contain the core genome of their bacterial species, they also carry a distinctive gene repertoire that allows them to adapt to non-intestinal conditions. Researchers have, therefore, suggested that they could represent “true” environmental strains of FIB, which have diverged genetically from the faecally-derived strains over long time frames of thousands to millions of years.

These findings raise the question of whether there are two groups of environmentally persistent strains of FIB: those strains of recent faecal origin that have adapted to persist/grow in the environment and the truly “naturalised” strains of FIB. The identification of any sources of environmental FIB, however, does have significant impacts on conventional methods of water quality monitoring and how those results impact on water management decisions and determination of health risk.

Sediments become an issue of concern for water managers when faecal indicator bacteria and pathogenic microorganisms are re-suspended from the sediments into the overlying water column such as during heavy rainfall events (Obiri-Danso and Jones, 2000; Shehane et al., 2005). Ibekwe et al. (2011) noted the prevalence of highly related isolates of *E. coli* associated with sediments compared with the overlying water column, which contained a greater diversity of *E. coli* subtypes suggestive of transient populations derived from recent faecal inputs. Based on these factors it would be expected that populations of FIB in a specific location could be in flux with subsequent impacts on the levels measured in water (Piorkowski et al., 2014a).

Survival rates

The survival rates in sediment and water of FIB derived from faecal inputs have been shown to be dependent on temperature effects, salinity, sunlight inactivation and the impact of predators and organic carbon (Garzio-Hadzick et al., 2010; Geldreich, 1966; Gilpin et al., 2013; Korajkic et al., 2013a; Korajkic et al., 2013b; Rozen and Belkin, 2001; Sinton et al., 2002). However, the magnitude of the impact of each of these variables in contributing to the persistence or decline in FIB populations has been shown to be dependent on the water type (fresh versus marine) and the matrix type (sediment versus water column) (Korajkic et al., 2013b). In addition, Korajkic et al. (2013b) also investigated the decline of *E. coli* concentrations in the presence/absence of natural microflora, which could act as competitors of *E. coli*. They noted a greater persistence of *E. coli* in all matrices and water types when competitors and predators were removed in comparison

with predators alone. This decreased persistence, reflects the detrimental impact on *E. coli* of competition with environmental aquatic microbes. Korajkic et al. (2013a) found that the source of the faecal contamination was the most significant factor in decay of FIB with lesser contributions from sunlight and natural microflora. FIB in faecal sources from cattle were more persistent compared with human sewage in all mesocosm treatments. The authors suggested that the differences in persistence of FIB between faecal sources may be attributed to differences in their gastrointestinal tract environments. The higher particulate content of cattle faeces may provide a richer nutrient environment and protective attachment properties for FIB.

Effects of light

Effects of ultraviolet (UV) and visible light in sunlight have been shown to have a detrimental effect on the viability of indicator bacteria (Davies-Colley et al., 1994; Sinton et al., 2007a; Sinton et al., 1999; Sinton et al., 2002). Sunlight inactivation rates in *E. coli* from sewage in mesocosms of marine and freshwater were noted to slow down after the first day (Gilpin et al., 2013). It was hypothesised that slower decay rates after Day 1 were attributed to the photo repair mechanisms of Deoxyribonucleic acid (DNA) activated by surviving *E. coli*. This activation of DNA repair mechanisms conferred greater resistance to sunlight exposure in the remaining two days of the experiment.

Debate has arisen because many light/dark experiments did not account for predator and bacterial competitor effects on FIB persistence. Removal of predator populations from light/dark water mesocosms have shown delayed rates of inactivation (Korajkic et al., 2013a; Korajkic et al., 2013b). However, Sassoubre et al. (2015) noted minimal impact of the presence of marine microbiota on sewage community composition in comparison to the greater deleterious effects of sunlight. Korajkic et al. (2014) suggested decay factors may be impacted by the length of the experimental period, with sunlight only being important in the early stages (first few days) of decay, after which predator/competitor relationships were the dominant contributors to decay. Although high decay rates of FIB associated with predation were noted by Dick et al. (2010), they queried the relevance of predation in the water column of a flowing river system. It is apparent from all of these experiments that multiple factors impact on the decline/persistence of FIB once discharged into the aquatic environment. Therefore, the impact of each of these factors will be dependent on the water type and natural environment of the receiving water (Wanjugi and Harwood, 2013).

How well do microbial indicators correlate with pathogen presence?

Drinking and recreational water have both been identified as complying with bacteriological criteria but still containing pathogenic viruses or protozoa. These findings suggest that a reliance on FIB may be inadequate for identifying risks associated with all pathogens (Craun et al., 1997; Leclerc et al., 2002; Thompson et al., 2003). For example, there have been examples of GI outbreaks due to drinking water where the bacteriological criteria were met and the water contained adequate levels of chlorination (Goldstein et al., 1996; Leclerc et al., 2002; Meinhardt et al., 1996). Bacteria such as the FIB are, in general, inactivated by water treatments such as chlorination, whereas protozoa and viruses are more resistant. This greater resistance can lead to the absence of FIB, while protozoa and viruses are still present in treated water (Codony et al., 2012; King et al., 1988). A study in the Netherlands investigating untreated recreational waterborne outbreaks over the period 1991-2007, identified that 85% of water samples tested were in compliance with the European bathing-water legislation but still led to outbreaks of gastroenteritis and/or skin infections (Schets et al., 2011).

There have been many studies investigating the correlation between detection of FIB and pathogens in a water sample with varying conclusions (Duris et al., 2013; Harwood et al., 2005; Reano et al., 2015). The discrepancies between studies of indicator-pathogen correlations were investigated by collating multiple studies ($n = >500$) over 40 years of research from many different water types (Wu et al., 2011). Important findings from this meta-analysis included that correlations were more likely where there were high numbers of samples tested (>30) and where at least 13 of those samples were positive for pathogens.

Faecal indicator bacteria do not identify the source of faecal inputs

Another limitation of microbial faecal indicators is that they provide little guidance on the source of faecal pollution because of their ubiquitous presence in the intestinal environment and therefore, the faeces of all animal types, including humans (Bettelheim et al., 1976). Moderate to low levels of *E. coli* that are close to the recreational water quality guidelines of ≤ 260 CFU/100 ml are difficult to interpret for water quality managers tasked with recommending beach closures where public health could be at risk. Faecal sources of contamination may not be apparent during routine sanitary surveys of waterways, and conventional FIB tests offer no guidance as to the source. More sophisticated tools are required to track the source(s) of faecal contamination (Sinton et al., 1998). In addition, the lag time between collection of water sample and the test result means that beach closures are based on FIB levels from the preceding day's sampling,

requiring the development of new indicators which have a faster detection time (Wade et al., 2006; Weintraub and Wright, 2008).

It has been suggested that with the advent of new technologies a new paradigm is required that shifts the reliance on conventional FIB to utilising different faecal indicators dependent on the target water body and its catchment, and on the question being asked (Wilkes et al., 2009; Yates, 2007). The conventional FIB, *E. coli* and enterococci will continue to be used as frontline tools but in association with a suite of other indicators and tools (Harwood et al., 2005) as discussed in the following section.

1.3 Identifying Faecal sources: Faecal source tracking (FST)

There is an expectation that when levels of indicator microorganisms exceed water quality guidelines, corrective action will follow. Characterisation of the faecal source is necessary for the establishment of best management practices to control the major pollution contributors to human health risk. Mitigation measures, however, require a focal point for remediation work and as explained, identification of the traditional microbial indicators does not provide faecal source information. Impacts of high faecal microbial loadings in waterways include beach closures and warnings against shellfish collection, which is detrimental to commercial and recreational harvesting. In the past most closures of beaches have occurred without identification of the pollution source (Santo Domingo et al., 2007). One response to this low discrimination power has been a growing interest in the development of faecal source tracking (FST) tools which differentiate between animal species (Harwood, 2014; Harwood et al., 2014; Tran et al., 2015). This has led to the identification of various chemical and microbial markers that help to discriminate between human and non-human faecal sources and also between the non-human species. These markers can be used in conjunction with traditional bacterial indicators and surveys of the surrounding environment to increase information leading to elimination of the faecal sources of pollution.

FIB are susceptible to industrial waste processes whereby chemical disinfectants, heat and toxic pollutants affect the viability and therefore detection of bacterial indicators (Switzer-Howse and Dukta, 1978). For the detection of treated waste discharge, therefore, this has required the development of assays such as chemical markers, which are more resistant to chemical and physical degradation. Chemical markers can be divided into the chemicals which are inherently detected in faeces such as faecal steroids and those which are strongly associated with faecal waste such as fluorescent whitening agents, caffeine and pharmaceuticals (Sinton et

al., 1998). The two chemical FST markers (faecal steroids and fluorescent whitening agents) employed in this thesis will be discussed in the following sections.

1.3.1 Chemical FST markers: Faecal Steroids

The structure of steroids

Steroids are a group of cyclic organic compounds arranged in four rings of which three of the rings contain 6 carbons and the other is a 5 carbon ring from which a carbon side chain is attached at carbon 17 (Hill et al., 1991) (Figure 1). Sterols are an important group of steroids, characterised by having a carbon 27 cholestane framework, and an hydroxyl group at the carbon 3 position (MacDonald et al., 1983). Faecal steroids are lipophilic in nature and bind strongly to particulate matter. Cholesterol, is an example of a major steroid found in higher animals (MacDonald et al., 1983). The use of cholesterol as an individual biomarker of faecal contamination is limited because of its widespread distribution in a variety of sources including animals, algae, marine plankton and sewage (Volkman, 1986). Instead, degradation products of cholesterol are used in faecal steroid analysis (Mudge and Duce, 2005).

Upon entering the digestive tract cholesterol is hydrogenated to stanols of various isomeric configurations by anaerobic bacteria (MacDonald et al., 1983). The reduced stanols are saturated sterols as they have no double bonds in the sterol ring structure. Cholesterol is the C₂₇ precursor to the 5 α and 5 β -C₂₇ stanols, cholestanol and coprostanol (respectively). Coprostanol is of particular interest in the detection of human faecal pollution as it is the principal steroid identified in human faeces where it comprises approximately 60% of the total steroid concentration (Leeming et al., 1996; Leeming et al., 1998b). Coprostanol is identified primarily in human faeces, in comparison, most other faecal steroids are found in a variety of organisms which include bacteria, algae, zooplankton and protozoa (Takada and Eganhouse, 1998). Coprostanol is identified in cats and pigs but at concentrations tenfold less than in humans (Leeming et al., 1998b). In comparison to 5 β -coprostanol, which requires transformation via anaerobic bacteria in the animal gut, 5 α -cholestanol is the thermodynamically more stable isomer of cholesterol and commonly occurs in pristine environments (Nishimura, 1982). Epicoprostanol is an epi-isomer of coprostanol and is a minor component of the steroid fraction in faeces (McCalley et al., 1981). Epi-isomers have a hydroxyl group (OH) in the α -configuration at carbon position 3 compared to the β -configuration at the same carbon for coprostanol and 24-ethylcoprostanol (MacDonald et al., 1983).

The steroid fingerprint for discriminating between animal species

Differentiation of human from herbivore (e.g. cows and sheep) faecal pollution relies on the high production of C₂₉ stanols, such as 24-ethylcoprostanol in herbivores compared to human faeces (Leeming et al., 1996). This is based on herbivore consumption of plant material, which contains predominantly C₂₉ sterol precursors such as 24-ethylcholesterol, which are reduced to the β -stanol, 24-ethylcoprostanol in the herbivore intestine. Different faecal steroids are not unique to a particular animal species, however, the faeces of each animal type has a distinguishing steroid fingerprint that is determined by three factors: an animal's diet; whether sterols are synthesised by the animal (e.g. humans synthesise cholesterol), and the transformations that are mediated by microorganisms resident in the host's digestive tract (Bull et al., 2002; Leeming et al., 1996). Faecal steroid analysis generates a lot of data, the interpretation of which can be quite complex. Guidelines to the ten steroids analysed in this thesis are described in Table 2.

Ratio analysis of steroids for discriminating between animal species

The absolute levels of each sterol or stanol in water or sediment can be dependent on many factors including dilution and partitioning between sediment and water (Bull et al., 2002). Steroids are hydrophobic and preferentially attach to particulate matter. In sediment, the steroid concentration is dependent on total organic carbon (TOC) (Hatcher and McGillivray, 1979). The TOC is related to the grain-size of the sediment due to the organic particles trapped within the fine grains of the sediment. There has been no consensus around the absolute concentration of coprostanol identified in sediments that correlates with human faecal pollution (Muniz et al., 2015). Ratios between steroids are, however, less concentration dependent, for example normalising coprostanol content to total faecal steroid content and representing it as the percentage of coprostanol/total steroids (Venkatesan and Kaplan, 1990). Ratio analysis, therefore, is the preferred interpretation of the relevance of the various sterol/stanol concentrations detected. Some of the ratios used for FST and the type of faecal pollution they indicate are outlined in Table 3.

Originally, steroid ratios were developed based on sediment and faeces but Grimault et al. (1990) and Furtula et al. (2012a) showed that, in general, ratios applied to both sediment and water particulate matter have similar values for discriminating between polluted and non-polluted environments. In addition, Furtula et al. (2012a) has analysed the same ratios in sewage influent/effluent and suggested some changes to threshold criteria for a few ratios, such as reducing the threshold for detection of human faecal contamination from >0.7 to ≥ 0.5 for the ratio coprostanol/(coprostanol + cholestanol).

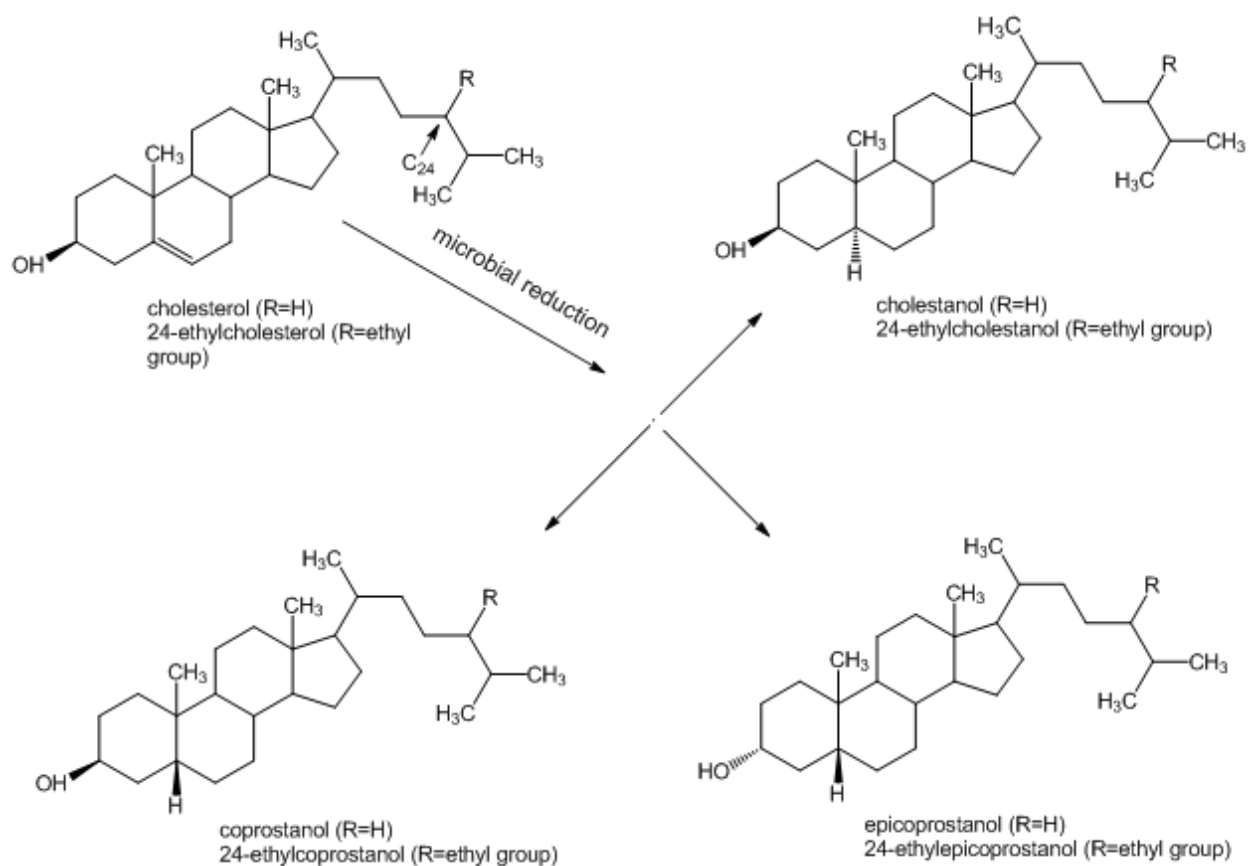


Figure 1: Biotransformation of cholesterol to various stanols adapted from Leeming et al. (1996). Substitution of H for an ethyl group at carbon number 24 on the coprostanol structure, produces 24-ethylcoprostanol. Similar substitutions are shown for the other stanols. Created by Darren Saunders as directed by the author.

Table 2: Faecal steroids analysed for faecal source tracking

Sterol/Stanol	Description	References
Coprostanol	Principal human biomarker, high relative amounts indicate fresh human faecal material. Constitutes up to 60% of the total steroids found in human faeces. Dogs and birds have either no coprostanol or only trace amounts, present in their faeces. Coprostanol is not found in unpolluted fresh or marine waters or in fully oxic sediments (only anaerobic bacteria can hydrogenate cholesterol to coprostanol). However under conditions of anoxia, small amounts can be found in sediments not contaminated by faecal pollution.	Leeming et al. (1996) Leeming et al. (1998b)
Epicoprostanol	Found in trace amounts (relative to coprostanol) in human faeces. Increases in relative proportions in digested sewage sludges, perhaps through conversion of coprostanol to epicoprostanol.	McCalley et al. (1981)
24-ethylcoprostanol	Principal herbivore indicator.	Leeming et al. (1996) Leeming et al. (1998b)
24-ethylepicoprostanol	Usually also present in herbivore faeces.	Derrien et al. (2011)
Cholesterol	Precursor to coprostanol and epicoprostanol. Also comes from domestic waste, food scraps, algae etc.	
Cholestanol	Most stable isomer, ubiquitous and occurs in pristine environments.	Nishimura (1982)
24-methylcholesterol	Plant sterol (also known as campesterol).	Nash et al. (2005)
24-ethylcholesterol	Precursor to 24-ethylcoprostanol and 24-ethylepicoprostanol (24-ethylcholesterol also known as β -sitosterol).	Volkman (1986)
Stigmasterol	Plant sterol.	
24-ethylcholestanol	Breakdown product of 24-ethylcholesterol	

Table 3: Steroid ratio analysis as indicators of the source of faecal pollution. Cop = coprostanol; Cholestan = cholestanol; 24-Echolestan = 24-ethylcholestanol; Epicop = epicoprostanol; 24-Echolesterol = 24-ethylcholesterol; 24-Ecop = 24-ethylcoprostanol; 24-E-epicop = 24-ethylepicoprostanol.

Type of ratio	Ratio	Steroid ratio	Criteria	References
General faecal	F1	cop/cholestan	>0.5 indicative of faecal source of steroids <0.3 non-polluted source	Leeming et al. (1997) Leeming et al. (1998a) Leeming et al. (1998b)
	F2	24-Ecop/24-Echolestan		
Human-associated	H1	% cop/total steroids	Ratio >5-6% suggests human source	Reeves and Patton (2001) Isobe et al. (2002)
	H2	cop/(cop+cholestan)	Ratio >0.7 suggests human source; <0.3 suggests non-polluted source and 0.3-0.7 uncertain source	Grimault et al. (1990) Fattore et al. (1996) Mudge et al. (2008)
Discriminates human and herbivore	H3	cop/24-Ecop	Ratio >1 suggests human source	Leeming et al. (1996)
	H4	cop/(cop + 24-Ecop)	Ratio >0.73 suggests 100% human source <0.38 suggests 100 % herbivore source	Leeming et al. (1998a) Leeming et al. (1998b)
	H5	%Human faecal contribution	If ratio is between 0.38 and 0.73 then: (Ratio value – 0.38) x 2.86 for human contribution	Bull et al. (2002)
Human-associated	H6	cop/epicop	Ratio criteria for identifying human contamination >1.5 No criteria for discriminating recent from aged sources	Fattore et al. (1996) Patton and Reeves (1999)
Herbivore	R1	%24-Ecop/total steroids	Ratio >5-6% suggests herbivore	Leeming et al. (1998a) Leeming et al. (1998b) Devane et al. (2015)
	R2	%Herbivore faecal contribution	Ratio <0.38 suggests 100% herbivore source If ratio is between 0.38 and 0.73 then: (0.73 - ratio value) x 2.86 for herbivore contribution	Leeming et al. (1998a) Leeming et al. (1998b) Bull et al. (2002)
Plant	P1	24-Echolesterol/24-Ecop	Ratio >4.0 suggests plant decay Ratio ≥7.0 is supportive of avian pollution	Nash et al. (2005) Devane et al. (2015)
Avian	Av1	24-Echolestan/(24-Echolestan + 24-Ecop + 24-E-epicop)	Ratio >0.4 suggests avian pollution	Devane et al. (2015)
	Av2	Cholestan/(cholestan + cop + epicop)	Ratio >0.5 suggests avian pollution	
Porcine	%H4	%cop/(cop+24-Ecop)	Ratio >60% suggests human source <60% suggests bovine or porcine	Gourmelon et al. (2010)
	R3	24-Echolestan/cop	Ratio >1.0 suggests bovine source Ratio <1.0 suggests human or porcine source	

1.3.2 Chemical FST markers: Fluorescent Whitening Agents

Fluorescent whitening agents (FWA) are used in laundry detergents, textile and paper industries because of their ability to whiten materials. The FWA absorb incident radiation in the 360 nm wavelength region and re-emit it at ~ 430 nm as visible blue fluorescence (Ganz et al., 1975) creating the visual whitening effect. The large carbon structure of the FWA causes a high binding efficiency to cellulosic fabrics such as cotton or those materials containing polyamides, for example, nylon (Burg et al., 1977). FWA are acidic and hydrophilic in nature (Shu and Ding, 2005) due to the addition of sulfonate groups, which increase the water solubility of the otherwise hydrophobic FWA (Stoll and Giger, 1998).

FWA are a minor component of laundry detergents forming only 0.15% of the final product (Managaki and Takada, 2005). In NZ, there is only one FWA used in the wash powder industry, DAS1 or 4,4'-bis[(4-anilino-6-morpholino-1,3,5-triazin-2-yl)-amino]stilbene-2,2'-disulfonate, (J. Scott, Ciba Specialty Chemicals Limited, Auckland, NZ, pers. comm. 1999). As a component of laundry detergents the excess unabsorbed FWA will be present in greywater, which in most household plumbing systems is discharged into the sewerage. Detection, therefore, of FWA in waste and receiving waters are indicative of human sewage.

DAS1 is a stilbene derivative and contains a single ethylene bond, which means it can undergo isomerisation due to twisting about the stilbene double bond (Canonica et al., 1997; Poiger et al., 1996). DAS1 can, therefore, form two isomers, the cis and trans forms. Only the trans isomer is fluorescent and is the FWA manufactured for addition to laundry detergents. The fraction of the trans isomer compared to the cis is 90% when adsorbed to cotton and exposed to sunlight (Canonica et al., 1997). Isomer transition occurs rapidly when the FWA is irradiated by sunlight and is called photoisomerisation, which is reversible. Isomerisation from trans to cis form is accompanied by a loss in fluorescence and lost affinity for cellulosic and particulate matter, such as sediment (Poiger et al., 1996). Thus because the trans isomers are more strongly adsorbed to particles, the transport of FWA in wastewaters and surface waters will be dependent on the parameters affecting photoisomerisation, which include turbidity and exposure to sunlight.

Studies on the toxicity to humans via the oral route and skin absorption have not indicated any hazard due to exposure to FWA (Burg et al., 1977). The predicted no effect concentration for FWA for DAS1 is 100 µg/L (Richner et al. (1997) cited in Poiger et al. (1998)). These figures were generally well above the reported FWA concentrations found in

Swiss rivers, which ranged from 6 to 1100 ng/L (Poiger et al., 1996; Poiger et al., 1998; Stoll and Giger, 1998).

1.3.3 Microbial source tracking and PCR markers

Microbial source tracking (MST) of faecal contamination is based on the bacteria harboured in the intestinal environment of each animal and bird species that are specific to that particular host (Harwood et al., 2014). This inherent specificity is due to differences in diet and digestive systems between species, which impacts on the intestinal microflora of humans, animals and birds (Roslev and Bukh, 2011). Microbes targeted for MST need to be identified in high concentration in the faecal outputs of their respective host. Microbial markers include culture-based methods which identify phenotypic (e.g. antibiotic resistance, or biochemical fingerprinting for enterococcus, (Patel et al., 2011)), or genotypic traits such as DNA hybridisation (Lynch et al., 2002) and fingerprinting techniques such as restriction fragment length polymorphism (Fogarty and Voytek, 2005). Phenotypic or genotypic fingerprinting methods for characterising bacteria require a comparison between the bacterial isolates from the water under investigation and faecal isolates from the surrounding environment. The library of isolates for comparison is usually geographically specific and >500 isolates per location have been assessed to evaluate accurate classification (Ahmed et al., 2007; Stoeckel et al., 2004). These library-dependent methods are time consuming and have reported a high rate of misclassification (Harwood et al., 2003; Stoeckel et al., 2004).

Polymerase Chain Reaction (PCR) Markers for MST

The limitations of library dependent MST methods have led to new developments focussing on molecular methods such as the Polymerase Chain Reaction (PCR) which can target both culturable and non-culturable microorganisms in the intestinal environment (Field et al., 2003b; Santo Domingo et al., 2007). Many of the non-culturable enteric population have anaerobic requirements, which is a useful characteristic for FST markers as it reduces the likelihood of their survival when excreted into the external environment (Savichtcheva and Okabe, 2006).

Numerous PCR-based markers for the detection of faecal contamination from specific sources have been described including examples for human faecal contamination (Gomi et al., 2014; Stachler and Bibby, 2014), ruminant (Bernhard and Field, 2000) including specifically for sheep (Schill and Mathes, 2008) and cows (Raith et al., 2013; Shanks et al., 2008), dogs (Green et al., 2014) feral animals such as possums (Devane et al., 2013), avian species (Green et al., 2012), and pigs (Heaney et al., 2015; Mieszkin et al., 2009).

Initial PCR markers for human and ruminants were designed based on the 16S ribosomal ribonucleic acid (16S rRNA) gene of the Bacteroidales, as this Order of bacteria is well represented in the intestine of mammals (Eckburg et al., 2005; Field et al., 2003a; Field et al., 2003b; Kildare et al., 2007). Bacteroidales are reported to be identified in higher concentrations in the gut than traditional microbial indicators such as *E. coli* (Salyers, 1984). An important consideration for an indicator of faecal contamination is that the Bacteroidales are obligate anaerobes, and therefore, less likely to replicate in the environment. The HF183 primer system designed by Bernhard and Field (2000) is an example of a human PCR marker that targets the 16S rRNA operon of the Bacteroidales.

Additional bacterial species have been targeted as candidates for microbial source tracking (MST) such as *Bifidobacterium adolescentis*, which has been used as a marker for human pollution (Matsuki et al., 2004). Avian species have been reported to carry low numbers of *Bacteroides–Prevotella* species used as PCR markers in other hosts (Fogarty and Voytek, 2005; Lu et al., 2008). Therefore, specific avian markers have been designed to amplify the DNA of *Helicobacters* (Green et al., 2012); *Catelicoccus* and *Streptococcus* (Ryu et al., 2012); and *Desulphovibrio*-like bacteria (Devane et al., 2007). All of the above assays target the 16S rRNA gene, which is an essential gene present in all bacterial genomes. 16S rRNA has hypervariable regions able to provide discrimination between closely related species. Another major advantage of this gene is the multiple copies of 16S rRNA that are produced per cell, which greatly increases the sensitivity of the PCR assay (McLellan and Eren, 2014).

Initially PCR for MST markers involved the endpoint detection of amplified DNA with amplicons separated on electrophoresis gels, using ethidium bromide under ultraviolet light to visualise DNA bands on the agarose gels (Bernhard and Field, 2000; Field et al., 2003a). PCR technology advanced with the advent of quantitative PCR (qPCR) where the DNA product is amplified and detected in “real-time” using a non-specific fluorescent reporting molecule such as SYBR Green or “probes” of fluorescent chromogens attached to DNA bases that are specific to the MST target (Roslev and Bukh, 2011; Savichtcheva and Okabe, 2006). Real-time monitoring of DNA amplification allows quantification of the target marker by comparison of the amplification cycle with that of known concentrations of target DNA, which have been used to generate a standard curve.

PCR and qPCR markers have gained interest as they outperformed other methods of faecal source tracking (FST) in an interlaboratory experiment (Griffith et al., 2003). In addition, with the advent of real time PCR methods, genetic markers are regarded as delivering results in a timely and cost-effective manner in comparison to other FST methods (Field et al., 2003a; Santo

Domingo et al., 2007). Another advantage of PCR markers is that they can be designed to target a particular animal/bird species. Multiple PCR markers targeting a range of potential sources can be assayed concurrently, meaning two or more different markers targeting a single source such as human pollution can be assayed at little additional cost, increasing confidence in the result (Savichtcheva and Okabe, 2006).

Evaluation of PCR markers is required to determine host distribution, sensitivity and specificity prior to implementing an assay for source attribution. The host-specific bacteria targeted by the genetic marker should be present in high abundance in the majority of the host type's individual faecal samples (Mieszkin et al., 2009; Santo Domingo et al., 2007). Sensitivity is defined as the percentage of host organisms that carry the host-specific marker. Specificity is defined as the percentage of non-host organisms in which the host-specific marker is not identified (i.e. true negatives). While it is recognised that all host specific markers have a false positive rate, this must be minimised. Amplification efficiency needs to be tested as theoretically, PCR reactions will double the number of DNA amplicons at every PCR cycle (Kildare et al., 2007). Environmental samples may contain organic matter such as humic acids which can inhibit the PCR assay and require methods for determining if inhibition is present to reduce false negative results (Cao et al., 2012; Haugland et al., 2005). The limit of detection has been used as a parameter to assess qPCR assay performance but difficulties with non-standardisation of methods limit inter-laboratory comparisons (Ervin et al., 2013; Wang et al., 2014). For example, lack of uniformity of the unit of measure such as either DNA or faecal mass, which is used to determine the limit of detection (LOD) of an assay.

The large number of PCR assays developed for MST and multiple targets for a particular species has led researchers to perform evaluations of blinded samples containing single faecal sources or doubletons (two sources) to ascertain the performance of individual PCR markers (Boehm et al., 2013; Raith et al., 2013; Sinigalliano et al., 2013; Stewart et al., 2013). Sensitivity and specificity of 41 PCR markers targeting human and a range of animals and birds was conducted in 27 laboratories by testing the markers against host and non-host target faeces (Boehm et al., 2013). The study provided useful information on those assays that consistently performed at >80% sensitivity and specificity in multiple laboratories such as the human PCR marker HF183 for both endpoint PCR (Bernhard and Field, 2000) and qPCR using SYBR Green (Seurinck et al., 2005). Inter- and intra-laboratory reproducibility of PCR markers was investigated in a multi-lab (n = 3 to 5) comparison (Ebentier et al., 2013). A lack of standardisation of reagents and protocols was noted to increase variability of results between laboratories and also within a laboratory. These studies highlighted the need for standardised

protocols between all laboratories performing MST PCR assays to allow for inter-laboratory comparisons and confidence in results for inclusion in USEPA methods for water quality monitoring.

Metagenomics: utilising next generation sequencing technologies

The massively parallel nature of next generation sequencing (NGS) enables amplification of millions of DNA molecules at the same time, and has revolutionised the mining of genetic information from organisms. NGS allows cost-effective sequencing of millions of sequence reads per environmental sample or bacterial colony isolate (Wooley et al., 2010). The revolution began with the sequencing of the entire genomes of single microorganisms (Edwards and Holt, 2013), and the targeted sequencing of the 16S rRNA of microbes to build up large databases such as GenBank containing identity information on multiple microbial species (Kuczynski et al., 2012). The progression of sequencing technology allows researchers to obtain genetic information directly from environmental samples without requiring the cultivation of individual microorganisms. This is a major advantage as the majority of microbial species (>99%) have not been isolated using cultivation procedures (Amann et al., 1995; Davis et al., 2005).

The addition of unique DNA barcodes to identify individual samples allows for the amplification of multiple samples simultaneously and the generation of very large datasets for analysis of different environments concurrently (Cardenas and Tiedje, 2008). The specific barcode sequences introduced during sample preparation allow sequences unique to a sample to be separated out and assigned to their original sample after sequencing. The analysis of the large datasets generated by NGS has been a limitation requiring the development of bioinformatic computational “pipelines” to assign taxonomic status and determine microbial biodiversity in a stepwise fashion (Gonzalez and Knight, 2012). Pipelines can trim, screen, and align sequences; calculate phylogenetic distances; assign sequences to operational taxonomic units; and describe the microbial diversity, all within a single software package. Some of these sequencing pipelines include Qiime (Quantitative Insights into Microbial Ecology) (Caporaso et al., 2010) MEGAN (Mitra et al., 2011) and Mothur (Schloss et al., 2009).

Sequence data amplified directly from all of the microbes in an environmental sample has been characterized as the metagenome. Metagenomic studies of the microorganisms present in a sample have been performed on many different environments, for example, soil (Fierer et al., 2007), human faecal material (Moore et al., 2015), and the ruminal fluid of cattle (Jami et al., 2013). Metagenomic investigations of environmental samples are a natural technological progression for microbial source tracking, which currently relies on PCR markers. Several

studies have investigated the bacterial communities in water and faeces to facilitate assignment of faecal contamination to sources (Staley et al., 2015; Unno et al., 2010). Cao et al. (2013) has shown a high degree of correct classification to faecal source when using three different genetic approaches (including NGS) to microbial community analysis.

Application of NGS to FST necessitates a library-dependent approach by developing a collection of known bacterial sequences for each animal faecal source and comparing the library with the bacterial sequences from water samples. Quantification of the contribution of each source to the water contamination based on the percentage of sequence reads attributed to individual sources may be possible in the future. Ahmed et al. (2015b), however, found variable agreement when comparing source contribution data between sequencing and conventional PCR markers, but highlighted the potential of NGS for faecal source tracking as part of a toolbox used in conjunction with PCR markers.

1.4 Factors affecting FST marker persistence in the environment over time

1.4.1 The persistence of faecal steroids in the environment

The fate of steroids as they undergo sewage treatment

Faecal steroids are non-polar, non-ionic, and water insoluble due to their hydrocarbon structure (Figure 1) and therefore become associated with fine grain particles and sediments. Due to the low solubility of steroids they are strongly associated with particulate matter in the final effluent of sewage (McCalley et al., 1981; Saad and Higuchi, 1965). Microbial action in the sludge digester environment may lead to conversion of cholesterol to coprostanol, similar to the transformation that occurs in the mammalian gut (MacDonald et al., 1983). In addition, the microbial action during the digestion process reduces the volume of organic matter by the partial conversion of its bulk to gaseous products. Therefore, because coprostanol is resistant to anaerobic degradation it increases in concentration relative to the remaining weight of sludge. An increased concentration of coprostanol during sewage digestion raises the likelihood of its detection as a biomarker when discharged into the environment.

Steroids undergo aerobic degradation by bacteria. The decay rate of coprostanol was measured in sewage sludges, both raw and diluted (Bartlett, 1987). Although the raw sewage remained anaerobic there was sufficient air bubbling through its bulk to enable aerobic decay of coprostanol, and coprostanol levels declined to 15% of its initial concentration after 29 days and then 9% by day 54, when the experiment was stopped. It was noted that the more dilute the sludge, the faster the decay rate of coprostanol. In a study of steroid degradation during sewage

treatment, steroid concentrations were noted to decrease by approximately 90% through the treatment process, however, the abundance of faecal steroids relative to each other remained the same, validating the use of steroid ratios to identify faecal sources (Furtula et al., 2012a).

The fate of faecal steroids after discharge into waterways

Similar to the findings for sewage effluent, aerobic degradation of faecal steroids occurs in the water column within 2 weeks (Switzer-Howse and Dukta, 1978). Once the steroids, are incorporated into sediments, then further degradation is limited. Therefore, if the faecal steroids associate with particulate matter, it is likely that they will enter the sediments prior to complete degradation and provide a long term signature of faecal contamination (Leeming et al., 1996).

Switzer-Howse and Dukta (1978) contrasted the degradation rates of steroids by natural microbial populations present in water with that of single bacterial species, which had been cultured on media containing coprostanol or cholesterol as their sole carbon source. They noted that biodegradation was most efficient with an assemblage of natural microbial populations as present in aquatic samples. The degradation of coprostanol in marine waters was investigated to determine its rate of decomposition in seawater (Marty et al., 1996). A mixture of human effluent and seawater was incubated at 15°C in the dark. In the particulate fraction, the percentage of 5 β -stanols (coprostanol and 24-ethylcoprostanol) compared with total steroid composition remained relatively constant throughout the 60-day incubation. The researchers concluded that particulate steroids retained their anthropogenic signature during the first two weeks of decomposition of organic matter in seawater confirming the reliability of 5 β -stanols as tracers of anthropogenic waste in coastal waters.

Bartlett (1987) investigated the degradation of coprostanol in artificial sediments overlaid with seawater to mimic marine sediments. Overall, steroid concentrations remained largely unchanged. Nishimura and Koyama (1977) showed that where sediments are anoxic, steroids are not expected to be degraded. The coprostanol content in pure sludge-derived sediment was determined to be 33% of the total steroid concentration. Reported coprostanol levels of 10-15% in sediments are suggestive of approximately half of the organic matter being derived from sewage inputs (Hatcher and McGillivray, 1979). The percent coprostanol can, therefore, provide a historical record of the degree of sewage contamination.

In conclusion, research results suggest that levels of coprostanol in the water column would be reduced by the following factors: aerobic degradation, dilution effects, the physical transport of water currents and incorporation into sediments. Bartlett (1987) suggests that after 20 days, only a continuous input of coprostanol would be detectable in the water column. If the

sediments were anaerobic, coprostanol would be expected to persist, with reduction in sediment levels attributed to physical transport processes. Furthermore, degradation rates of individual steroids in sewage and aquatic environments are similar to each other, thus maintaining the faecal steroid signature used for source tracking.

1.4.2 The persistence of Fluorescent Whitening agents in the environment

FWA are susceptible to photodegradation, which is preceded by the faster photoisomerisation reaction from the adsorptive, fluorescent trans form to the non-adsorptive cis isomer (Kramer et al., 1996). Kramer et al. (1996) noted the UV shielding effect of dissolved natural organic material reducing photodegradation rates of FWA. It has been shown that alcohols are the major photo-oxidation products of DAS1. Unlike their precursor, the photolysis products of DAS1 are biodegradable (Guglielmetti, 1975). FWA do not come into contact with light during the washing process and transportation through the sewers, and it is only once the raw sewage reaches the treatment plant that isomerisation plays a role. During the primary treatment process there is too much particulate matter present for light to penetrate the water column, therefore, it is only in the secondary effluent that the isomers reach a steady state, which has been reported as 75% cis and 25% trans (the fluorescent/adsorptive form) for DAS1 (Poiger et al., 1996). Under summertime sunlight conditions, therefore, most of the DAS1 would be in a non-adsorptive isomer form and less likely to be removed by sedimentation in sewage (Poiger et al., 1996).

FWA are degraded by chlorine products, including household hypochlorite bleach, resulting in the decomposition of their fluorescent structure (Burg et al., 1977). The high molecular weight of FWA makes it unlikely that they would volatilise to a significant degree, therefore, gas exchange is not expected to be a route for removal of FWA (Poiger et al., 1999; Stoll et al., 1998). Adsorption to cellulosic substrates such as tissue paper and faeces, however, is a major source of removal of FWA onto wastewater solids. Concentrations of dissolved FWA in raw and primary treated effluent had similar ranges (Ganz et al., 1975; Hayashi et al., 2002; Poiger et al., 1996; Poiger et al., 1998). Concentration of DAS1 in raw sewage has been reported as mean 10.5 ± 2.8 $\mu\text{g/L}$, and 6.9 ± 2.2 $\mu\text{g/L}$ in primary effluent. This concentration, decreased significantly in secondary effluent treatment to 2.4 ± 0.3 $\mu\text{g/L}$ (Poiger et al., 1998). Levels of FWA found in anaerobically digested sewage sludges are in the range 85-170 mg/kg dry matter (Poiger et al., 1993).

Poiger et al. (1998) observed a lack of FWA biodegradation in sludge under any atmospheric conditions in both the aerobic activated sludge system, and after six weeks of anaerobic digestion. They concluded that adsorption to sludge was the main route for removal of

FWA from wastewater. The fate of FWA in soil is unknown, but their strong adsorption to sludge and lack of biodegradation suggest they might be adsorbed to soil and thus accumulate with repeated application of sewage sludge to land (Poiger et al., 1998).

FWA concentrations in receiving waters

Under natural sunlight in freshwater, the isomer distribution will favour the non-adsorptive isomer of DAS1 (>80%) (Canonica et al., 1997). River and lake studies have shown that the main processes of removal of FWA were photolysis and photodegradation in the top few metres of the water surface and partitioning of FWA between the water column and sediment (Poiger et al., 1993; Poiger et al., 1999; Stoll et al., 1998). Macrophytes may also reduce the efficiency of photolysis. Although there is no data available, it is assumed that FWA adsorb to macrophytes in rivers as well as sediments (Poiger et al., 1999). Stoll et al. (1998) established through mathematical modelling that hydrolysis of FWA in lake water was negligible and did not account for its removal.

The lack of biodegradability of FWA results in large percentages of FWA being discharged from rivers and transported through estuaries and deposited in coastal and open-ocean sediments. The FWA are preserved in these sediments under relatively stable conditions. DAS1 concentrations in estuarine sediments ranged from 0.02-1.55 µg/g (Managaki and Takada, 2005), which was similar to concentrations (3.3 µg/g, DAS1) reported for river sediments in Switzerland (Poiger et al., 1993). In a study evaluating FWA discharges into Tokyo Bay, the trend in FWA concentrations was for a decrease in concentration with distance from the shore (Managaki and Takada, 2005).

Based on the stability of FWA in the environment, due to their resistance to biodegradation and hydrolysis, it has been suggested that they could be used as molecular markers of wastewater produced from manufacturing plants and municipal communities (Stoll and Giger, 1998). In conclusion, the main routes for removal of FWA in fresh, estuarine and marine waters are likely to be dilution effects, adsorption to particulate matter such as sediments, and the process of photodegradation. Below the photic zone, it is expected that FWA will persist in sediments and water.

1.4.3 PCR marker persistence in the environment

An increasing number of studies on the decay of PCR markers have consistently shown that reduced temperature, higher salinity, lower sunlight inactivation and reduced predation are factors that contribute to the persistence of PCR markers in the aquatic environment (Bell et al., 2009; Dick et al., 2010; Gilpin et al., 2013; Green et al., 2011; Kreader, 1998; Okabe and

Shimazu, 2007; Schulz and Childers, 2011; Silkie and Nelson, 2009). Inhibition of bacterial predation increased the persistence of PCR markers targeting *Bacteroides distasonis* from 1-2 days to at least 14 days at 24°C (Kreader, 1998). In the same study, temperature effects showed that PCR markers were detected for 14 days at 4°C, decreasing to one day at 30°C. In general, water temperatures below 16°C have been noted to contribute to extending the DNA signal from bacterial targets.

The impact of sunlight on the persistence of PCR markers has been investigated by comparing dark and sunlit microcosms over periods of up to 28 days with a mixture of results. The studies of Bae and Wuertz (2009) and Walters and Field (2009) concluded that there was no significant sunlight-induced degradation of the human-specific Bacteroidales marker. Walters et al. (2009) and Gilpin et al. (2013) did identify a significant decrease in detection of human Bacteroidales in sunlit versus dark microcosms. Korajkic et al. (2014) concluded that sunlight and aquatic microflora are both important factors in degradation of FIB and PCR markers within a few days of discharge, but after this period, aquatic microflora (predation and bacterial competition) have the major influence on decay rates.

Salinity effects resulting in increased persistence of PCR markers has been attributed to inactivation of predators in saline conditions (Okabe and Shimazu, 2007). In one study, the effect of saline conditions showed that the persistence of seven Bacteroidales and one enterococci PCR marker was longer in marine compared with freshwater microcosms (15 L) exposed to natural sunlight (Green et al., 2011). This finding was different to Bae and Wuertz (2015), where the lag phase was on average 3.1 days longer in marine water, but after the lag phase, decay was more rapid in the marine water.

There has been conflicting evidence about similar decay rates for the Bacteroidetes/Bacteroidales markers (general and host-specific markers), which would preclude using ratios between PCR markers to apportion the contribution of human associated faecal pollution (Dick et al., 2010; Green et al., 2011; Silkie and Nelson, 2009). Dick et al. (2010) observed differences in the persistence of Bacteroidales host-specific PCR markers associated with sediments. Re-suspension of the sediments at the end of their experiment returned general Bacteroidales PCR marker in overlying water to 50% of the initial concentration, compared with 1% for the specific host-markers. From these experiments it can be concluded that a thorough understanding of the faecal source(s) and catchment under investigation is required to understand the persistence of MST markers and the potential impacts of environmental conditions on MST results.

1.5 Limitations of current methods of faecal identification

An important area of research identified for MST is the persistence of PCR markers in the environment. Research has shown the ability of conventional indicator bacteria to survive in environmental reservoirs (Anderson et al., 2005; Halliday and Gast, 2011) and the focus is now shifting to the survival and persistence of the bacteria used as targets for host specific MST (Dick et al., 2010; Green et al., 2011). Bacterial communities belonging to the large Bacteroidetes group used as PCR targets for general, non-specific sources of faecal contamination have been identified in association with the green alga, *Cladophora* (Olapade et al., 2006). A study by Whitman et al. (2014) also found free-living *Bacteroides* species associated with *Cladophora* mats, which were not genetically, closely related to enteric *Bacteroides* species but would still be amplified by the general faecal PCR markers. However, the study suggested that this group of free-living *Bacteroides* may not impact the assessment of host-specific markers, which target narrower bacterial groups.

1.5.1 Correlations between FIB, FST markers and pathogens

The variable persistence of microbial and FST indicators in the environment as outlined in previous sections confounds their role as indicators of a fresh faecal event when they are identified in a waterway. The presence of potential pathogens associated with the indicators needs to be ascertained to understand the potential for serious health implications to water users. However, the ability to predict pathogens in aquatic environments has been investigated by researchers with mixed results for indicator-pathogen combinations, using both traditional FIB, and FST markers (Harwood et al., 2014; Kapoor et al., 2013; Nshimyimana et al., 2014; Savichtcheva and Okabe, 2006; Savichtcheva et al., 2007; Wu et al., 2011).

There is agreement that no one indicator is sufficient to predict all pathogens (bacteria, viruses and protozoa) because of the varying environmental characteristics of water bodies and differences in survival/persistence of microbes in sediments and water. Pathogen concentrations also vary due to the source of faecal contamination and temporal carriage in the host community, hence the practicality of combining faecal source tracking with identification of contamination inputs (Mulugeta et al., 2012; Wu et al., 2011). Savichtcheva et al. (2007) noted a predictive relationship in water between human-specific *Bacteroides* PCR markers and pathogenic *E. coli* and *Salmonella*. This relationship was significantly improved when the PCR marker concentration was greater than 10^3 copies/100 mL.

Harwood et al. (2014) reviewed four epidemiological studies where rates of illness of bathers was correlated to microbial indicators/pathogens detected by conventional microbial

indicators and PCR (including qPCR) markers of human pollution. Despite surveying large numbers of people (n = 1,000-21,000), few correlations were observed with bathers compared with the control groups. More success has been achieved using qPCR markers for enterococci to predict the case numbers of swimming-related illnesses (Wade et al., 2008; Wade et al., 2006; Wade et al., 2010).

1.5.2 Faecal ageing

An important parameter to establish when investigating a potential faecal contamination event is whether the *E. coli* or enterococci levels measured are related to a recent faecal input or historical inputs due to persistence or survival of the FIB in the environment. Below is a review of some of the proposed methods for assessing faecal aging.

The ratio between coprostanol and epicoprostanol has been investigated as a way to distinguish between fresh and aged/treated human sewage. Epicoprostanol is present in low amounts in human faeces, however both cholesterol and coprostanol are converted to epicoprostanol during the treatment process of anaerobic sludge digestion (McCalley et al., 1981; Seguel et al., 2001). A high ratio of coprostanol to epicoprostanol, therefore, is indicative of fresh and/or untreated human pollution, whereas a low ratio is suggestive of treated human waste and/or aged pollution (Mudge and Duce, 2005).

Dyes such as propidium monoazide (PMA) can be used to assess bacterial viability because they intercalate with DNA after exposure to light and prevent amplification of the DNA by PCR (Kacprzak et al., 2015; Nocker et al., 2007). If the cell membrane is compromised, PMA diffuses into the cell inactivating the DNA, resulting in no MST signal. Implementation of methods of qPCR which utilise PMA to inhibit amplification of DNA from non-viable cells or extracellular DNA are gaining momentum as potential indicators of fresh faecal inputs (Bae and Wuertz, 2009; Bae and Wuertz, 2012; Bae and Wuertz, 2015). Bae and Wuertz (2009) describe a simple equation for determining faecal age:

$C_{p \text{ PMA}} / C_{p \text{ cycle}} - C_{p \text{ without PMA}}$, where $C_{p \text{ PMA}}$ is the cycle threshold number (C_p) for the qPCR marker with PMA treatment, $C_{p \text{ cycle}}$ is the total number of cycles in the qPCR (for example 40 cycles) and $C_{p \text{ without PMA}}$ is the C_p of total DNA as measured by normal qPCR.

Ratio of atypical colonies and total coliforms (AC/TC)

The ratio between numbers of atypical colonies and total coliforms (AC/TC) as measured by the standard method of total coliform detection has been used as an indication of the age of the faecal input to a river system (Brion, 2005; Brion et al., 2002b; Chandramouli et al., 2008; Nieman and Brion, 2003; Reed et al., 2011; Ward et al., 2009). The AC/TC ratio is measured by

the membrane filtration method as outlined in Standard Methods for the Examination of Water and Wastewater (18th Edition) for enumeration of total coliforms.

Nieman and Brion (2003) reported that an influx of fresh faecal material into a river system results in an increase in the numbers of total coliforms derived from sewage, which displace the background microflora normally associated with the waterway. The group of bacteria called Total Coliforms (TC) is comprised of facultative anaerobic and aerobic non-spore forming bacteria that are gram-negative and rod-shaped and able to ferment lactose, resulting in the production of aldehydes and gas, within 24 hours at 35°C (APHA, 1998). Faecal coliforms (FC) are a thermotolerant subset of TC and include the genus, *Escherichia coli*. FC produce gas from lactose when incubated at the higher temperature of 44.5°C. Bacteria in the TC grouping belong to the family of Enterobacteriaceae. TC are identified as those microbes that produce a red colony with a green metallic sheen when cultured on an Endo-type medium for 22-24 hours at 35°C. The endo medium contains a fuchsin-sulphite indicator which turns red when the coliforms utilise the carbon in the indicator.

Atypical colonies may be detected alongside coliforms on endo medium and are characterised as those colonies that appear as dark red/pink with no metallic sheen when incubated under the same conditions as total coliforms (APHA, 1998). The atypical red colonies were considered to be a nuisance when using endo medium for detection of total coliforms, however it was hypothesized that a large proportion of atypical colonies (AC) are indigenous to waterways (Brion and Mao, 2000; Brion et al., 2000). This indigenous group of river microflora has been shown to be relatively stable in comparison to TC levels in rivers, although numbers may fluctuate dependent on seasonal variation and nutrient inputs. Brion and Mao (2000) characterised atypical colonies on endo medium and identified AC colonies belonging to the microbial species of *Aeromonas*, *Salmonella*, *Pseudomonas* and *Vibrio*. The presence of AC on media used for counting TC has been used as a useful internal reference for assessing inputs of TC relative to the normal background count of the river microflora.

AC/TC ratios in fresh manure start at values <1 and increase with faecal aging. For fresh human sewage the AC/TC values are <1.5. Care is required, however, when evaluating ratios associated with domestic sewage which is a composite of faecal material varying in age. Table 4 shows a variety of environmental studies examining the values of the AC/TC ratio in relation to faecal contamination events/sources. In water, AC/TC ratios of <5.0 suggest the input of fresh faecal material (Brion, 2005) due to high numbers of total coliforms. The ratio rises over time as the faecal material ages and total coliforms die-off in the aquatic environment. In addition, there may be an increase in AC as a result of nutrient influx associated with sewage inputs. The aged

faecal material gives off a higher AC/TC ratio (>20) than for fresh faecal material indicating the passage of time.

Table 4: AC/TC ratios associated with faecal contamination events and sources

Source	Event	Average AC/TC ratio	Reference
Kentucky River	Heavy rainfall	3.0	Brion et al. (2002b); Nieman and Brion (2003)
	Day 3 after storm	10.0	
	Day 7 after storm	79.0	
Fresh cow manure	Day 1	<1.0	Brion (2005)
	Day 14	2.9	
Fresh horse manure	Day 1	<1.0	
	Day 14	11.8	
Fresh human sewage	Day 1	<1.0	
Impounded suburban runoff		103.0	
Flowing suburban		18.9	
Human sewage		3.9	
Flowing agricultural		10.0	
Urban watershed		25.1	Brion and Mao (2000)
Mixed-use watersheds		23.4 and 15.5	
Human sewage		1.5	



Figure 2: Swimming in Aotearoa, Taihape, North Island/Te Ika a Māui. Photo credit: Greg Devane

1.6 Research aims

The aims of the research presented in this thesis were to identify limitations of existing microbial water quality indicators, and to refine and/or develop alternative, improved indicators for determining the source of faecal contamination in urban and rural surface waters.

Objectives:

- 1) To determine the temporal and spatial correlations between FST markers (fluorescent whitening agents, faecal steroids and PCR markers), FIB and pathogens in an urban waterway impacted by discharges of untreated human sewage.
- 2) To determine the temporal correlations between existing FIB and FST markers (faecal steroids and PCR markers) in rural faecal pollution sources and determine rates of mobilisation decline for FST PCR markers in bovine faecal sources.
- 3) To identify cost-effective refinements to current tools and alternative, practical approaches for improved water quality monitoring by assessing microbial and FST methods and their best application to both urban and rural environments. To validate/identify faecal ageing markers for inclusion in the FST toolbox to enable discrimination between recent and historical faecal inputs to urban and rural waterways.

The first objective of this thesis (Chapter Three) was an evaluation of the faecal source tracking (FST) markers and their associations with current microbial indicators of faecal contamination and pathogens. This objective was undertaken in an urban river, which was receiving major discharges of raw human sewage after the 2010-2012 earthquakes in Christchurch, NZ. The urban river study assessed indicators in the water and sediment to identify refinements for improved water quality monitoring. To fulfil the spatial and temporal evaluation of indicators and pathogens, samples of water and sediment were collected from three sites along the river during the discharges and for half a year post-discharge, followed by additional sampling a year later. Hereafter, this objective will be referred to as the “urban river study”.

The second objective (Chapter Four) focused on rural pollution sources and involved a temporal evaluation of the FST signal as cowpats aged under field conditions. Cowpats were subjected to irrigation treatments, and mobilisation of *E. coli* and FST markers was effected by simulated flood and rainfall events. This objective will be referred to as the “rural study”. The overall hypothesis was that changes in the microbial and chemical composition of ageing cowpats would impact the FST signature from PCR markers and faecal steroids. The hypothesis was investigated by monitoring analytes of the FST markers for changes in concentration/ratio which would affect interpretation of faecal source signatures derived from the cowpat. Shifts in the microbial community of the decomposing cowpats were illustrated using an amplicon-based

metagenomic sequencing approach to identify members of the microbial community mobilised from the cowpat. Furthermore, microbial and FST markers were monitored in both the urban river and rural cowpat experiments for potential markers that would signal a change to an aged faecal environment.

The third objective was to integrate the findings of Objectives One and Two to provide a cohesive framework of recommendations for improving interpretations of current water quality tools in urban and rural settings, and provide supplementary indicators for discrimination between recent and historical faecal sources. These recommendations for improved water quality tools are outlined in Chapter Five.

1.7 Overview of the thesis structure

Chapter One is an introduction to the health hazards associated with faecal contamination and its impact on aquatic environments. It discusses the current conventional microbial methods of indicating a faecal contamination event, and the more sophisticated tools for faecal source tracking, with a discussion of each of their limitations. Chapter Two outlines the analysis methods used to evaluate the microbial indicators, pathogens and FST toolbox and validate/identify faecal ageing markers.

Chapter Three encompasses the urban river study, and evaluated the correlation between current faecal source tracking (FST) markers, pathogens and conventional microbial indicators in an urban river environment impacted by continuous discharges of raw human sewage. This unusual situation provided the location for monitoring the levels of faecal markers and pathogens in sediments and the overlying water column during sewer overflows, and post-discharge. Evaluation post-discharge included tracking the fate of indicators and pathogens in sediments to understand the contribution of sediments as a source of pathogens and indicator markers with the potential to confound health risk assessment and water quality monitoring, respectively.

One paper was written from the urban river study and published in *Science of the Total Environment*. It presented the results on the microorganisms in sediment and water and the correlation between microbial indicators and pathogens:

Devane ML, Moriarty EM, Wood D, Webster-Brown J, Gilpin BJ. The impact of major earthquakes and subsequent sewage discharges on the microbial quality of water and sediments in an urban river. *Sci Total Environ* 2014; 485-486: 666-80.

Chapter Four represents the rural study and reports on the changes in the microbial community and FST markers mobilised from the cowpat under the influence of various methods

of generating runoff. It evaluated the hypothesis that the faecal source signature from cowpats, as measured by FST markers, would change as the cowpat deteriorated on pasture over a five and a half month period. This rural study was composed of two trials investigating mobilisation of FIB and FST markers (PCR markers and faecal steroids) from the decomposing cowpats. It was hypothesised that oscillations in the microbial and chemical composition of the ageing cowpats would occur as the cowpat microbial population fluctuated with nutrient status and differences in water activity. Mobilised FST analytes from the cowpats were, therefore, assessed for changes in their faecal signature which might impact on interpretation of source determination.

Chapter Five is a review of the human health risk associated with different sources of non-human and human faecal pollution, so that specific recommendations for a particular faecal source can be made to guide water quality management. Chapter Six summarises and discusses the main results and presents recommendations for improving water quality monitoring of faecal contamination while suggesting additional research initiatives.

Author's contributions

The papers generated by this thesis are multi-authored, which reflects the team approach of my half-time employer, Environmental Science and Research Ltd (ESR). The design of the Avon/Otākaro River experiment (Chapter Three) was an integration of my contributions and ideas from Brent Gilpin and Elaine Moriarty based on direction from our funders. I had particular input to the FST strategy applied to this urban river study. Sampling of water and sediment from the Avon/Otākaro River was performed by the ESR team, including myself. The design and implementation of the two cowpat faecal ageing experiments was my own. I was assisted in the field with sampling by my work colleagues.

For the urban river study, I performed sediment processing in preparation for microbial analyses. Protozoa in water were sub-contracted to the Massey University. For both studies, in conjunction with the team, I performed microbial analyses of *E. coli* and the faecal ageing ratio AC/TC, and the extraction of water and faeces for PCR markers. Technical analysis of PCR markers was performed by Beth Robson and I carried out data analysis of PCR markers. Faecal steroid analysis and fluorescent whitening agents were analysed under sub-contract by the Food Chemistry team at ESR. I performed laboratory procedures on cowpat DNA extractions in preparation for sequence analysis and metagenomic analyses of the bacterial sequences. I carried out the data analysis, the writing of papers and this dissertation with the guidance of my supervisors.

2 Chapter Two

Analytical Methods

This methods section contains all of the analytical methodology for microbial indicators, pathogens and faecal source tracking markers (faecal steroids, PCR markers and fluorescent whitening agents (FWA)) used in the urban river and rural studies. This chapter also includes the methods used for the amplicon-based metagenomic analysis of the mobilised fraction from decomposing cowpats in the rural study. The site locations, strategies for sampling and experimental design for the urban river and rural studies are found in Chapters Three and Four, respectively. Also included in these experimental chapters are the data collection of relevant physical parameters and the statistical approaches used for the individual studies.

The urban river study involved the concurrent collection of water and underlying sediment from three sites along the river to investigate the relationships between microbial indicators, FST markers and pathogens. Sampling occurred over approximately seven months during the continuous sewage discharges and then continued post-discharge with the last collection eighteen months after cessation of discharges. Water and sediment were analysed for the microbial indicators, *Escherichia coli*, *Clostridium perfringens* and F-RNA phage; FST markers (quantitative Polymerase Chain Reaction (in water only), and faecal sterols and fluorescent whitening agents (FWA)), the faecal ageing ratio of atypical colonies/total coliforms (AC/TC), and the pathogens, *Campylobacter*, *Giardia* and *Cryptosporidium*.

The rural study was composed of two trials, conducted over separate summer periods and investigated mobilisation of *E. coli* and FST markers (PCR markers and faecal steroids) from decomposing cowpats under simulated flood and rainfall conditions. Changes in the concentrations of water quality analytes and their mobilisation from the ageing cowpats were evaluated to determine their impact on faecal source identification. Mobilisation of analytes from cowpats was initiated under 1) conditions that simulated re-suspension (termed the supernatant) of the cowpats as occurs during a flood event, and 2) the impact of simulated rainfall. Trial 1 investigated the re-suspension of irrigated and non-irrigated cowpats on mobilisation of analytes. In contrast, Trial 2 compared mobilisation rates from non-irrigated cowpats subjected to either a re-suspension event or simulated rainfall.

2.1 Microbial analysis

All dilutions for microorganisms from water and cowpat runoff samples were performed in 0.1% Peptone water (Fort Richards Laboratories, Otahuhu, NZ). In the urban river study, microorganisms in the sediments were measured after re-suspension of a known amount of sediment into a sterile diluent of ¼ strength Ringers Solution (Merck, Darmstedt, Germany), rather than directly analysing the sediment. The sediments were allowed to stand for 30 min to allow the bulk sediment to settle, and then overlying water was decanted and discarded. Sediment samples were mixed to ensure a homogenous suspension and a subsample of 10 g placed in a sterile bottle. Ringers Solution (¼ strength) was added to sediment to create a 10-fold dilution. The suspension was mixed by hand for 2 min, allowed to settle (< 5 min) and a volume of the supernatant was eluted and further 10-fold dilutions using Peptone water were undertaken. All sediment concentrations were reported as counts per gram dry weight of sediment.

2.1.1 *E. coli*

E. coli was the only microorganism tested in both the urban river and the rural study. For the urban river study, duplicate samples (1 mL) from tenfold dilutions of water or sediment suspension were analysed by the standard pour plate technique (APHA, 2005) using Chromocult® *E. coli* agar (Merck). The plates were inverted and incubated at 30°C for 4 h, followed by 37°C for 20 h. The blue-violet colonies of *E. coli* were counted and the detection limit of the method was <50 CFU/100 mL and <10 CFU/g dry weight.

For Trial 1 and 2 of the rural study, duplicate samples of either re-suspended cowpat (supernatant) and rainfall runoff were analysed neat and/or diluted tenfold and 1 mL of appropriate dilutions (n = 4) were filtered in 99 mL of sterile water through 47-mm, 0.45-µm cellulose ester membrane filters (Millipore, France). In the latter stages of the trials, when mobilised *E. coli* concentrations were reduced, up to 200 mL of supernatant or rainfall runoff was filtered. Following filtration, membranes were incubated on Brilliance *E. coli* agar (Oxoid, Basingstoke, UK) at 44°C for 24 hours. The blue-violet colonies of *E. coli* were counted and the detection limit of the method was <1 CFU/100 mL.

2.1.2 *F-RNA phage*

F-RNA phage analysis of water and sediment samples was by overlay pour plating of 1 mL volumes of serial dilutions according to the Male-Specific Coliphage assay protocol described in APHA (2005) using the host strain *E. coli* HS(pFamp)R and agar preparation as described in Debartolomeis and Cabelli (1991). The *E. coli* HS(pFamp)R is a non-pathogenic *E. coli*, which

carries a plasmid under ampicillin selection, which encodes pilus production, and is therefore, host specific for F-specific bacteriophage. This *E. coli* HS(pFamp)R strain is resistant to streptomycin sulphate and somatic coliphages T2 to T7 and ϕ X174 (Debartolomeis and Cabelli, 1991). Plates were inverted and incubated for 18 ± 2 h at 35°C in the dark. Plates were examined by eye for slightly opaque plaques and were expressed as plaque forming units (PFU)/100 mL. Phage limit of detection was <50 PFU per 100 ml and <10 PFU/g dry weight.

2.1.3 *Clostridium perfringens*

To quantify spores of *C. perfringens* (Bisson and Cabelli, 1979), river water and sediment suspension (20 mL of 10 g/100 mL) were brought to 65°C and held there for 15 min, then serially diluted in 0.1% peptone water. Duplicate samples (1 mL) were filtered through 47 mm diameter, 0.45 μ m cellulose ester membrane filters (Millipore, France) and placed onto modified Tryptose Sulfite Cycloserine (TSC) agar (Merck) containing 4-methylumbelliferyl phosphate (MUP), 100 mg/L disodium salt (Sigma Aldrich, St. Louis, MO, USA) and 400 mg/L D-cycloserine (EMD Chemicals Inc. San Diego, USA). Plates were incubated in a modified atmosphere of less than 1% oxygen and 9 – 13% carbon dioxide using MGC AnaeroPack System (Mitsubishi Gas Chemical Company, Inc., Japan) and an anaerobic indicator strip (Oxoid) at 44°C for 24 h. Following incubation, those colonies which were black in colour and fluoresced under long wave UV light (365 nm) were enumerated. Limit of detection of *C. perfringens* was <50 CFU/100 mL and <10 CFU/g dry weight.

2.1.4 *Campylobacter* spp.

Campylobacter spp. were enumerated using a 3 \times 3 most probable number (MPN) procedure. Samples of water (1, 10, 100 mL), and 1 g of sediment and appropriate 1 mL dilutions of 10 g/100 mL sediment suspension ($n = 3$) were filtered in triplicate through 47 mm, 0.45 μ m cellulose ester membrane filters (Millipore). Enrichment and enumeration of campylobacters in m-Exeter Broth (Fort Richards Laboratories) followed the procedure of Moriarty et al. (2012). Limit of detection was <1 MPN/100 mL and <0.3 MPN/g dry weight.

2.1.5 Analysis of Protozoa in water

River water samples were processed according to USEPA 1623 (USEPA, 2001) for the detection of *Cryptosporidium* and *Giardia*. Water samples (≤ 30 L) were filtered through a woven cartridge (Filta-Max, IDEXX laboratories, Maine, USA) and the cartridge was processed by MicroAquaTech (Palmerston North, NZ). The limit of detection is dependent on the recovery rate from the samples. Typical recovery rates in these sample matrices were 40%.

Isolation of *Cryptosporidium* spp. and *Giardia* spp. from river sediment

River sediment samples (20 g) were weighed out into sterile glass bottles (100 mL capacity). Eighty mL of Phosphate Buffered Saline (BR14; Oxoid) containing 0.1% of Tween 20 (Sigma Aldrich) (PBST) was added to the sediment. The bottle was shaken by hand for 5 min. Following shaking the sample was filtered through a sterile 45 mm stainless steel sieve. The filtrate was collected via a funnel into two sterile 50 mL centrifuge tubes (Corning, UK). A further volume (10 mL) of PBST was added to the glass bottle. The bottle was shaken and the contents poured into the centrifuge tube via the sieve and funnel. This process was repeated to elute any remaining (oo)cysts. The tubes were centrifuged at 2500 gravitational force (g) for 15 min with high acceleration in the absence of a brake during deceleration. Following centrifugation approximately 30 mL of supernatant was aspirated from each of the centrifuge tubes. The contents of the tubes were amalgamated into one centrifuge tube. PBST (10 mL) was added to the empty centrifuge tube and it was vortexed for one minute. The contents were added to the centrifuge tube containing the sample and the tube was centrifuged again using the same conditions as before. Following centrifugation, the supernatant was aspirated until approximately 5 mL of sample remained. The sample was re-suspended using a sterile Pasteur pipette and transferred to a sterile labelled Leighton tube (Dynal, Biotech ASA, Oslo, Norway). PBST (5 mL) was added to the centrifuge tube and vortexed for 1 minute. The liquid was added to the Leighton tube and immunomagnetic separation (IMS) for *Cryptosporidium* and *Giardia* was carried out according to the manufacturer's instructions (Dynal Biotech ASA, G-C Combo kit, Oslo, Norway). Following IMS, prepared slides were enumerated using fluorescein isothiocyanate (FITC) labelled antibodies for the detection of *Cryptosporidium* spp. and *Giardia* spp. (Waterborne Inc., New Orleans, LA, USA) according to the manufacturer's instructions. The entire surface of the slide was viewed using an epifluorescent microscope and any suspect (oo)cysts were recorded, photographed and measured.

Rate of recovery of *Cryptosporidium* and *Giardia* from sediment

Sediment samples (20 g) were weighed out into sterile glass bottles (100 mL capacity). PBST (80 mL) was added to the sediment along with a vial of Colorseed (BTF, Australia) containing 100 inactivated *Cryptosporidium* and *Giardia* (oo)cysts. The sample was processed as normal including IMS and FITC staining. During enumeration of the slide all suspect (oo)cysts were viewed initially under FITC and then under Texas red filter (580 nm excitation wavelength, 615 nm emission wavelength). (oo)Cysts that were stained apple green under FITC filter and red under Texas red filter were enumerated as the spiked Colorseed (oo)cysts. Those which were

only fluorescent under FITC and not Texas red were enumerated as non-spiked *Cryptosporidium* and *Giardia* naturally present in the sample. The rate of recovery was calculated as the number of (oo)cysts enumerated as a percentage of those added to the sample. Typical recovery rates from these sediment samples were 10%.

2.1.6 Faecal ageing ratio: AC/TC

The AC/TC ratio was evaluated in both the urban river and the rural study. In the urban river study, ten-fold dilutions of each water or re-suspended sediment sample were prepared in 0.1% peptone water as appropriate (n = 4). Duplicate samples (1 mL) from neat water and/or appropriate dilutions of water or sediment suspension were filtered through 47 mm diameter, 0.45 µm cellulose ester membrane filters (Millipore) and placed onto modified (m-Endo) agar (Fort Richard Laboratories). Plates were incubated at 35°C (±1°C) for 22 (± 2) hours. After incubation, atypical colonies were enumerated by counting pink/red colonies and Total coliforms were enumerated by counting colonies with a green metallic sheen. The AC/TC ratio was calculated by dividing AC (CFU/100 mL) counts by TC (CFU/100 mL) counts.

In Trial 2 of the rural cowpat studies, to provide a background river microflora for the AC counts, the cowpat supernatant and rainfall runoff were diluted 1:10 into freshly collected water from a local stream to simulate overland flow of cowpat runoff into a waterway. The procedure for AC/TC then followed the same protocol as the urban river water study. A blank of appropriate aliquots or dilutions (n = 4) of freshly collected stream water was included in sampling runs and TC counted. To evaluate only the cowpat derived TC, prior to calculating the AC/TC ratio, the concentration of TC in the stream water (blank) was subtracted from the concentration of TC in the cowpat plus stream water.

Quality control for microbial analyses

All microbial analyses included incubation of appropriate positive and negative controls of bacterial species on the appropriate media and with sterility controls for each media type to confirm the performance and non-contamination of media.

2.2 Dry weight analysis

For cowpat Trial 1, a one kg equivalent (half of the cowpat) was used for dry weight analysis by splitting it into duplicate analyses. For Trial 2, one cowpat was used for dry weight analysis with 3 replicates of 100 g each. For each trial, samples of cowpat were distributed into pre-weighed foil trays, and weighed, before placing in 105°C oven for 2-3 days. Samples were then reweighed on consecutive days until changes in weight were within 0.06 g. Dry weight of

sediment from the urban river study was determined in a similar manner by drying a subsample of sediment (10–20 g) in a 105°C oven until there was no significant weight change (APHA, 2005).

2.3 PCR markers

Quantitative PCR (qPCR) assays used in this study are presented in Table 5, alongside their animal, human or avian host. Also indicated in the table is the bacterial gene targeted, and whether the qPCR assay was based on a Probe assay or a SYBR Green assay. Specificity testing of the PCR primers used in this study are provided in Devane et al. (2013) and Devane et al. (2007) and/or outlined in Table 6.

2.3.1 DNA extraction methods

DNA was extracted from urban river water samples according to the protocol of Dick and Field (2004). In brief, 150 ml river water samples were filtered through a Supor 200, 0.2 µM Polyethersulfone (PES) filter (Pall Corp. Washington Port, NY, USA). The filter(s) were immersed in 1 mL of guanidine isothiocyanate (GITC) buffer (5 M GITC, 0.1 M EDTA, 10% sarcosyl) and vortexed, after which they were frozen at -20°C. After thawing and repeated vortexing of the filter, DNA was extracted using the Qiagen DNeasy Kit (QIAGEN, Valencia, CA). Briefly, 700 µl AL buffer (supplied by manufacturer) was added to the filter and the mixture was vortexed and incubated for 5 min at room temperature. The supernatant was added to a spin column from the DNeasy kit, and the column centrifuged for 1 min at 15,700 g. The flow-through was discarded. This step was repeated until all of the supernatant was transferred to the spin column. The filter was then washed using the kit's reagents and the DNA eluted in 100 µl of elution buffer.

In the rural cowpat studies, the ZR Fecal DNA Kit™ (#D6010 Zymo Research, Orange, California, USA) was used to extract DNA from supernatant and rainfall extracts from Trials 1 and 2. During the initial stages of the cowpat trials when supernatant and rainfall runoff were dilute faecal slurries, 0.3-2 mL of cowpat supernatant were centrifuged at 4500 g for 10 min. Supernatant was discarded and the faecal residue (approximately 150 mg) was weighed and transferred into bead beater tubes with 750 µL of lysis buffer containing β-mercaptoethanol (Sigma-Aldrich Co., St. Louis, MO). In the following stages of each experiment when less faecal material was re-suspended in the supernatant and runoff, then 50-600 ml was filtered through 47-mm, 0.45-µm cellulose ester membrane filters (Millipore, France) and resuspended in mini bead beater tubes from the ZR Fecal DNA Kit™ with 750 µL of lysis kit buffer (containing β-

mercaptoethanol) and kit instructions followed. In brief, DNA was extracted using the protocol of the ZR Fecal DNA Kit™, including processing faeces and/or filter(s) in a bead beater (MixMate, EppendorfAG, Hamburg, Germany) for 5 min at 2000 g. The DNA was isolated and purified using the kit's series of Fast-Spin columns, DNA was eluted in 100 µl of elution buffer. The exception for the eluant volume was for the Trial 2 study, when due to dilute samples, 50 µL of elution buffer was used from Day 50 onwards.

2.3.2 PCR amplification conditions

PCR amplifications were performed in a total volume of 25 µl using 2 µl of DNA template for the urban river water samples and Trial 1 cowpat supernatants. For Trial 2 cowpat supernatants and runoff, 5 µL of DNA template was used in each PCR reaction to maximise detection of PCR markers. PCR conditions for the SYBR Green assays were as follows, 2 x LightCycler 480 SYBR Green I Master mix (Roche Diagnostics Ltd, Penzberg, Germany), 0.25 µM of each primer and 0.2 mg/ml of bovine serum albumin (BSA) (Sigma-Aldrich, Missouri, USA). Addition of BSA helps to mitigate inhibition of samples by organic substances such as fulvic and humic acids commonly found in environmental samples of water and faeces (Kreader, 1996). PCR conditions for the probe based assays were as follows: 2 x LightCycler 480 Probes Master mix (Roche Diagnostics Ltd), 100 nM of probe, 500 nM of each primer and 0.2 mg/ml of BSA (Sigma-Aldrich). All primer sets in this study used an annealing temperature of 60°C (Devane et al., 2013) and followed the protocol outlined for amplification.

Thermal cycling conditions for the LightCycler 480® (Roche Diagnostics Ltd) started with an initial denaturing cycle at 95°C for 5 min, followed by 45 cycles at 95°C for 10 s and 60°C for 10 s, and elongation at 72°C for 20 s. Standard curves were generated from 10-fold serial dilutions of the appropriate target cloned into *E. coli* DH5α (Invitrogen, Carlsbad, California, USA) using the pGEM-T Easy cloning kit (Promega, Fitchburg, Wisconsin, USA). A NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA), determined the DNA concentration and allowed for calculation of the copy number of target DNA extracts from plasmid constructs. Quantification cycle thresholds (C_p) were translated into gene copy (GC) numbers using single or master standard calibration models (Sivaganesan et al., 2010).

Quality Control for PCR analysis

At the time of each sample extraction event, a blank of sterile water (150 mL for urban river study and 200 mL for rural study) was filtered and the filter was extracted to monitor for potential DNA contamination. Each assay run included a positive control of faecal DNA extract

derived from a composite of approximately five faecal specimens from the target species, a non-template control (NTC) and the sample extraction blank.

Melting curve (T_m) analysis of SYBR assays began with a pre-incubation step at 95°C for 5 s, then 1 min at 65°C, followed by an increase in the temperature from 65°C to 97°C at a ramp rate of 0.11°C/s, and a cooling period at 40°C for 10 s. All amplicons were within 0.3°C of the plasmid standards on each LightCycler 480® run. If the T_m of duplicates was not within $\pm 0.3^\circ\text{C}$ of the standard T_m , or the C_p of duplicates for the probe assays was not within $\pm 1 C_p$, then another replicate of the DNA extract was analysed by qPCR, and the result scored as two out of three. Samples that registered a C_p value above 40 were recorded as not detected.

The amplification efficiency of the PCR marker assays was determined by collating the results of the single or master standard curves generated using 10-fold serial dilutions of known amounts of the PGemT easy plasmid carrying the cloned unique host target sequence. The slope of the standard curves was used to calculate the amplification efficiency (E) using the following formula: $E = 10^{-1/s} - 1$, where s is the slope. The amplification efficiency of the PCR assays was considered acceptable at >90% for PCR targets, and the coefficient of determination (r^2) at ≥ 0.92 for assays.

The limit of quantification (LOQ) of PCR markers was calculated as the lowest standard consistently detected in the standard curve, which was 20 gene copies per reaction for all qPCR markers. In the urban river study, where 150 mL of water was filtered, the PCR marker LOQ was 667 gene copies/100 mL for all PCR markers. In the rural study of cowpats, LOQ was based on gene copy (GC) per PCR reaction because the volume of the supernatant or runoff sample varied over time as the matrix became more dilute and higher volumes of sample were required. If the PCR marker was less than the LOQ it was reported as zero, except in the case of the urban study, where PCR markers with levels between 600 to 666 GC/100 mL were reported if detection of the faecal source was supported by steroid analysis.

For the Trial 2 experiment, a PCR inhibitor control using Cal Fluor orange 560 nm fluorescence detection (Bioline Reagents Ltd, London, UK) was used to verify presence/absence of inhibition in DNA extracts. Samples were monitored to see if the PCR inhibitor control registered a $C_p 31.0 \pm 1.0$. If the C_p was outside of this range, then inhibition was suspected and dilutions of 1:10 to 1:100 were performed and DNA samples without inhibition were used to calculate concentrations. In the urban river and Trial 1 study, dilutions were only performed if non-detection of the GenBac3 PCR marker suggested inhibition.

Table 5: PCR markers used in this study. The GenBac3 marker was used in both urban river and rural studies. The gray shading indicates the PCR markers used only in the urban river study, whereas the green shading indicates those markers used only in the rural study.

Target Host	PCR Assay	Bacterial target and/or gene target	Type of qPCR	Reference
General faecal indicator	GenBac3	Bacteroidetes (16S rRNA)	Probe	Siefring et al. (2008)
Human	B. adol	<i>Bifidobacterium adolescentis</i> (16S rRNA)	SYBR Green	Matsuki et al. (2004)
	HumBac (HF183)	Bacteroidales (16S rRNA)	SYBR Green	Bernhard and Field (2000)
	HumM3	Putative sigma factor	Probe	Shanks et al. (2009)
Ducks and other aquatic avian spp.	E2	<i>Desulphovibrio</i> -like sp. (16S rRNA)	SYBR Green	Devane et al. (2007)
Canine dominant	Dog	Bacteroidetes (16S rRNA)	SYBR Green	Dick et al. (2005)
Ruminant	BacR	Bacteroidales (16S rRNA)	Probe	Reischer et al. (2006)
Bovine	CowM2	Bacteroidales-like: protein involved in energy metabolism and electron transport	Probe	Shanks et al. (2008)

Table 6: Sensitivity and specificity of PCR markers used in the urban river and rural studies

Host associated PCR Markers	The percentage of of non-target host faecal samples positive for each PCR marker (n = number of samples tested)								
	Sensitivity	Possum n = 10	Human n = 16	Sewage n = 4	Cow n = 20	Sheep n = 20	Pig n = 10	Duck n = 10	Black swan n = 10
GenBac3	99%	100	100	100	100	100	100	100	90
HumM3	60%	100	63	50	0	0	0	0	0
HumBac (HF183)	65%	100	69	50	0	0	0	0	0
B. adol	60%	30 [‡]	43 (n =14)	100 (n = 6)	0	0	0	35 (n =23)	8 [‡] (n = 12)
*Ruminant specific BacR	100%	100	0	50	100	100	0	10	20
**Bovine Specific CowM2	60%	0	0	0	60	0	0	0	0
Avian (E2) qPCR	50%	0 (n = 3)	0 (n = 9)	0 (n = 3)	0 (n = 16)	0 (n = 7)	66 (n = 3)	50	NT
Avian (E2) Conventional PCR	42%	0 (n = 23)	0 (n = 13)	0 (n = 13)	0 (n = 9)	0 (n =3 [¥])	0 (n = 1)	76 (n = 42)	20
Canine	90 (n = 20)	0 (n = 7)	0 (n = 15)	60 (n = 5)	0	0	0 (n = 6)	0 (n = 14)	0

*Non-specific reactions for BacR at least three orders of magnitude below cows and sheep

**Recent (Dec 2015) studies of deer faeces have noted that 50% of 20 NZ deer faecal extracts were positive for CowM2, calling into question its bovine-specific status in NZ. It may be more appropriate to term CowM2 as ruminant-associated, however it was not detected in the ruminants: goats and sheep.

[‡] all Cp >36.7; [¥] composite faecal samples (n = 3 to 5 individual samples)

2.4 Metagenomic studies on irrigated cowpat faecal DNA extracts

As a pilot study, the DNA extracts from irrigated cowpat supernatant faecal samples in Trial 1 were analysed by next generation sequencing methods to identify changes in the microbial community in the cowpat as it aged over the course of the trial. One of the aims of this amplicon-based metagenomic analysis was to detect potential PCR marker candidates for ageing the faecal runoff from cowpats. In particular, the identification of bacteria that were present in high copy number in fresh faecal material and the identification of bacteria that were present in high number in aged faecal inputs. It was hypothesised that there would be a demarcation in time between the identification of these two bacterial targets. The irrigated cowpat supernatants were chosen for this analysis because of the similarities in PCR marker degradation between the two irrigation treatments. The triplicate supernatant samples from each of the ten sampling events were analysed separately to give 30 samples subjected to metagenomic analysis.

2.4.1 Amplicon preparation and sequencing

Genomic DNA extracted from irrigated cowpat supernatant faecal samples was used as templates for the amplification of the V1-V3 region of the 16S rRNA gene, using eubacterial primers Bac8F (5'-AGAGTTTGATCCTGGCTCAG-3') and Univ529R (5'-ACCGCGGCKGCTGGC-3') (Baker et al., 2003; Fierer et al., 2007). The V1-V3 region was chosen for amplification of the 16S rDNA in the rural study as it has been shown to provide a deep richness in the numbers of taxa identified with a high degree of classification accuracy, combined with less bias towards dominant taxon groups (Handl et al., 2011; Vilo and Dong, 2012; Wang et al., 2007). Unique eight nucleotide barcode sequences (Bystrykh, 2012) were incorporated at the 5' end of both primers as sample identifiers. Amplicons were prepared by PCR using the following cycling conditions: initial denaturation (95°C, 2 min), followed by 25 cycles of denaturation (98°C, 20 s), annealing (68°C, 15 s) and extension (72°C, 15 s). A final extension step followed the 25 cycles (72°C for 15 s). Each 50 µl PCR reaction contained 1x PCR buffer, 2 mM Mg²⁺, 0.3 mM dNTPs, 0.5 U Kapa High Fidelity polymerase (Kapa Biosystems, USA), 0.3 µM of each barcoded primer (Invitrogen, USA) and 2 µl of template DNA. Each PCR reaction was performed in duplicate, and pooled prior to purification. Amplicons were purified using Agencourt AMPure-XP beads (Beckman Coulter, USA). Amplicon samples were prepared by pooling samples in an equimolar ratio as quantified by the Qubit dsDNA HS Assay kit (Invitrogen, USA) and 10 ng/µL of DNA per sample was sent off for sequencing by Macrogen Inc. (Seoul, Korea) on a Roche 454 GS FLX platform.

Quality Control for amplicon-based metagenomic PCR amplification

Each PCR amplification assay of the V1-V3 region included a positive control of faecal DNA, and a non-template control and the sample extraction blanks from each sampling event to analyse for potential contamination.

2.4.2 Data analysis of cowpat faecal DNA sequences

Ligation of 454 sequencing adaptors and sequencing of the pooled PCR products was provided by Macrogen Inc (Korea), using a 1/8 region plate of a Roche 454 GS FLX platform for each sequencing sample. The raw data provided by Macrogen Inc., Korea, already had the sequencing adaptors removed and they provided both the FASTA and quality files. These files were used to initiate the QIIME pipeline (Quantitative Insights Into Microbial Ecology). QIIME is a Linux-based open source software package designed for comparison and analysis of microbial community data obtained from NGS amplicon sequencing (Caporaso et al., 2010). QIIME provides a pipeline that takes raw sequencing data through the filtering of data and demultiplexing, initial analyses, such as picking operational taxonomic units (OTUs), taxonomic assignment against established databases, such as the Ribosomal Database Project (RDP) classifier (Wang et al., 2007) and construction of phylogenetic trees. The RDP classifier assigns 16S rRNA sequences to bacterial taxonomy, based on the RDP naïve Bayesian rRNA Classifier, using the RDP 16S rRNA training set 9 (Cole et al., 2009). Chimera checking using ChimeraSlayer (Haas et al., 2011) was performed to remove false sequences derived from multiple taxonomies i.e. those sequences containing multiple parent sequences, which can have a significant impact on diversity (Kunin et al., 2010; Schloss et al., 2011). QIIME also provides statistical analyses and visualisations of this data, such as rarefaction curves and diversity plots. QIIME makes use of other open source tools as part of many of its pipeline processes, including Uclust (Edgar, 2010), PyNAST (Caporaso et al., 2009) and FastTree2 (Price et al., 2010).

QIIME 1.6.0 was set up on an 8-core Windows 2008 R2 system with 24 GB of RAM, using a Virtual Box (VirtualBox 4.2.8 for Windows hosts, www.virtualbox.org/wiki/downloads). The QIIME Virtual Box is a virtual machine based on Ubuntu Linux, which comes pre-packaged with QIIME's dependencies. Greengenes 16S (DeSantis et al., 2006) alignment and Lanemask files were downloaded into QIIME prior to starting.

A tab-delimited mapping file was set up, which contained the information required to perform the data analysis, and included the name of each sample, the barcode sequences, the primer sequences, and any metadata information about the samples that could be used for sorting

the data. Two mapping files were created, a Forward and a Reverse file; the reverse file swapped the two primers around to allow for sequences being in the opposite orientation.

The first step of the QIIME pipeline took the raw sequencing data in FASTA format, as well as a quality file, and split the sequences up based on their barcodes into the appropriate sampling day. The QIIME default parameters for filtering data were kept, with a minimum/maximum length of 200/1000 base pairs, minimum quality score of 25, maximum length of homopolymers of 6, no ambiguous bases allowed and no mismatches allowed in the primer sequence. Because the sequences were all partial sequences, a bootstrap cutoff confidence threshold of 60% was used for classifying. It has previously been shown that a bootstrap cutoff of 50% or greater is sufficient to accurately classify sequences at the genus level for partial sequences of length shorter than 250 base pairs (Claesson et al., 2009). Both the forward and reverse primers were removed, as well as the barcode sequence, to ensure these sequences did not interfere with later analyses such as OTU picking and taxonomic assignments.

Picking of OTUs was performed by clustering samples based on sequence similarity using Uclust (Edgar, 2010), followed by selecting a representative sequence set from each OTU and assigning taxonomic identities to each representative OTU sequence using the RDP classifier. The representative sequences were aligned with PyNAST (Caporaso et al., 2009), and the sequences filtered to remove gaps and excessively variable locations using the default Lanemask file. A Newick phylogenetic tree of the representative OTUs was assembled, which was required for downstream analysis using FastTree2 (Price et al., 2010). The final step was construction of an OTU map to produce a readable matrix of the OTU abundance in each sample. This script was run using QIIME defaults, and generated an OTU table in biom format for further downstream analysis. The taxa were summarised via a script which generated a variety of tables and plots, which assigned sequences to different taxonomic levels. OTUs were grouped based on species information provided in the metadata file.

2.4.3 Microbial community diversity

The microbial diversity within (alpha (α)-diversity) and between (beta (β)-diversity) samples was assessed within QIIME, to describe the diversity within the study. α -Diversity statistics and rarefaction plots were generated for a number of diversity metrics. The default settings were used, which included the Chao1 index for qualitative species richness, observed species to give the count of unique OTUs in each sample, and Phylogenetic diversity which is a divergence based metric of diversity.

β -diversity is the comparison of different samples based on microbial community composition. QIIME analysis produced Principal Coordinate Analysis (PCoA) plots for each β -diversity metric. The default settings were used, consisting of weighted and unweighted Unique Fraction metric (UniFrac) phylogenetic measures (Lozupone and Knight, 2005; Lozupone et al., 2007). The OTU table, mapping file and phylogenetic tree were all required for both α -and β -diversity workflows.

2.5 Steroid analysis of water, sediment and cowpat runoff samples

2.5.1 Extraction of faecal steroids from environmental matrices

Analysis of faecal steroids followed the methods outlined in Devane et al. (2015). In brief, faecal steroids (Table 7) were extracted directly from urban river sediment samples (1-2 g wet weight (ww)), which were spiked with deuterated internal standard of d5-coprostanol and d5-epicoprostanol and refluxed with 6% methanolic KOH (BDH, VWR, Radnor, Pennsylvania, USA) for 4 hours (Mudge and Norris, 1997). Steroids were partitioned into 25 ml hexane (Scharlau, Sentmenat, Spain) and dried with a small quantity of NaSO₄ (BDH). After removal of hexane, steroids were reconstituted into methyl-tert-butyl-ether (Merck & Co., Darmstadt, Germany) and evaporated to dryness under a stream of nitrogen. Each sample was derivatised by addition of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco, Sigma-Aldrich, Missouri, USA), vortexed and heated at 50°C overnight. The system monitoring compound (cholestane, Steraloids Inc., Newport, Rhode Island) was added and analysed by gas chromatography with mass spectrometric (GCMS) detection. Sediments were reported as ng/g dry weight (dw).

Water samples from the urban river study (up to 4 litres), and runoff supernatant (up to 215 mL) and rainfall samples (up to 1250 mL) from the rural study were analysed by the same method excepting that surface water/runoff was filtered through one or two GF/F filter papers (Whatman, GE Healthcare Services, Buckinghamshire, UK) and the filter(s) were treated as for the sediment samples.

2.5.2 GCMS protocol for analysis of steroids

Quantitative analyses for steroids were performed on a GC-2010 Gas Chromatography instrument (Shimadzu, Kyoto, Japan) equipped with a J&W DB-5MS capillary column coupled to a Shimadzu QP2010 Plus mass spectrometer operating in selected ion monitoring (SIM) mode. The steroids quantified are presented in Table 7, alongside their International Union of

Pure and Applied Chemistry (IUPAC) names, for each compound, and the mass to charge ratio (m/z) fragments used for identification (reference ion) and quantification.

Quality control for analysis of faecal steroids

Quantification was achieved using a calibration curve for each steroid based on standard solutions ranging from 100–16,000 ng/mL. Each of the steroids was quantified by comparing the integrated peak areas of quantification ions with those of the appropriate internal standard (Table 7). Identification of steroids was based on retention times and the ratio of qualitative/quantitative ion. Standards were injected at the beginning and at the end of each batch. Final concentration of steroids in samples was adjusted for the volume or weight of water/runoff or sediment extracted because the raw result of steroid concentration was based on calculations assuming a 1.0 g or 1.0 mL sample.

Quality control measures of blanks (containing GF/F blank filters) and spiked blanks were extracted and analysed for each run. Six batches were analysed for the extraction efficiency of each of the steroids by analyzing the recovery of the standard whose concentration for each of the steroids ranged between 1,500–2,100 ng/mL and had been added to a blank sample containing the GF/F filter. Extraction efficiencies for all of the steroids analysed were greater than 91%.

The limit of detection (LOD) of each steroid was estimated by calculating the signal to noise ratio (S/N) for five standard solutions containing the target steroids in the range of 100–2,200 ng/mL. Each standard concentration was analysed 12 times over 6 runs. The LOD was defined as the concentration where the S/N ratio was greater than three. The LOD of each steroid/stanol is presented in Table 7.

When working with cowpat supernatants, it was difficult to predict volumes from which we could obtain steroid concentrations within the range of the standard concentrations. In addition, repeat analyses were prohibitively expensive. On occasion for Trial 1, particularly during Days 77 and 105, a nine standard concentration curve had to be applied for sample concentrations of individual steroids above the standard range.

2.6 Fluorescent whitening agents (FWA)

The FWA (4,4'-bis[(4-anilino-6-morpholino-1,3,5-triazin-2-yl)-amino]stilbene-2,2'-disulfonate) is used in NZ laundry detergents, and is the analyte tested in this thesis. This FWA (93.1% purity) was obtained from Ciba Speciality Chemicals, Grenzach-Wyhlen, Germany.

FWA were extracted from 100 mL water samples and analysed by High Pressure Liquid Chromatography (HPLC). The primary FWA standard (1g/L) was dissolved in 50 ml of

dimethylformamide (BDH) prior to making to volume with deionised water. All FWA standards were stored at 5°C and wrapped in aluminium foil to avoid photodegradation. A working standard of 1000 µg/L was prepared fresh for each analysis. The standard curve was prepared by dilution of the working standard with mobile phase, (60:40) methanol (HPLC Grade Burdick & Jackson, Honeywell International Inc., Michigan, USA), 0.1 M ammonium acetate (BDH), to give concentrations in the range of 0.5 – 50 µg/L. The standard curve was linear across this range.

FWA were extracted from water samples under vacuum, by elution onto a C-18 disc Sep-Pak cartridge (Maxi-Clean Cartridges 300 mg C18 Grace Alltech, Maryland, USA), pre-wet with methanol and de-ionised water. The FWA were eluted from the Sep-Pak cartridge with 5 ml of the mobile phase.

All analyses were performed using a Shimadzu Liquid Chromatograph LC-10ATVP equipped with a Shimadzu System Controller SCL-10AVP and Shimadzu Auto-injector SIL-10ADVP. FWA were detected using a Hitachi F1000 Fluorescence detector (Hitachi High-Technology Corporation, Tokyo, Japan). Fifty microlitres of eluate was injected onto a reverse phase Phenomenex RP-18 column (100 x 4.6 mm) (Spheri-5 ODS Column, Applied Biosystems, Foster City, California) and eluted with the mobile phase at a flow rate of 1.5 ml/min. The FWA were detected by fluorescence (350 nm excitation wavelength and 430 nm emission wavelength).

The method described above was adapted to detect FWA in sediments after freeze-drying of the sediment. Five grams of dried sediment was accurately weighed into a 50 ml centrifuge tube and made to the 45 ml mark with mobile phase containing methanol and ammonium acetate. The sediment was shaken by hand for 2 minutes, allowed to settle before removal of a portion of the supernatant for analysis by HPLC.

Quality control for FWA analysis

Quality controls included in each run were a blank of deionised water and a sample spiked with 1000 µg/L standard to give a theoretical concentration in the sample of 2.5 µg/L FWA. Recovery was >80%. The limit of detection in water samples was 0.01 µg/L FWA. Identification of 0.1 µg/L of FWA in water is suggestive of human faecal pollution. Limit of detection of FWA in sediment is 2.0 µg/kg and FWA identified above this level is indicative of human faecal pollution.

Table 7: Characteristics of analysed steroids used for quantification

Common Names	Abbreviations	IUPAC name	Mass/charge ratio		Internal standard	Limit of Detection (ng/mL)
			Reference ion	Quantitative ion		
Coprostanol	Cop	5 β -cholestan-3 β -ol (β -stanol)	355	370	d5-coprostanol	1
24-ethylcoprostanol	24-Ecop	24-ethyl-5 β -cholestan-3 β -ol (β -stanol)	383	398	d5-coprostanol	5
epicoprostanol	epicop	5 β -cholestan-3 α -ol (β -stanol)	355	370	d5-epicoprostanol	5
cholesterol	chol	cholest-5-en-3 β -ol	458	368	d5-epicoprostanol	10
Cholestanol	Cholestan	5 α -cholestan-3 β -ol (α -stanol)	460	445	d5-epicoprostanol	10
24-methylcholesterol (campesterol)	24-Mchol	24-methylcholest-5-en-3 β -ol	472	382	d5-epicoprostanol	15
24-ethylepicoprostanol	24-E-epicop	24-ethyl-5 β -cholestan-3 α -ol (β -stanol)	383	398	d5-epicoprostanol	10
stigmasterol	stigmast	24-ethylcholesta-5,22(<i>E</i>)-dien-3 β -ol	394	484	d5-epicoprostanol	22
24-ethylcholesterol (β-sitosterol)	24-Echol	24-ethylcholest-5-en-3 β -ol	357	396	d5-epicoprostanol	10
24-ethylcholestanol (β-sitostanol)	24-Echolestan	24-ethyl-5 α -cholestan-3 β -ol (3- β ,5- α -stigmastan-3-ol) (α -stanol)	383	488	d5-epicoprostanol	30
Internal standards and monitoring compounds						
cholestane		System monitoring compound	372	357		*N/A
d5-coprostanol		Internal standard	360	375		N/A
d5-epicoprostanol		Internal standard	360	375		N/A

*N/A, not applicable

3 Chapter Three:

Indicators and pathogens in urban river water and sediments after significant discharges of human raw sewage

3.1 Introduction

In 2010 and 2011, the province of Canterbury in NZ experienced a series of damaging earthquakes. All four earthquakes (magnitude 6.0 -7.1) and aftershocks were shallow (5-11 km depth), which increased the damaging effect of the ground movement. The damage caused extreme disruption to water, wastewater and stormwater infrastructure throughout much of the city of Christchurch (population 436,000) (Statistics NZ, 2013, <http://www.stats.govt.nz>). The municipal sewage treatment plant, sited in the east of the city (Figure 3) and pump stations in the eastern area, suffered major damage after the February 2011 earthquake (magnitude 6.3) due to liquefaction and physical disturbance. This resulted in the discharge of large volumes of raw sewage (up to 38,000 m³/day) directly into the city's rivers, the Avon/Otākaro and Heathcote/Ōpāwaho, and the Avon-Heathcote/Ihutai Estuary from February 2011 until September 2011.

Microbial indicators of faecal contamination

Wastewater can contain a number of pathogenic organisms including *Campylobacter* spp., *Escherichia coli* O157, *Cryptosporidium* spp., and *Giardia* spp. and enteric viruses, which when ingested can cause severe illness and, in some cases, death (Leclerc et al., 2002). When untreated sewage contaminates rivers or oceans, waterborne transmission of pathogens can occur to those who participate in swimming, boating, fishing and shellfish-gathering activities (Cornelisen et al., 2011). Microbial water quality is assessed primarily by testing for the indicator bacteria *E. coli* and enterococci in freshwater and enterococci in saline waters. These conventional indicator bacteria usually do not cause disease themselves, but they are prevalent in faecal material and sewage, and therefore indicate the presence of pathogenic organisms that can be transmitted by the faecal-oral route (Yates, 2007). Methods for the detection of these indicator organisms in waters are timely, simple and relatively cheap to perform in the laboratory (Yates, 2007).

In fresh, untreated sewage, *E. coli* and enterococci are considered to be good indicators of potential risk to human health from pathogenic bacteria and protozoa (USEPA, 1996). Once sewage discharge occurs into receiving waters, however, a range of physical and environmental factors including dilution, movement within a river, storage in sediments, and the intrinsic characteristics of the microorganisms may, over time, alter the relationship between these indicator bacteria and the pathogens of concern (Sinclair et al., 2012).

As outlined in Chapter One, studies have shown that even in the absence of recent faecal inputs, the faecal indicator *E. coli* can occur in soil, sediment, vegetation and algal mats in waterbodies as part of the natural microflora (Byappanahalli and Fujioka, 2004; Byappanahalli et al., 2003b; Chandrasekaran et al., 2015; Whitman et al., 2005). These factors call into question *E. coli*'s ability to perform as an indicator of faecal contamination, when environmental sources of *E. coli* re-suspended from sediments and macrophytes; and from vegetative and soil run-off, may impact a watercourse confounding the correlation between indicator and pathogen.

Due to this persistence and potential for growth of *E. coli* in the environment, additional indicators have been recommended as surrogates for sewage contamination such as *C. perfringens* and coliphages for monitoring of tropical aquatic environments (Fujioka, 2001; Vithanage et al., 2011). F-RNA phage have also been proposed as useful indicators of fresh faecal contamination in tropical waters (Fung et al., 2007; Vergara et al., 2015). It has been suggested that better predictability of pathogens may require a suite of indicator organisms (Harwood et al., 2005) to minimise false-negative tests where bacterial indicator concentrations are low in the presence of detectable pathogens (Wilkinson et al., 2006).

Faecal source tracking (FST)

The Avon/Otākaro River had been monitored by the local authorities over many years, and prior to the first 2010 earthquake, levels of *E. coli* often exceeded the action level of 550 colony forming units (CFU)/100 mL for secondary recreational water contact (Ministry for the Environment, 2003). In 2009, the local authorities initiated a faecal source tracking study to investigate the potential faecal sources of *E. coli*. The results indicated that the majority of the pollution detected was derived from avian faecal pollution during base river flows, with a greater proportion attributed to runoff from canine faecal sources after heavy rainfall events (Moriarty and Gilpin, 2009).

The tools used to investigate faecal sources during this urban study of the Avon/Otākaro River were the chemical markers: faecal steroids and fluorescent whitening agents (FWA) and the Polymerase Chain Reaction (PCR) markers. Faecal steroids are used as biomarkers of human and animal faecal pollution (Leeming et al., 1996). Differences in sterol and stanol

concentrations between warm-blooded species allows the generation of a steroid fingerprint based on the ratios between the individual faecal steroids. For example, the ratio of coprostanol to 24-ethylcoprostanol has been widely used to discriminate between herbivore and human faecal sources (Ahmed et al., 2011; Leeming et al., 1996). FWA are added to laundry detergents to whiten clothes and excess FWA is removed in the grey water, which mixes with the wastewater and enters the sewerage system. FWA, therefore, act as specific chemical indicators of human faecal contamination (Hayashi et al., 2002; Managaki et al., 2006). Polymerase Chain Reaction (PCR) markers are useful indicators of faecal input from a particular animal species because these markers target the DNA of microorganisms that specifically reside in the intestine of that animal species (Bernhard and Field, 2000; Shanks et al., 2009; Shanks et al., 2010), or amplify the mitochondrial DNA of the target animal itself (Martellini et al., 2005; Schill and Mathes, 2008).

Discriminating between fresh and historical/treated human faecal inputs to water

The persistence of FIB in the environmental reservoirs outside of the animal host (Byappanahalli et al., 2006a; Byappanahalli et al., 2012b), may confound the role of FIB as indicators of a fresh faecal event (Nevers et al., 2014). A ratio comparing the high numbers of Total Coliforms (TC) found in fresh sewage with the background microflora of the river (identified as Atypical Coliforms (AC)) on the same media as TC, has been used as an indicator of fresh faecal inputs to a waterway (Black et al., 2007; Brion, 2005). A low AC/TC ratio (<1.5) has been identified in raw sewage, and when discharged into a waterbody the ratio of AC/TC increases over time as TC numbers decrease due to die-off after excretion into the environment. The faecal steroid ratio between coprostanol and epicoprostanol has also been investigated as a way to distinguish the treatment status and age of human faecal inputs in sediments. The very low levels of epicoprostanol present in human faeces (Leeming et al., 1998b) increase during anaerobic sludge digestion as both cholesterol and coprostanol are converted to epicoprostanol (McCalley et al., 1981). It is postulated that the same conversion occurs in sediment, therefore, a high ratio of coprostanol to epicoprostanol in sediment is indicative of fresh untreated human pollution (Carreira et al., 2004).

Tracking the fate of microorganisms in the river system

Effective wastewater treatment removes or inactivates wastewater-derived pathogens before they enter natural waterways. However, the severe damage to the wastewater system in Christchurch after the February 2011 earthquakes led to the initial discharge of up to 38,000 m³/day of raw sewage into the Avon/Otākaro River (personal communication, Mike Bourke, Christchurch City

Council), with volumes decreasing over the ensuing six months. The Avon/Otākaro River rises from a groundwater spring in the west within the city boundary and traverses the urban environment of Christchurch City providing a popular destination for recreational and tourist activities. The microorganisms in the discharged sewage may have remained suspended in the water column to eventually reach the Avon-Heathcote/Ihutai Estuary (Figure 3), or may have been deposited into the riverbed sediment.

The process of deposition and re-suspension of microorganisms to and from sediments is poorly understood. There is also limited information about the rates of microbial survival in sediments, although reduced oxygen levels and protection from sunlight may allow microorganisms to survive longer in sediments than in the water column (Anderson et al., 2005; Davies et al., 1995; Pachepsky and Shelton, 2011). Many recreational water activities can disturb sediments, mobilising microorganisms from the riverbed, as can heavy rainfall, increased river flows, and the presence of animals (Chandrasekaran et al., 2015; Curriero et al., 2001; Nagels et al., 2002; Wilkinson et al., 2006). Disturbance of sediment microbial reservoirs is also likely to progressively enrich downstream sediments with microorganisms, even after sewage discharges cease (Brookes et al., 2004).

The unanticipated discharge of large volumes of untreated human sewage into Christchurch's rivers, although unfortunate, did provide an opportunity to increase our understanding of the relationship between, and the behaviour and fate of, indicator microorganisms (*E. coli*, *C. perfringens* and F-RNA phage) and pathogens (*Campylobacter*, *Giardia* and *Cryptosporidium*) and FST markers (faecal steroids, FWA and PCR markers) in river environments during active sewage discharges. It also allowed a comparison of relationships between microbial and FST indicators and pathogens following cessation of such sewage discharges, and the evaluation of faecal ageing tools to discriminate fresh discharges from historical inputs. In addition, the role of riverbed sediments as a reservoir for faecal indicators and pathogens was investigated to understand the potential for their re-mobilisation during future disturbance events.

3.2 Methods

3.2.1 Site Location

The Avon/Otākaro River has a length of 14 kilometres (km) and traverses Christchurch City in a west to east direction before exiting the built environment at the northern entrance to the Avon-Heathcote/Ihutai Estuary. It arises as a spring within the city boundaries and therefore has little exposure to pollution from agricultural sources. In the estuary, the Avon/Otākaro River mixes with the Heathcote/Ōpāwaho River prior to flowing into the Pacific Ocean via Pegasus Bay (Figure 3).

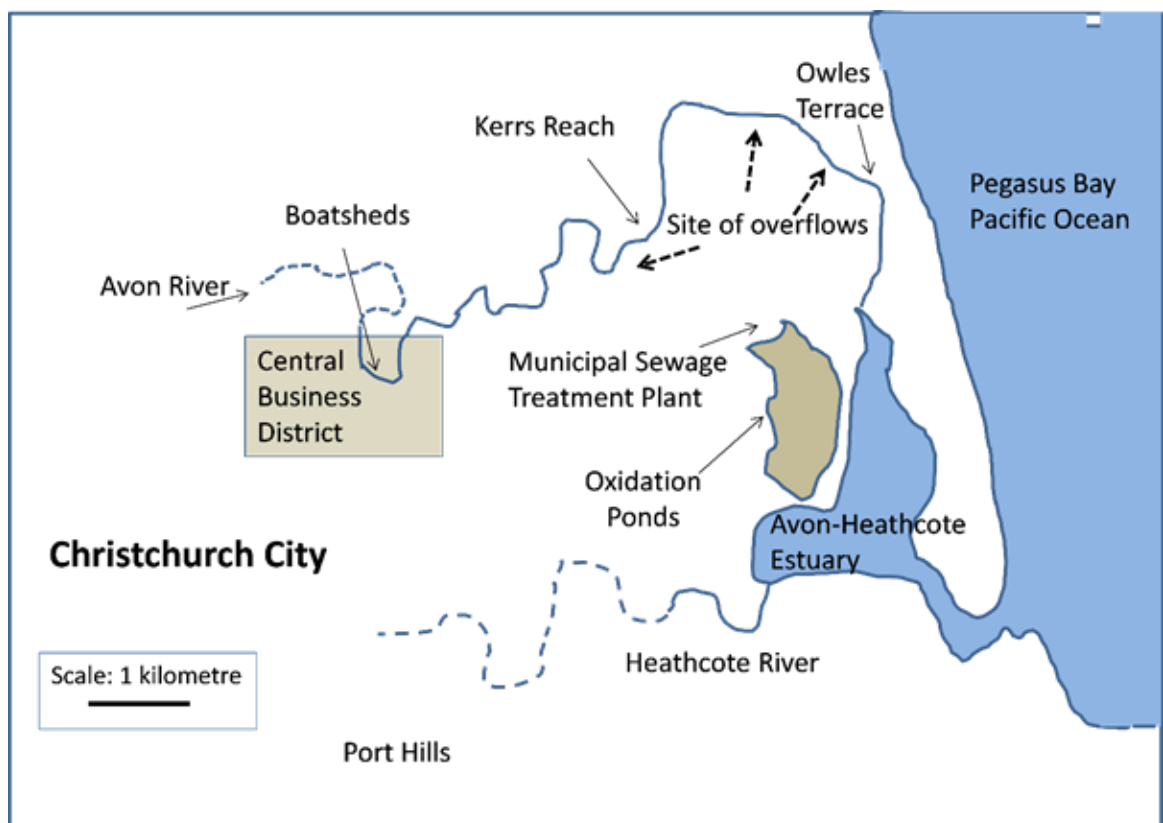


Figure 3: Map of the sampling sites and their location on the Avon/Otākaro River

Three sites were chosen along the Avon/Otākaro River for collection of water and underlying sediment on 16 sampling occasions in the period: March 2011 to March 2012 and three occasions during March and April, 2013. The most upstream site was the Boatsheds (BS), which received no known sewage discharges and was not influenced by tidal effects (Figure 3). Further downstream, below the central business district (CBD) were the two sampling sites, Kerrs Reach (KR) and Owles Terrace (OT), which were receiving continuous discharges of raw sewage due to the failure of pump stations after the damage caused by the earthquakes. These

stations were unable to pump sewage to the municipal treatment station, because of sewer pipe breakages and pump failure, therefore, the sewage was re-routed to discharge directly into the Avon/Otākaro River from sites downstream of BS but upstream of both KR and OT. OT was tidally influenced, being situated approximately 2 km upstream of the entrance to the Avon-Heathcote/Ihutai Estuary. KR had less tidal influence being approximately 5 km upstream of OT. The period of major continuous discharge was termed the active discharge phase and occurred between February and September, 2011. There were low volume, intermittent sewage discharges affecting all sampling sites during the post-active discharge phase October 2011 – March, 2012 and March-April, 2013 due to on-going aftershocks and a fragile sewerage system.

3.2.2 Collection of river water and sediment

All water samples collected from the Avon/Otākaro River for analysis were taken as grab samples (6 L) from the river bank. Collection of samples occurred early in the morning within an hour either side of low tide as measured at Lyttelton Harbour, Christchurch. For sediments, a 250 mL sterile container was attached to a Mighty Gripper (The Mighty Gripper Company, Whangarei, NZ) and lowered into the water. A grab sample of sediment was collected from the top two centimetres of the surface layer along a one metre transect, thereby targeting the recently deposited, surficial sediments. Water and sediment samples were kept chilled during transport to the laboratory and analysed within 24 h of collection for PCR markers and microorganisms, with the exception of protozoa in sediment which were stored at 4°C until analysis within two weeks. Samples for FWA and steroid analysis were stored at 4°C in the dark for up to one week prior to analysis, or in the case of steroids, sediments and filtered water samples were stored at -20°C until analysis. Water bottles used to sample FWA were wrapped in aluminium foil to avoid photodegradation of FWA. Direct testing of FWA levels in sewage prior to discharge and dilution in the Avon/Otākaro River was performed by collecting three replicates each at two discharge locations near Kerrs Reach and Owles Terrace.

Dates of analysis of individual indicators and markers

- *E. coli* and AC/TC were analysed in water and sediment for each sampling event.
- In water and sediment, *C. perfringens* was analysed only on the eleven occasions during 26 April 2011 and March 2012.
- In water and sediment, protozoa were analysed on ten occasions during 26 April 2011 and March 2012. In sediment, samples were analysed for protozoa on three occasions in 2013.
- F-RNA Phage and *Campylobacter* were analysed in water and sediment from 26 April 2011 till March 2012 and in March and April, 2013.

- In general, PCR markers were analysed in water on all samples except for the first sampling on 8 March 2011. Other individual exceptions for PCR markers can be viewed in the Appendix: Table 30 to Table 32. PCR markers were not analysed in sediment.
- Faecal steroids were analysed in water and sediments on 8 and 23 March 2011 and then from 26 April till March 2012 at all sites. Faecal steroids were analysed in water at all sites on the three occasions in 2013, but for sediments only at KR on 25 March and at KR and BS on 8 April 2013. Steroids in sediment samples were not analysed at OT during 2013.
- FWA analysis of water and sediment was performed on ten occasions from 26 April 2011 till March 2012. During 2013 water samples were tested for FWA at only KR on 25 March and at KR and BS on 8 April 2013. FWA in sediment samples were not analysed at any sites during 2013.

3.2.3 Analysis Methods

Details of analytical methods for microbial analyses and FST markers in water and sediment are presented in Chapter Two. Speciation of pathogens was not within the scope of this study and therefore, *Campylobacter* and the protozoa, *Cryptosporidium* and *Giardia* are referred to as potential pathogens.

3.2.4 Physical and chemical water parameters

Water temperature (°C), pH, dissolved oxygen (DO, mg/L), turbidity (Nephelometric Turbidity Units or NTUs) and conductivity (milliSiemens/cm) were measured using a Hydrolab Quanta® Water Quality Monitoring System (Hach Environmental, Loveland Colorado, USA). Due to equipment failure water parameters were only measured during the active discharge phase.

Rainfall data were obtained from a weather station close to the city centre

(www.cliflo.niwa.co.nz). Provisional data on daily mean river flows at the Gloucester Street Bridge on the Avon/Otākaro River were provided by the Regional Council, Environment Canterbury. Details of the volume and discharge location of untreated sewage into the environment were provided courtesy of the Christchurch City Council. Details of significant earthquakes were obtained from the Geonet website (www.geonet.co.nz).

3.2.5 Statistical analysis

Statistical analysis was undertaken using SigmaPlot version 11.0 (Systat Software, San Jose, California, USA, 2008) and XLSTAT (2007.6) to calculate inferential statistics. Significance was characterised at the α -level of 0.05 for all statistical analyses. All counts were expressed as arithmetic means. Non-parametric statistical analyses were performed because the distribution of

much of the data failed the Shapiro-Wilkes normality tests. In addition, the analytes in the urban river study were identified in a wide range of concentrations from 1 cyst/100 litres to 10^5 CFU/100 mL. There were no concentrations above the upper detection limits for the MPN analysis of *Campylobacter*. Values below the limit of detection for microbial assays were assumed to be zero; therefore, analysis was based on observations where there was numerical data for all determinants. Application of non-parametric statistical analyses such as Spearman ranks test where variables were ranked based on concentration, meant that using the value of zero for non-detects did not affect statistical outcomes. A sensitivity analysis was performed on the regression analyses to determine if using half of the detection limit in place of zero for log transformation of each of the samples would change the regression between indicators and pathogens. Comparison of the range of the slope and of the y-intercept for *Campylobacter* with *E. coli* showed that inclusion of the non-detects in the data set did not change the range of the 95% confidence interval and also the range of the slope did not cover zero suggesting that there was a valid relationship between the concentration of *Campylobacter* and that of *E. coli*. A similar analysis of *Campylobacter* and F-RNA phage with inclusion of the non-detects also showed similar ranges for slope and for the y-intercept when the data set included or excluded the non-detect data. However, in the case of the regression analysis for *Campylobacter* and F-RNA phage for both data sets in/exclusive of non-detects, the range of the slope did cover the zero value suggesting that the two variables were not related and prediction of *Campylobacter* concentration based on F-RNA phage was not valid.

The non-parametric Spearman ranks test (Spearman rho, r_s) was used to test if there was a relationship between the FST variables and microbes, with correlation values $r_s \geq 0.75$ reported as strong; r_s 0.50-0.74 as moderate; and below r_s 0.50 as weak. In addition, in the urban river study, FST markers were analysed by Principal Component Analysis (PCA) (using Spearman ranks in XLSTAT) as a method to reduce the number of variables to see if the data could be explained by a subset of FST markers. Correlation and PCA were performed on the data from 2011-2013 for all combined sites where there was data for all variables including additional pathogen testing. Statistical analyses of during discharge and post-discharge excluded 2013 data (unless stated in the text) and concentrated on data where there was information collected on all pathogens (April, 2011 - March, 2012). Analysis of the two discharge phases employed non-parametric statistical methods including Mann Whitney Rank Sum test and Kruskal-Wallis analysis of variance. In some cases, additional analyses were performed on individual sites or the combined two discharge sites, but this is stated in the text.

Linear regression was employed to quantitatively evaluate relationships between log transformed concentrations of microbial indicators and pathogens in water. Logistic regression was performed using XLSTAT and converting the human PCR marker and human steroid ratio data to binary values of one and zero to compare the concordance between the detection of human faecal inputs by each of these FST markers. Binary detection of human inputs by steroid ratio analysis was scored as 1.0, based on the three steroid ratios H1, H2 and H3 (Table 3). H1 (%coprostanol) had to be >5% threshold, and H3 >1.0 to ensure that pollution was human derived, not primarily herbivore inputs. In addition, H2 had to be ≥ 0.7 to confirm coprostanol was derived from human sources rather than environmental sources such as algae (H2 <0.3). Detection of human inputs by PCR was scored as 1.0 if at least two of the three Human PCR markers were detected in a water sample. There were 47 observations where both steroid and PCR data were available for analysis for logistic regression. Cohen's kappa statistical method was used to assess the concordance between the PCR and steroid FST methods. Binary data for (non-)detection of human contamination by both PCR markers and steroid data was used to calculate the kappa statistic (<https://www.niwa.co.nz/services/statistical>). To account for sampling error a one-sided hypothesis test was performed using a simple 95% confidence interval approach as outlined in McBride (2005) using the kappa criterion of >0.6 to establish if there was a substantial strength of agreement between the two FST methods (Landis and Koch, 1977). FWA values were also converted to binary data for evaluation with *E. coli* concentrations with a score of 1.0 when the FWA level was ≥ 0.1 $\mu\text{g/L}$ in water; and ≥ 2.0 $\mu\text{g/kg}$ in sediment.



a) Raw sewage discharging to Avon/Otākaro River upstream from Owles Terrace sampling site



b) Boatsheds sampling site upstream from central business district



c) Sampling at Kerrs Reach. Note the high level of sedimentation due to liquefaction generated by earthquakes



d) Lateral movement of land at Kerrs Reach caused by earthquakes



e) Sampling at Kerrs Reach



f) Sampling from the jetty at Owles Terrace

Figure 4: Sampling sites along the Avon/Otākaro River; Owles Terrace (a,f), Boatsheds (b) and Kerrs Reach (c,d,e). Photo credit: Brent Gilpin, Institute of Environmental Science and Research Ltd (ESR).

3.3 Results

This study describes the water quality at three sites in an urban river, variously impacted by faecal contamination after a series of large earthquakes. Each site was sampled for water and underlying sediments on 19 occasions, nine occurring during active sewage discharges into the Avon/Otākaro River (8 March to 7 September, 2011), and six occurring post-discharge (27 September, 2011 to March, 2012) and three occurring in March and April, 2013. Not all variables were analysed on every occasion as outlined in the methods and tables in the Appendix (Table 30 to Table 34).

In the month preceding the February 2011 earthquake, volumes of discharged sewage were recorded up to 23% of the volume of Avon/Otākaro River flow. The average river flow during February was 2022 L/s (range 1688 to 3961). The percentage contribution of sewage to the river flow decreased over the following months to an average of $6.5\% \pm 1.4$ as the sewerage network was remediated. Average river flow during this period was 1794 ± 380 L/s. However, in June 2011, another significant earthquake (magnitude 6.4) resulted in a second maximum of 16% of river flow attributed to sewage discharge (river flow 2052 L/s). This contribution from sewage discharge decreased to an average of $5.6\% \pm 2.3$ of river flow (average 1885 ± 602 L/s) until cessation of all major discharges in late September 2011.

During the active discharges, the mean temperatures and pH values of the water were similar at all sites ranging from 11.8 to 12.1°C and pH 7.2 to 7.4, respectively. The greatest variation in water quality occurred at OT which was two kilometres upstream of the opening into the estuary and received the highest volume of sewage discharges. This greater discharge is reflected in the higher turbidity at OT (mean = 22.4 NTU \pm SD 5.8), followed by KR (mean = 11.1 \pm SD 3.1 NTU). The BS site was not receiving any major discharges during the active discharge phase. A similar conclusion can be reached for the dissolved oxygen (DO) values which were lower at the two sites receiving active discharges. Overall, values of DO were approximately 5.0- 6.0 mg/L at the two discharge sites, which is bordering the recommended levels for the health of freshwater fish (www.water-research.net/Watershed/dissolvedoxygen). These lower values were in comparison to DO values of 7.8 ± 0.25 mg/L at BS during the active discharge period. OT was the sampling site most influenced by tides which accounts for the higher values and wider variations for conductivity (mean 1.02 mS/cm, range 0.4-2.5 mS/cm) compared with the other two sites, which both had means of $0.2 \pm$ SD 0.02 mS/cm.

There were only three occasions where significant rainfall (>5 mm) occurred 48 hours prior to sampling of the river water: 26 April 2011, 28 June 2011 and 22 November 2011.

Rainfall in the 48 hours prior to 28 June 2011 (17.6 mm), may have contributed to the increase in *E. coli* concentration ($3.1 \times 10^4/100$ mL) noted at OT, however, increases in *E. coli* levels were not reported at the other two sites (Figure 5). In addition, this elevation of *E. coli* occurred during the active discharge phase and two weeks after the June 13 2011 earthquake when further damage occurred to the sewerage network. Rainfall on the sampling of 22 November 2011 (14.4 mm in the 24 hours prior), may have contributed to the elevated *E. coli* levels recorded at all three sites on this occasion.

3.3.1 Water: Determining the source of faecal contamination

PCR markers identified in river water at the three sites are presented in Figure 6 for human-associated PCR markers and Figure 7 for the general and animal-associated markers. The chemical FST markers are presented in Table 8 to Table 10 with a summary of the faecal sources identified by all FST methods.

Contamination sources at the Boatsheds (BS)

FST analysis of the water samples collected before 16 May, 2011 showed wildfowl and dogs were the major sources of faecal contamination at BS. The wildfowl PCR marker was detected in water at BS on 17 of the 18 sampling events where processing for PCR markers was performed. From 16 May until October 2011 at BS, human faecal contamination was detected by the three human PCR markers and steroid ratios, in association with *Campylobacter* and protozoa on most sampling occasions (Figure 6 and Figure 8). The dog PCR marker was identified at BS, seven from eight occasions during the active discharge phase but only two occasions post-discharge.

Contamination sources at the active discharge sites: Kerrs Reach (KR) and Owles Terrace (OT)

At the two active discharge sites, KR and OT, before the official cessation of major discharges in mid-September 2011, coprostanol levels (H1) were on average 21% (standard deviation (SD) 8%) of the total steroids identified and ratios of H2, and H3–H5 confirmed human sources of coprostanol.

At KR and OT, two of the three human PCR markers were detected in most samples with the HumM3 marker detected on all occasions. At the first post-discharge sampling on 27 September 2011, *E. coli* levels were still >5000 CFU/100 mL and %coprostanol was 26-28% at the active discharge sites, with all three human PCR markers detected. Thereafter, there were notable decreases in both *E. coli* and %coprostanol for both sites with mean 8% coprostanol (SD 3%) up to and including March 2012, and mean 3% (SD 1%) in March and April, 2013. In

addition, human PCR marker detection was intermittent, which was supported by steroid ratios not always indicating human sources. The exception, for this downward trend was at KR during 2013 where *E. coli* levels in water were between 1,000 and 5,000 CFU/100 mL on all three occasions but FST markers were suggesting wildfowl faecal sources dominated.

Avian and dog PCR markers

The wildfowl PCR marker in water was detected frequently at BS and on 11 from 18 occasions at KR and only two occasions at OT (Figure 7). The dog PCR marker was identified at all sites on almost every occasion during the active discharge phase, but on only three occasions post-discharge (two times at BS and once at OT). In 2013, avian sources predominated at all sites with, in general, elevated levels of *E. coli* >1000 CFU/100 mL at BS and KR. Borderline human levels of 4.5% coprostanol were identified at OT on 8 April 2013, but this steroid marker was not supported by PCR markers or *E. coli* (240 CFU/100 mL).

FWA levels in water

FWA levels in water were tested from April 2011-March 2012 period (n = 11 per site) and intermittently during 2013 (Table 8 to Table 10). Low levels of FWA in water (mean 0.06 SD 0.08 µg/L, range 0.01-0.40 µg/L) were identified throughout the study. Levels of FWA in the river water strongly indicated (>0.2 µg/L) human faecal pollution on only two sampling occasions, with four occasions where levels were in the range suggestive of human faecal inputs (0.1 – 0.2 µg/L). Apart from these occasions, FWA levels in water at the two active discharge sites, were below the threshold for human faecal contamination. Due to the very low levels of FWA during times when other FST markers indicated human pollution, direct testing of FWA levels in sewage prior to discharge and dilution in the river was performed with, on average, 0.84 (SD 0.10) µg/L at a discharge location near KR and 2.53 (SD 1.18) µg/L near OT.

Differences in FST marker concentrations between discharge phases

Kruskal-Wallis analysis of variance of total steroids in water during the active discharge (March-8 September, 2011) versus post-discharge (27 September – March 2012) revealed a significant difference in concentration between BS and the other two sites ($p < 0.001$) with higher steroid concentrations at KR and OT attributed to the continuous loads of human sewage discharging into the river upstream of these sites. The steroid concentrations at these two sites showed a significant reduction after active discharges ceased in mid-September 2011 (KR, $p = 0.018$ and OT, $p = 0.008$) while maintaining the same human signature. Similar results were found for the general faecal and human PCR marker concentrations at KR and OT. There were significant reductions in PCR marker concentrations post-discharge ($p < 0.05$), although the human

signature at KR was, in general, the same as during active discharges. Only one human PCR marker (*B. adol*) was detected at OT after early November 2011, however, human steroid markers were present until March 2012.

At the two sites receiving continuous discharges, the copy number of the *B. adol* and HumBac PCR markers (Figure 6) were, in general, tenfold less than the general faecal PCR marker (Figure 7), and the HumM3 PCR marker was approximately two orders of magnitude less than the other two human PCR markers. During the active discharge phase, the general faecal PCR marker was identified at concentrations of $\geq 10^6$ gene copies (GC)/100 mL at all three sites with highest levels at KR and OT. At BS, levels of the general faecal PCR marker were 10^6 GC/100 mL even when wildfowl and dog were the dominant sources. Similar levels of this general PCR marker continued to be detected at BS during the post discharge phase including in 2013, however at the other two sites, levels decreased by tenfold (Appendix, Table 30 to Table 32).

In comparison, the concentrations of the human PCR markers at the two discharge sites showed more marked decreases, for example, at KR, the *B. adol* marker decreased by tenfold between the active discharge and post-discharge phases and further decreased tenfold in 2013. OT showed even greater decreases in *B. adol* over the post-discharge phases including 2013. During 2013, the other two human PCR markers were not detected at any of the sites, although levels of the general PCR marker were still 10^5 to 10^6 GC/100 mL. This high general PCR marker at OT during 2013, was in association with non-detection of the wildfowl and dog PCR markers (Figure 7) although steroid analysis suggested wildfowl and on one occasion low level human (Table 10). At the other two sites during 2013, the wildfowl PCR marker was detected in conjunction with the general PCR marker.

3.3.2 Water: microbial indicators and potential pathogens

Microbial indicators

All data for microorganisms detected in river water can be found in Table 30 to Table 32 in the Appendix. *E. coli* levels in water were elevated at all sites throughout the study (Figure 5). Prior to the cessation of active discharges (mid-September, 2011) with the exception of one water sample (BS, 8 September, 2011) all *E. coli* results exceeded the NZ recreational water guidelines Action level (550 CFU/100 mL). Following cessation of active discharges, a general reduction in levels of *E. coli* was observed from September 2011 to March 2012, although all samples continued to exceed the Alert level (260 CFU/100 mL), and at KR all samples exceeded the Action level. River water samples analysed during March and April, 2013 had elevated levels of

E. coli (maximum 5000 CFU/100 mL), with the exception of a sample from OT (240/100 mL) collected on 8 April 2013. *E. coli* levels were highest at KR during 2013, and notably higher than the general level recorded following the cessation of active discharges at KR.

F-RNA levels at KR and OT averaged 2,230 PFU/100 mL (range 450-3,950) during the active discharge period, compared to a mean of 425 PFU/100 mL (range 50-1,050) at BS (Figure 5). Once discharges ceased, mean levels of F-RNA phage were similar at all three sites (<160 PFU/100 mL). During 2013, F-RNA phage were identified intermittently in the range of 50-200 PFU/100mL, which was one to two orders of magnitude lower than during the active discharge phase. *C. perfringens* was present in almost all samples during 2011-2012 sampling, with levels at BS between 50–150/100 mL, while at KR concentrations ranged from 50–550/100 mL, and the highest levels were recorded at OT (50–1,200/100 mL). Due to its ubiquitous presence, *C. perfringens* was not sampled during 2013.

Potential pathogens

Campylobacter spp. were detected in water from at least one of the three river sampling sites on each of the ten sampling occasions throughout 2011-2012 and on all three sampling occasions at all three sites in 2013 (Figure 8). At BS, *Campylobacter* was detected at less than 10 MPN/100 mL of river water. *Campylobacter* was detected in concentrations ranging from 0.4 to \leq 110 MPN/100 mL at KR and OT. On 8 April 2013, KR had the highest concentrations of *Campylobacter* (46 MPN/100 ml) seen since active sewage discharges ceased.

In river water, the protozoa were sampled on only ten occasions from 26 April till 6 March 2012 (Figure 8). Low levels (20 oocysts/100 L) of *Cryptosporidium* spp. were detected in water at all three sites on three sampling occasions during active discharges (April – June, 2011). Once active discharges decreased and finally ceased, *Cryptosporidium* was not detected. In contrast, *Giardia* was detected from April 2011 till March 2012 at all three sites. The concentration of *Giardia* decreased markedly following the cessation of active discharges to the river from a high of 750 cysts/100 L of water in September, 2011 at both KR and OT, to 9 and 3 cysts/100 L (respectively) on the last sampling occasion in March 2012.

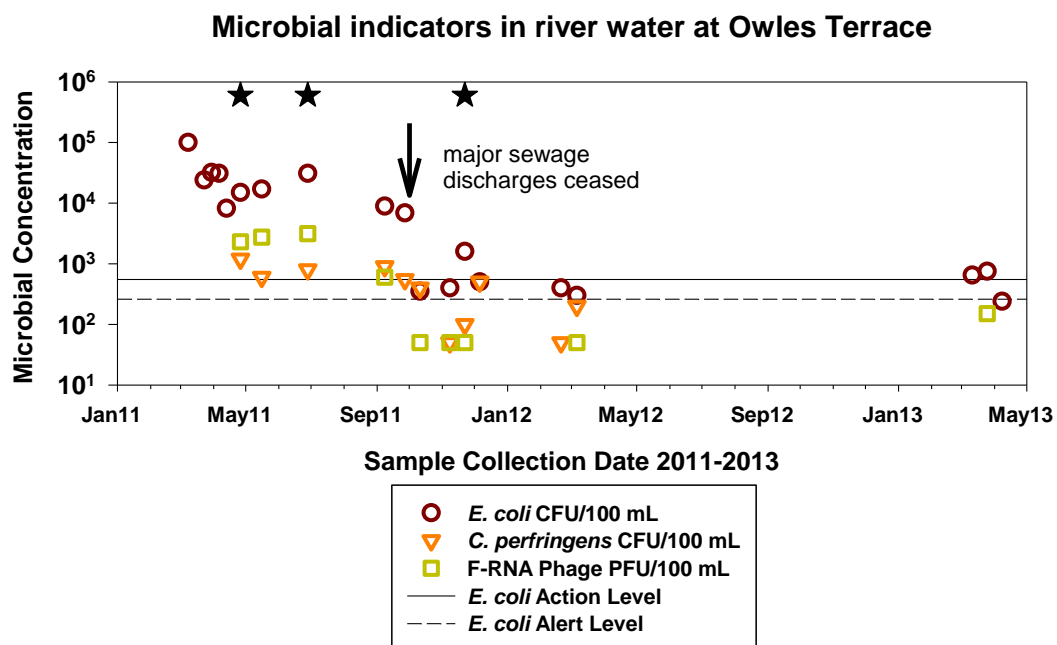
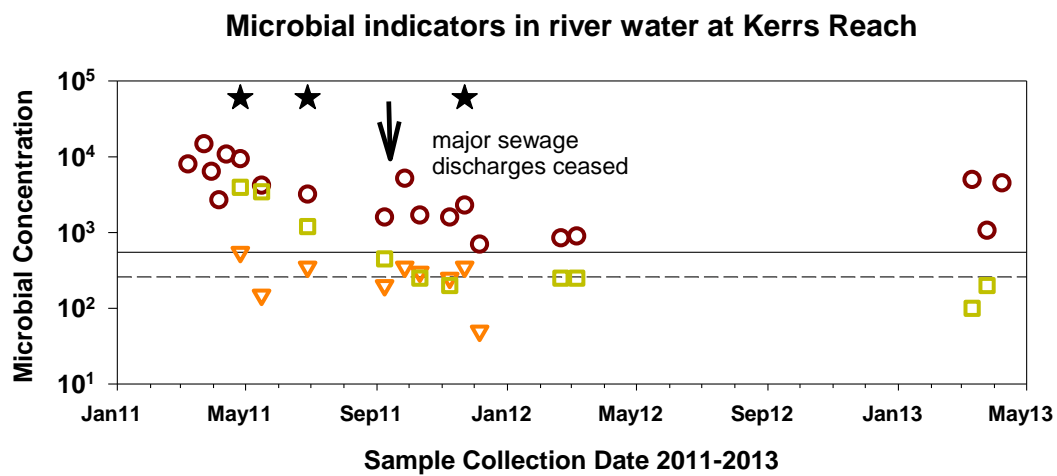
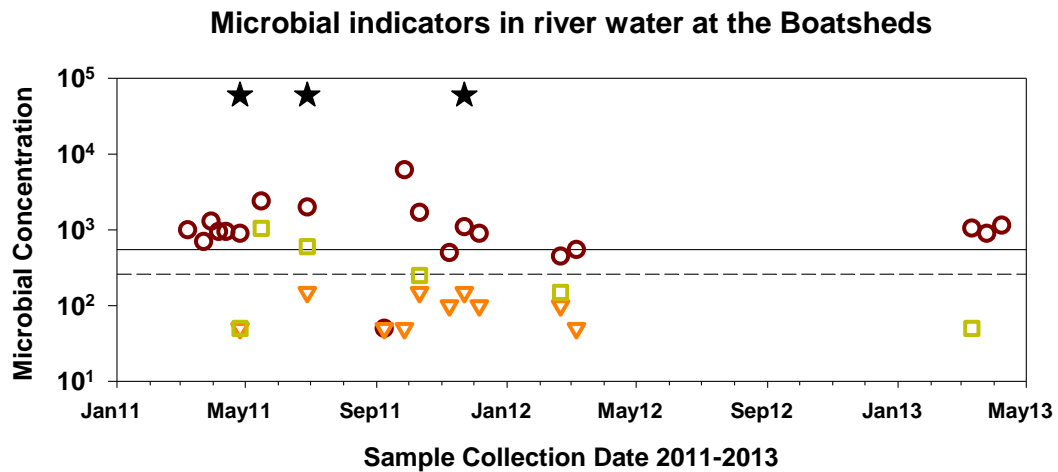
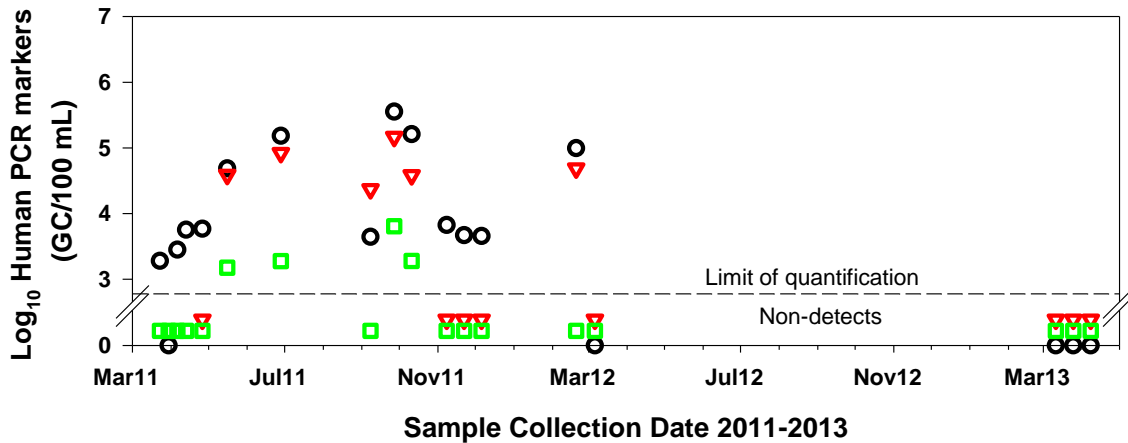
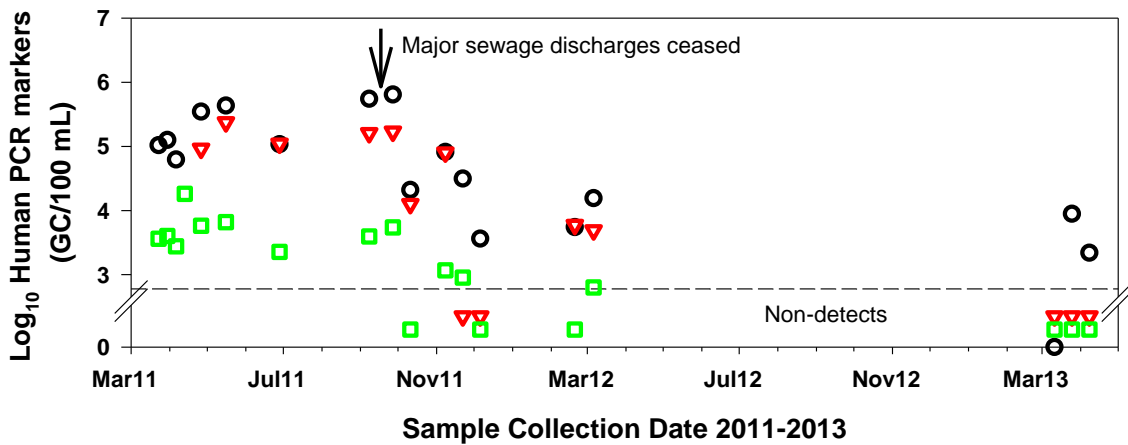


Figure 5: Microbial indicator concentrations in river water from 2011-2013. *E. coli* data only was collected prior to 26 April 2011. In 2013, *E. coli* and F-RNA phage only were tested in river water. Stars on graphs depict the days where rainfall was >5 mm in the 48 h prior to sampling.

Human PCR markers in river water at the Boatsheds



Human PCR markers in river water at Kerrs Reach



Human PCR markers in river water at Owles Terrace

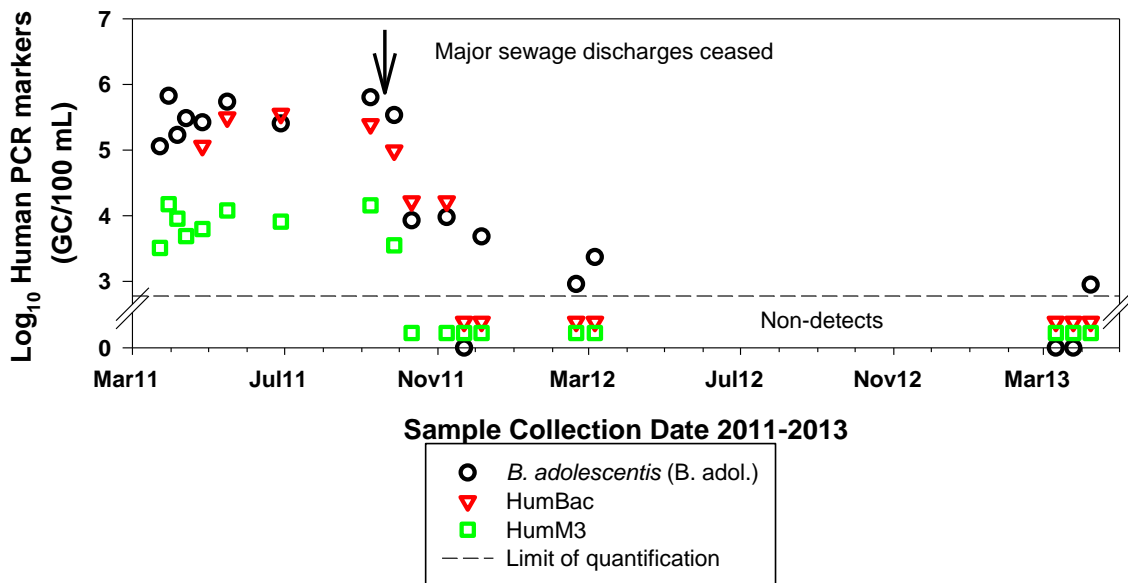
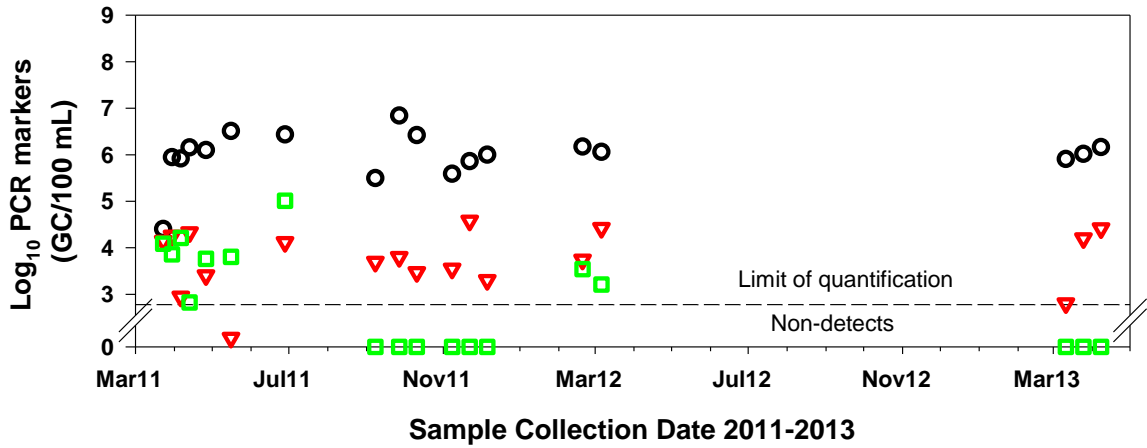
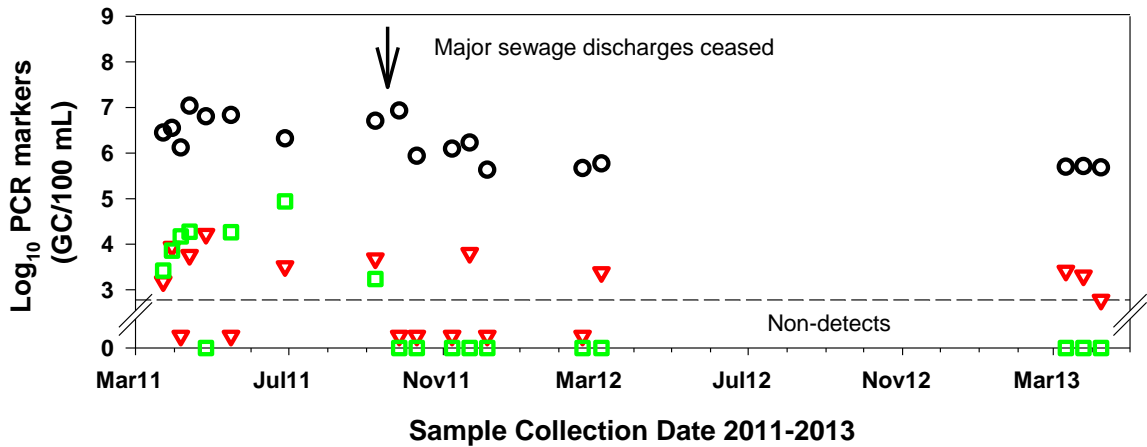


Figure 6: Human PCR marker concentrations in river water. PCR markers were analysed on most sampling occasions except for the first sampling on 8 March 2011, and HumBac PCR marker was not analysed on the first five sampling events. Icons below limit of quantification line were not detected for that particular PCR marker.

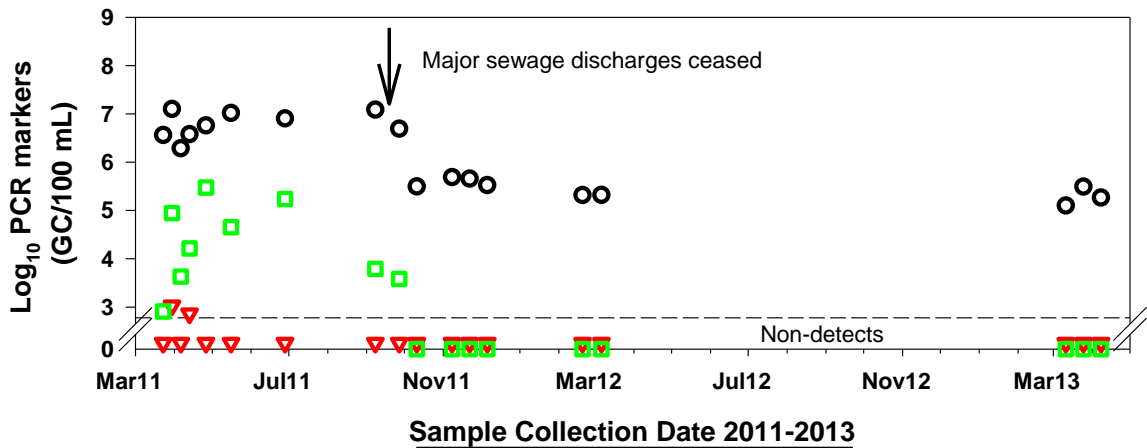
PCR markers in river water at the Boatsheds



PCR markers in river water at Kerrs Reach



PCR markers in river water at Owles Terrace



Sample Collection Date 2011-2013

- General PCR marker GenBac3
- ▼ Wildfowl-associated PCR marker
- Dog-associated PCR marker
- Limit of quantification

Figure 7: General and animal PCR markers in river water. In general, PCR markers were analysed in water on all sampling events, except for the first sampling on 8 March 2011. Icons below limit of quantification line were not detected for that particular PCR marker

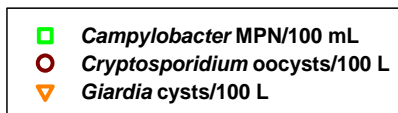
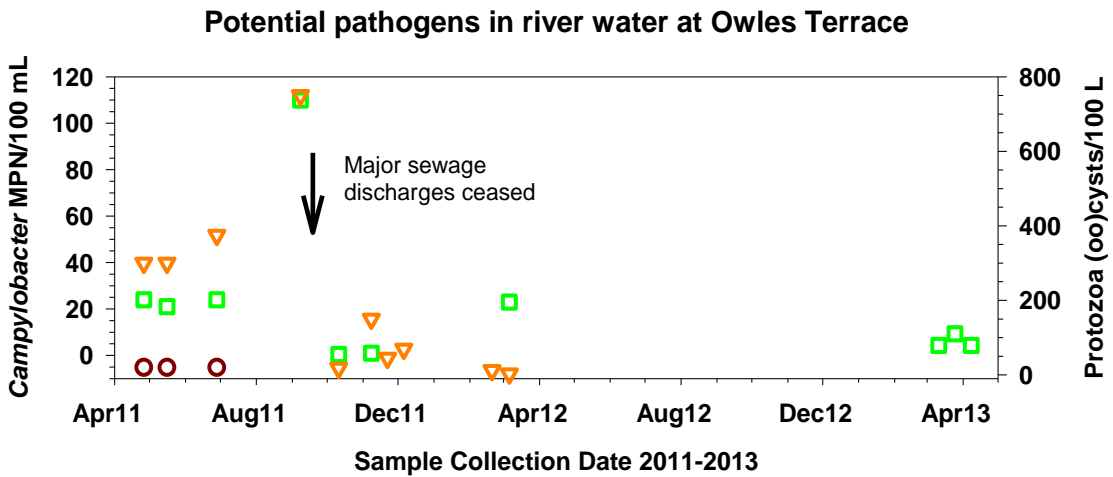
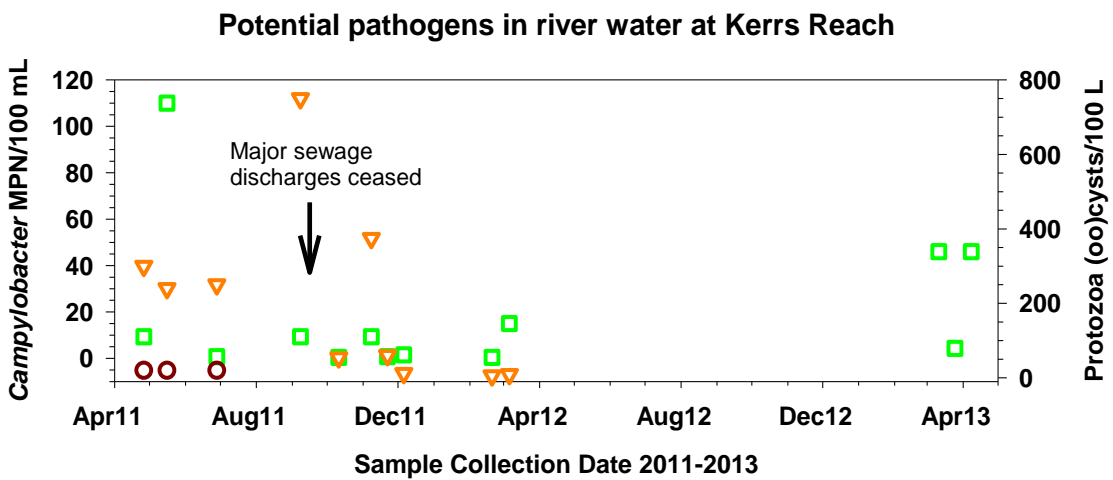
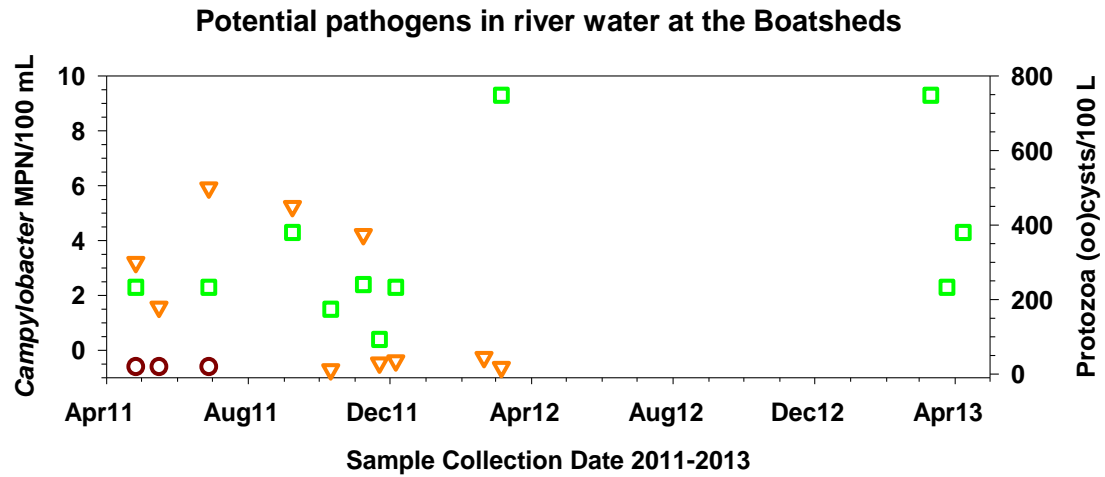


Figure 8: Potential pathogen concentrations in river water during 2011-2013. *Campylobacter* scale at BS is approximately ten-fold lower than for KR and OT. No pathogen data was collected before 26 April 2011 and only *Campylobacter* were tested in 2013.

Table 8: Chemical FST markers in river water at the Boatsheds. Detection of herbivore steroid ratio (R1) in the presence of human steroid ratios H1 >5% and H3 >1.0 indicates that human pollution is the source of mammalian stanols, coprostanol and 24-ethylcoprostanol

WATER BS	Total steroids (ng/L)	Human-associated steroid ratios								Herbivore	Avian-associated steroid ratios			FWA	**Summary of all FST markers including PCR markers
		F1 >0.5	F2 >0.5	H1 >5%	[‡] H2 >0.7	H3 >1.0	H4 >0.37	H5 %	H6 >1.5	R1 >5%	[¥] P1 >4.0	Av1 >0.4	Av2 >0.5	µg/L ≥0.1	
8-Mar-11	1,000	0.3	1.0	0.6	0.25	0.6	0.38	1	5.8	0.9	46.0	0.49	0.72	*NT	Wildfowl
23-Mar-11	1,673	0.7	1.6	2.5	0.40	0.7	0.43	14	6.2	3.4	9.7	0.36	0.57	NT	Wildfowl, dog
6-Apr-11	860	0.1	2.6	1.4	0.11	0.2	0.20	0	5.3	5.7	6.2	0.26	0.88	NT	Wildfowl, dog, herbivore
26-Apr-11	1,188	1.4	5.8	4.2	0.58	0.6	0.38	0	9.6	6.9	4.1	0.14	0.40	0.01	Wildfowl, dog, herbivore
16-May-11	1,871	4.5	9.5	13.3	0.82	1.1	0.53	43	38.2	11.8	2.4	0.09	0.18	0.01	Human, dog
28-Jun-11	2,189	9.4	11.5	25.0	0.90	2.1	0.67	84	66.2	12.1	1.4	0.08	0.09	0.01	Human, wildfowl, dog
8-Sep-11	1,003	2.7	7.4	7.0	0.73	1.1	0.53	43	17.6	6.2	5.3	0.12	0.26	0.01	Wildfowl, herbivore and human
27-Sep-11	3,316	9.6	16.3	25.1	0.91	1.6	0.61	67	151.3	15.8	0.8	0.06	0.09	0.01	Human, wildfowl
11-Oct-11	2,623	9.3	1.7	7.3	0.90	4.4	0.82	100	29.8	1.7	36.9	0.36	0.09	0.01	Human, wildfowl
8-Nov-11	5,189	1.6	1.7	2.9	0.62	1.3	0.56	52	16.1	2.2	27.7	0.37	0.37	0.01	Wildfowl
22-Nov-11	1,382	2.1	2.4	4.9	0.68	1.4	0.58	58	19.4	3.5	10.0	0.29	0.31	0.01	Wildfowl, borderline human
6-Dec-11	662	2.0	1.8	6.2	0.67	1.8	0.64	75	10.3	3.5	10.0	0.35	0.31	0.01	Low level human, wildfowl
20-Feb-12	1,745	3.9	4.1	14.3	0.80	2.3	0.70	91	26.9	6.1	3.7	0.19	0.20	0.01	Human, wildfowl, dog
6-Mar-12	2,119	0.8	2.6	6.1	0.45	1.1	0.53	43	7.5	5.3	5.3	0.26	0.52	0.01	Herbivore, wildfowl, dog, unconfirmed human
11-Mar-13	5,303	0.5	0.4	3.9	0.35	1.3	0.57	54	5.4	2.9	15.4	0.68	0.61	NT	Wildfowl
25-Mar-13	2,185	0.9	1.2	3.2	0.48	0.9	0.47	27	6.6	3.6	11.8	0.43	0.49	NT	Wildfowl
8-Apr-13	1,776	1.1	2.2	4.3	0.52	0.7	0.41	9	6.4	6.1	5.9	0.30	0.45	0.03	Herbivore and wildfowl

[‡]if ratio is between 0.3 and 0.7 this suggests a mix of human and environmental sources of coprostanol,

[¥]P1 ≥ 7.0 is supportive of avian pollution (Devane et al., 2015); *NT, Not tested; **Colour code for type of faecal pollution detected:

faecal pollution detected human herbivore avian

Table 9: Chemical FST markers in river water at Kerrs Reach. Detection of herbivore steroid ratio (R1) in the presence of human steroid ratios H1 >5% and H3 >1.0 indicates that human pollution is the source of mammalian stanols, coprostanol and 24-ethylcoprostanol

WATER KR	Total steroid (ng/L)	Human-associated steroid ratios								Herbivore R1	Avian-associated steroid ratios			FWA µg/L	**Summary of all FST markers including PCR markers
		F1 >0.5	F2 >0.5	H1 >5%	‡H2 >0.7	H3 >1.0	H4 >0.37	H5 %	H6 >1.5		‡P1 >4.0	Av1 >0.4	Av2 >0.5		
8-Mar-11	7,429	4.1	3.9	9.7	0.8	2.3	0.69	90	52.7	4.3	8.7	0.20	0.19	*NT	Human
23-Mar-11	5,028	4.5	7.9	21.5	0.82	3.5	0.78	100	95.2	6.2	3	0.11	0.18	NT	Human, wildfowl, dog
6-Apr-11	2,242	2.2	8.6	11.8	0.69	1.0	0.50	33	56.8	12.1	1.9	0.1	0.3	NT	Human, herbivore, dog
26-Apr-11	4,251	11.6	12.8	27.1	0.92	3.4	0.77	100	104.8	7.9	2.0	0.07	0.08	0.06	Human, wildfowl
16-May-11	6,581	12.8	9.2	25.1	0.93	3.2	0.76	100	169.2	7.8	2.4	0.10	0.07	0.09	Human, dog
28-Jun-11	5,288	13.2	14.3	28.3	0.93	3.3	0.77	100	95.4	8.5	1.4	0.06	0.07	0.10	Human, wildfowl, dog
8-Sep-11	3,662	3.7	5.0	7.0	0.79	2.5	0.72	96	47.8	2.8	16.5	0.17	0.21	0.04	Human, wildfowl, dog
27-Sep-11	6,039	11.4	16.1	27.8	0.92	1.9	0.66	80	239.6	14.3	0.8	0.06	0.08	0.04	Human
11-Oct-11	2,189	3.3	5.7	8.8	0.77	2.4	0.71	94	27.0	3.6	8.8	0.15	0.22	0.03	Human
8-Nov-11	2,718	8.3	5.4	14.8	0.89	2.7	0.73	99	50.2	5.5	5.4	0.16	0.11	0.04	Human
22-Nov-11	3,110	3.6	2.8	7.2	0.78	1.9	0.65	78	19.3	3.8	7.3	0.26	0.21	0.01	Human, low level wildfowl
6-Dec-11	1,636	0.9	1.8	4.5	0.47	1.1	0.52	40	14.1	4.2	7.9	0.36	0.51	0.01	Unconfirmed wildfowl
20-Feb-12	1,355	1.7	2.1	8.0	0.63	2.6	0.72	99	20.6	3.0	9.0	0.32	0.36	0.01	Low level human
6-Mar-12	1,987	1.8	3.0	7.7	0.64	2.2	0.68	87	12.5	3.6	7.2	0.24	0.34	0.40	Human, wildfowl
11-Mar-13	1,338	0.2	0.6	1.3	0.2	1.0	0.51	37	7.0	1.3	25.8	0.61	0.78	NT	Wildfowl
25-Mar-13	1,186	0.7	1.4	3.1	0.41	1.4	0.59	60	14.6	2.1	11.8	0.41	0.57	0.01	Wildfowl
8-Apr-13	1,341	0.3	4.1	3.4	0.22	1.0	0.51	37	13.2	3.3	8.8	0.19	0.77	0.03	Wildfowl

‡if ratio is between 0.3 and 0.7 this suggests a mix of human and environmental sources of coprostanol,

‡P1 ≥ 7.0 is supportive of avian pollution (Devane et al., 2015); *NT, Not tested; **Colour code for type of faecal pollution detected:

faecal pollution detected
 human
 herbivore
 avian

Table 10: Chemical FST markers in river water at Owles Terrace. Detection of herbivore steroid ratio (R1) in the presence of human steroid ratios H1 >5% and H3 >1.0 indicates that human pollution is the source of mammalian stanols, coprostanol and 24-ethylcoprostanol

WATER OT	Total steroids (ng/L)	Human-associated steroid ratios								Herbivore	Avian-associated steroid ratios			FWA	**Summary of all FST markers including PCR markers
		F1 >0.5	F2 >0.5	H1 >5%	‡H2 >0.7	H3 >1.0	H4 >0.37	H5 %	H6 >1.5	R1 >5%	‡P1 >4.0	Av1 >0.4	Av2 >0.5	µg/L ≥0.1	
8-Mar-11	22,376	6.3	9.3	14.6	0.86	1.9	0.65	79	64.1	7.7	3.5	0.10	0.14	*NT	Human
23-Mar-11	7,287	6.8	6.7	21.0	0.87	3.2	0.76	100	80.5	6.6	2.0	0.13	0.13	NT	Human, dog
26-Apr-11	9,503	13.5	17.5	24.9	0.93	3.0	0.75	100	107.7	8.3	1.1	0.05	0.07	0.14	Human, dog
16-May-11	11,127	15.0	16.6	30.6	0.94	2.9	0.75	100	142	10.5	1.2	0.06	0.06	0.21	Human, dog
28-Jun-11	4,498	19.0	18.4	31.7	0.95	3.1	0.75	100	146	10.3	1.1	0.05	0.05	0.18	Human, dog
8-Sep-11	6,233	7.2	9.3	20.1	0.88	1.8	0.65	76	64.9	11	1.9	0.10	0.12	0.14	Human, dog
27-Sep-11	8,264	12.1	16	26.5	0.92	2.4	0.71	94	125.1	11	1.4	0.06	0.08	0.06	Human, dog
11-Oct-11	3,067	4.7	9.3	11.7	0.83	2.1	0.68	86	21.2	5.5	5.8	0.10	0.17	0.03	Human
8-Nov-11	3,738	6.2	6.1	10.7	0.86	2.2	0.69	88	39.1	4.9	4.5	0.14	0.14	0.03	Human
22-Nov-11	1,819	3.3	4.0	8.6	0.77	1.8	0.64	74	13.6	4.9	4.0	0.20	0.22	0.02	Human
6-Dec-11	2,913	3.1	1.5	6.3	0.75	3.1	0.76	100	15.3	2	11.9	0.39	0.23	0.02	Human
20-Feb-12	1,621	1.4	2.3	6.8	0.58	2.1	0.67	84	9.5	3.3	3.7	0.30	0.4	0.01	Low level human
6-Mar-12	1,913	1.3	2.5	4.6	0.56	1.9	0.65	77	8.7	2.4	5.3	0.27	0.41	0.16	Borderline human
11-Mar-13	1,391	0.4	0.5	1.9	0.27	1.7	0.64	73	4.3	1.1	18.3	0.61	0.69	NT	Wildfowl
25-Mar-13	2,082	0.4	1.0	1.2	0.29	1.1	0.53	44	4.4	1.1	11.4	0.45	0.67	NT	Wildfowl
8-Apr-13	918	1.2	3.0	4.5	0.54	1.6	0.62	67	7.2	2.8	6.9	0.24	0.42	NT	Borderline human

‡if ratio is between 0.3 and 0.7 this suggests a mix of human and environmental sources of coprostanol,

‡P1 ≥ 7.0 is supportive of avian pollution (Devane et al., 2015); *NT, Not tested;

**Colour code for type of faecal pollution detected:

faecal pollution detected human herbivore avian

3.3.3 Water: comparisons between discharge and post-discharge concentrations

The microbial water quality data from the three locations were combined, and summarised results were presented in Table 11 as a high level overview of microbial concentrations during each discharge phase. The mean concentrations were greatest for *E. coli* followed by F-RNA phage, *C. perfringens*, *Campylobacter*, *Giardia*, and then *Cryptosporidium*. This pattern was consistent across all three locations.

The observed levels of *E. coli* at the three river sites were compared using the Kruskal-Wallis analysis of variance, both during active and post-discharge (2011-2012). There was a significant difference in *E. coli* between the three sampling sites ($p = 0.017$) during active discharge when the two downstream sites were receiving continuous discharges of sewage, compared with the intermittent nature of broken sewer pipes impacting the upstream, BS site. In comparison, after the major discharges ceased at the two downstream sites, there was no statistically significant difference detected in *E. coli* levels ($p = 0.129$) between the three sites. The observed mean levels of all microorganisms at KR and OT were higher during the period of the discharges, than after discharges ceased. At BS, above which only intermittent sewage discharges occurred, *E. coli*, *Campylobacter* and *C. perfringens* levels were highest in the post-discharge phase period. In comparison, F-RNA phage and the two protozoa (*Giardia* and *Cryptosporidium*) were detected at higher concentrations at BS prior to October 2011. When the results from all three sites were combined, a Mann-Whitney test indicated that, with the exception of *C. perfringens*, there were significant differences in the levels of all microorganisms between the two discharge periods ($p < 0.05$). Further analysis of the Mann-Whitney test on the individual sites showed that the differences between the two discharge phases were only significant at OT for all microbes, including *C. perfringens* (Table 12). In addition, F-RNA phage was the only microbe that showed a significant difference between the two discharge phases at KR. The low sample number for the individual sites may have reduced the power of the test to discriminate significant differences between the active discharge and post-discharge phases for the other microbes.

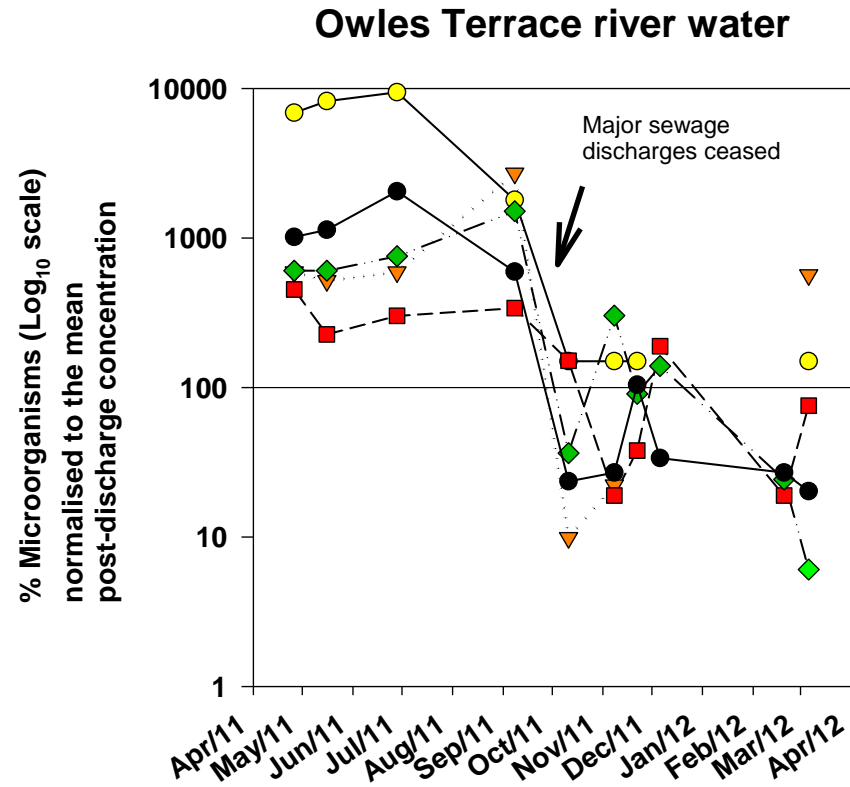
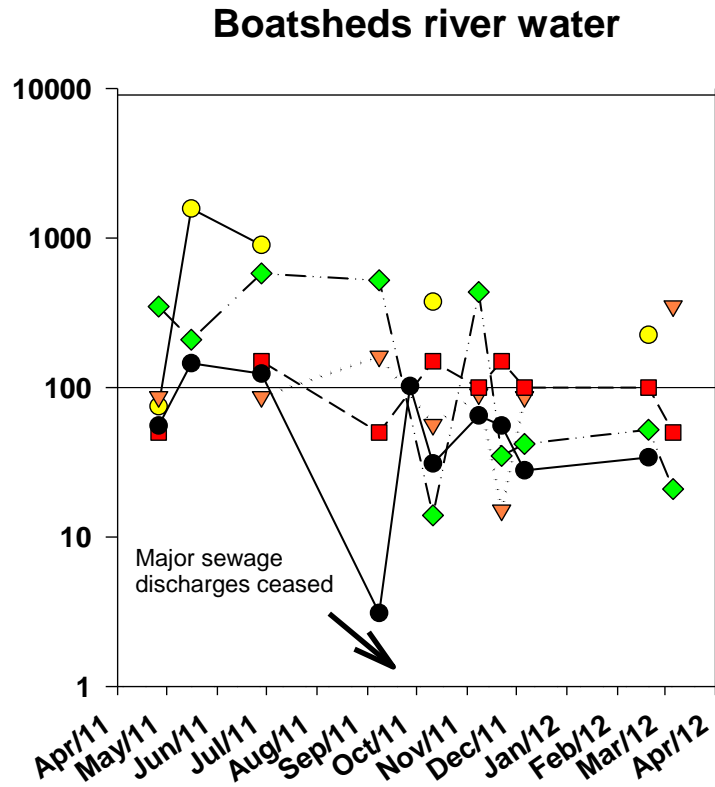
To further demonstrate the differences between the two discharge phases, the levels of indicators and pathogens were normalised against the mean post-discharge (late September, 2011-2012 data) level of each microorganism at OT and at BS, which acted as a comparison site where there were no significant differences between the two discharge phases. Figure 9 illustrates the impact of a major discharge at OT compared with the intermittent human and animal discharges that occurred at BS showing that the levels of pathogens and indicators were

more similar during the discharge and post-discharge phases at BS. KR was not included in this analysis as there was also no evidence of significant differences between discharge and post-discharge. In comparison, the levels of all microorganisms at OT during active discharge, were well above the mean level post-discharge.

Table 11: Mean levels (\pm standard deviation) of microorganisms in water during active discharges (April –8 September), post-discharge (27 September 2011-March 2012) and March-April 2013.

Discharge Phase	Sample numbers /site	Boatsheds (BS)	Kerrs Reach (KR)	Owles Terrace (OT)
<i>E. coli</i> CFU/100 mL				
Active discharge	9	1,138 (\pm 697)	6,789 (\pm 4,358)	29,678 (\pm 27,963)
Post-discharge	7	1,629 (\pm 2,063)	1,893 (\pm 1,566)	1,493 (\pm 2,427)
Mar-Apr 2013	3	1,035 (\pm 126)	3,523 (\pm 2,139)	547 (\pm 270)
Overall <i>E. coli</i>	19	1,303 (\pm 1,305)	4,469 (\pm 3,897)	14,694 (\pm 23,725)
F-RNA phage PFU/100 mL				
Active discharge	4	425 (\pm 497)	2,263 (\pm 1,700)	2,200 (\pm 1,122)
Post-discharge	6	67 (\pm 108)	158 (\pm 124)	33 (\pm 26)
Mar-Apr 2013	3	17 (\pm 29)	100 (\pm 100)	50 (\pm 87)
Overall F-RNA phage	10	165 (\pm 316)	792 (\pm 1,331)	704 (\pm 1,181)
<i>C. perfringens</i> CFU/100 mL				
Active discharge	4	63 (\pm 63)	313 (\pm 180)	875 (\pm 250)
Post-discharge	7	100 (\pm 41)	186 (\pm 163)	264 (\pm 215)
Mar-Apr 2013	0	*NT	NT	NT
Overall <i>C. perfringens</i>	11	86 (\pm 50)	232 (\pm 172)	486 (\pm 376)
<i>Campylobacter</i> MPN/100 mL				
Active discharge	4	2 (\pm 2)	32 (\pm 52)	45 (\pm 44)
Post-discharge	6	3 (\pm 3)	5 (\pm 6)	4 (\pm 9)
Mar-Apr 2013	3	5 (\pm 4)	32 (\pm 24)	6 (\pm 3)
Overall <i>Campylobacter</i>	13	3 (\pm 3)	19 (\pm 32)	17 (\pm 30)
<i>Giardia</i> cysts/100 L				
Active discharge	4	358 (\pm 146)	385 (\pm 245)	431 (\pm 215)
Post-discharge	6	86 (\pm 142)	86 (\pm 144)	50 (\pm 55)
Mar-Apr 2013	0	NT	NT	NT
Overall <i>Giardia</i>	10	195 (\pm 195)	206 (\pm 235)	202 (\pm 237)
<i>Cryptosporidium</i> oocysts/100 L				
Active discharge	4	15 (\pm 10)	15 (\pm 10)	15 (\pm 10)
Post-discharge	6	0	0	0
Mar-Apr 2013	0	NT	NT	NT
Overall <i>Cryptosporidium</i>	10	6 (\pm 10)	6 (\pm 10)	6 (\pm 10)

*NT, Not tested



Date 2011-2012

Date 2011-2012

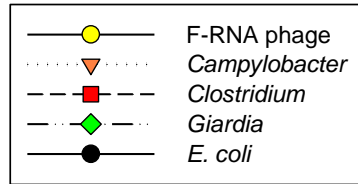


Figure 9: A comparison of normalised levels of pathogens and indicators at Owles Terrace and the Boatsheds during the discharge (April-mid September, 2011) and post-discharge (late September, 2011- March, 2012) phases. Mean value for post-discharge phase is set at 100%. KR was not included in this analysis, as similar to BS, there was no evidence of significant differences between discharge and post-discharge for indicators and pathogens. *Cryptosporidium* was not included in the figure as it was only detected during the discharge phase.

Table 12: Statistically significant differences identified between microbial concentrations during discharge and post-discharge at individual sites on the Avon/Otākaro River. Bold italics indicate significant difference with α values less than 0.05.

2011-2012 data Microorganism	<i>p</i> values for comparison of discharge and post-discharge microbial concentrations at each site		
	Boatsheds	Kerrs Reach	Owles Terrace
<i>E. coli</i>	0.968	0.127	<i>0.011</i>
F-RNA phage	0.257	<i>0.013</i>	<i>0.011</i>
<i>C. perfringens</i>	0.333	0.364	<i>0.011</i>
<i>Campylobacter</i>	0.886	0.229	<i>0.019</i>
<i>Giardia</i>	0.114	0.809	<i>0.014</i>
<i>Cryptosporidium</i>	<i>< 0.001</i>	<i>< 0.001</i>	<i>< 0.001</i>

3.3.4 Water: relationships between microbial indicators and pathogens

E. coli had significant correlations with all pathogens, including a moderate correlation with *Cryptosporidium* and weaker correlations with *Giardia* and *Campylobacter* (Table 13). The three microbial indicators all had moderate and significant correlations with each other, with the exception of a weaker correlation between F-RNA phage and *C. perfringens*. F-RNA phage had a moderate correlation with *Cryptosporidium*, a weak correlation with *Giardia*, and a non-significant correlation with *Campylobacter*. This lack of correlation between F-RNA phage and *Campylobacter* will be investigated in more detail during the regression analysis below, as it differs from the result reported in Devane et al. (2014). *Giardia* was the only pathogen that had a significant correlation (weak) with *C. perfringens*.

The NZ Microbiological Water Quality Guidelines for Marine and Freshwater Recreational Areas (Ministry for the Environment, 2003) specify that water with ≤ 260 *E. coli*/100 mL, is generally acceptable for recreational use. There were too few samples analysed in this study with less than 260 *E. coli* (in fact only two), to assess pathogen presence in samples with less than 260 *E. coli* per 100 mL. There were, however, 47/57 samples in this study with >550 *E. coli* (Action Mode), including *E. coli* always identified in concentrations >550 CFU/100 mL at KR. Thirty-two of these samples with >550 *E. coli* were also tested for microbes and had higher mean levels of *Giardia*, *Campylobacter* and F-RNA phage, than samples with less than 550 *E. coli*. *Cryptosporidium* were only detected in samples with >550 *E. coli*. Samples with between 260 and 550 *E. coli* also contained *Campylobacter* (5/8 samples), F-RNA phage (4/8) and *Giardia* (all samples) at lower levels. Table 14 shows the mean values for microorganisms present when *E. coli* was less than ≤ 550 and >550 CFU/100 mL. Although all

microbes had higher mean concentrations in samples containing *E. coli* >550, this was only statistically significant for F-RNA phage. *Cryptosporidium* was excluded from this analysis as it was only detected in samples with >550 *E. coli*.

Table 13: Correlation Matrix (Spearman, r_s) between indicators and pathogens in river water over the period April 2011 to March 2013.

Variables	<i>E. coli</i> (n=39)	<i>Campylobacter</i> (n=39)	F-RNA phage (n=22)	<i>C. perfringens</i> (n=33)	<i>Giardia</i> (n=30)
<i>Campylobacter</i>	0.396^a				
F-RNA phage	0.646	0.293			
<i>C. perfringens</i>	0.544	0.338	0.428		
<i>Giardia</i>	0.468	0.413	0.395	0.428	
<i>Cryptosporidium</i>	0.677	0.308	0.700	0.274	0.458

^a Values in bold italics are different from 0 with a significance level $\alpha = 0.05$.

Table 14: Comparison of microbial concentrations in the presence of *E. coli* concentrations above and below the water quality guidelines Action level of 550 CFU/100 mL for 2011 to 2013 data. *Cryptosporidium* were only detected in samples with >550 *E. coli*, and therefore, were not included in this analysis.

Mean <i>E. coli</i>	<i>Campylobacter</i> MPN/100 mL	<i>C. perfringens</i> CFU/100 mL	F-RNA phage PFU/100 mL	<i>Giardia</i> Cysts/100 L
Mean > 550 CFU/100 mL (n = 32)	16	306	734	233
Mean ≤ 550 CFU/100 mL (n = 10)	4	167	30	127
<i>P</i> ratio	<i>p</i> = 0.125	<i>p</i> = 0.272	<i>p</i> = 0.009	<i>p</i> = 0.286

Regression analysis of relationships between microbes in water

Regression analysis of log transformed data from 2011-2013 suggested there was a weak ($R^2 = 0.214$) but significant ($p = 0.006$) relationship between *E. coli* and *Campylobacter* concentrations (Figure 10A). This statistically significant relationship between *E. coli* and *Campylobacter* was also seen in Table 13 when using Spearman's rank correlation coefficient. The observed *E. coli* concentrations ranged from 50 to 100,000 CFU/100 mL. The full regression equation is $\text{Log } \textit{Campylobacter} = -1.13 + 0.57 * \text{log } \textit{E. coli}$. Predicted levels of *Campylobacter* based on observed levels of *E. coli* are presented in Table 15.

Devane et al. (2014) using the data collected in 2011-2012, noted that there was a similar significant correlation between F-RNA phage and *Campylobacter* as between *E. coli* and *Campylobacter*, therefore, the relationship between F-RNA phage and *Campylobacter* was investigated further. There was a relationship between log transformed F-RNA phage and

Campylobacter data ($p = 0.033$). The regression coefficient was 0.605 (95% Confidence Interval (CI) 0.055 to 1.156) and the regression equation was $\log \text{Campylobacter} = -0.866 + 0.605 * \log \text{F-RNA phage}$. However, with additional data ($n = 9$) collected in 2013 for F-RNA phage and *Campylobacter*, the relationship was no longer significantly correlated (r_s 0.30; $p = 0.07$) (Table 13) including when using the linear regression for log transformed F-RNA phage and *Campylobacter* ($p = 0.085$) (Figure 10B).

There were several samples where *Campylobacter* and/or F-RNA phage were not identified in a water sample. The regression analyses for *Campylobacter* with *E. coli* and F-RNA phage excluded data where one or more of the variables was not detected, which reduced the number of matched variables for the data sets ($n = 34$ and 22 , respectively) from a maximum of 39 samples. A sensitivity analysis was, therefore, performed on the regression analyses to determine if using half of the detection limit in place of zero for log transformation of each of the samples would change the outcome of the regression (data not shown). Comparison of the range of the slope and of the y-intercept for *Campylobacter* with *E. coli* showed that inclusion of the non-detects in the data set did not change the range of the 95% confidence interval and also the range of the slope did not cover zero suggesting that there was a valid relationship between the concentration of *Campylobacter* and that of *E. coli*. A similar analysis of *Campylobacter* and F-RNA phage with inclusion of the non-detects also showed similar ranges for slope and for the y-intercept when the data set included or excluded the non-detect data. However, in the case of the regression analysis for *Campylobacter* and F-RNA phage for both data sets in/exclusive of non-detects, the range of the slope did cover the zero value suggesting that the two variables were not related and prediction of *Campylobacter* concentration based on F-RNA phage was not valid.

The relationship between *E. coli* and *Giardia* is shown in Figure 10C. There was a significant relationship between the log transformed *E. coli* and *Giardia* data with a regression coefficient of 0.508 (95% CI 0.105 to 0.911). The full regression equation is $\log \text{Giardia} = 0.321 + 0.508 * \log \text{E. coli}$. Predicted levels of *Giardia* based on observed levels of *E. coli* are presented in Table 15. Regression analysis identified a relationship between *Giardia* and *C. perfringens*. However, the wider prediction intervals in Figure 10D suggested that *C. perfringens* was not as good as *E. coli* as a predictor of *Giardia*.

The relationship between *Cryptosporidium* and *E. coli* (not shown) cannot be quantified because the *Cryptosporidium* data are based on presence-absence values. *Cryptosporidium* was present in nine out of twelve water samples when active discharges were occurring but was not present in any of the samples collected after active discharges ceased. A Mann-Whitney test

($p < 0.001$) indicated *Cryptosporidium* was present when levels of *E. coli* were high and absent when it was low. The analysis indicated a relationship between log transformed *E. coli* data and the presence/absence of *Cryptosporidium* (P chi square = 0.009). The logistic regression equation for the probability of finding *Cryptosporidium* was given by:

$Prob(\text{Cryptosporidium present}) = \frac{1}{1+e^{-x}}$, where $x = 4.1 \log(E. coli) - 14.3$ and the coefficient 4.1 has a 95% confidence interval of 1.0 to 7.2. The expected probability of finding *Cryptosporidium* based on the observed level of *E. coli* is presented in Table 15.

Table 15: Predicted pathogen concentrations based on relationships with *E. coli*

Observed level of <i>E. coli</i>	Predicted level of pathogen based on observed level of <i>E. coli</i>		Probability of identifying pathogen based on observed level of <i>E. coli</i> ^a
<i>E. coli</i> (CFU/100 mL)	<i>Campylobacter</i> (MPN/100 mL)	<i>Giardia</i> (cysts/100 L)	<i>Cryptosporidium</i> (%)
30,000	26	394	98.30
10,000	14	225	89.09
1,000	4	70	11.92
500	3	49	3.79
100	1	22	0.22

^a Probability is based on presence/absence data for *Cryptosporidium*.

3.3.5 Water: relationships between FST markers and microbes

FWA levels in water were very low, and therefore, were excluded from the correlation analyses. Moderate to strong (range, r_s 0.55 to 0.92), significant ($p \leq 0.004$), positive correlations were observed between human PCR markers and human-associated steroid ratios. There were also moderate to strong, positive correlations (range, r_s 0.57 to 0.84, $p < 0.001$) between *E. coli* and the human FST markers in water samples with the highest correlation with the HumM3 PCR marker. F-RNA phage had similar moderate to strong correlations with human FST markers (r_s 0.62 to 0.84, $p < 0.001$) with the highest correlations to human PCR markers, HumBac and HumM3. In comparison, *C. perfringens* had weaker significant correlations (r_s 0.44 to 0.64, $p \leq 0.011$) with human FST markers. The Dog PCR marker, also had mostly moderate, positive correlations with human FST markers ($p < 0.003$).

The human-associated steroid ratios (F1, F2, H1, H2 and H6) had very strong, positive correlations with each other (r_s 0.83 to 0.95; $p \leq 0.001$). The H3-H5 ratios, which discriminate between human and herbivore sources, were not correlated with the herbivore ratio (R1), but showed moderate to strong positive correlations with the human-associated ratios and total steroids ($p \leq 0.001$).

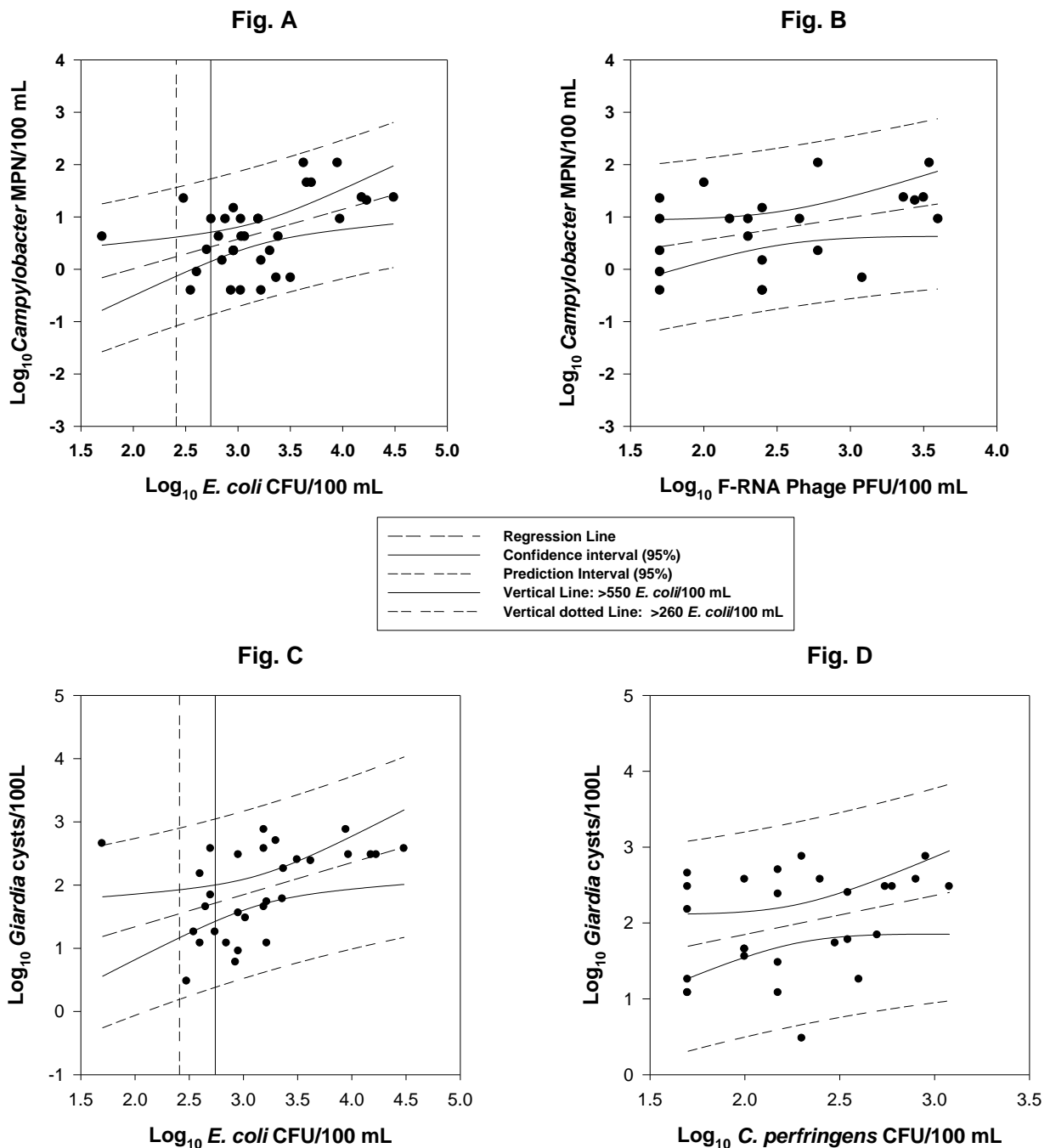


Figure 10: Regression analysis of indicators and pathogens in river water (2011-2013 data). A) *Campylobacter* and *E. coli*, based on 34 observations; B) *Campylobacter* and F-RNA phage, based on 22 observations; C) *E. coli* and *Giardia*, based on 30 observations, and D) *C. perfringens* and *Giardia*, based on 27 observations.

Principal component analysis (PCA) of all variables in water

Analysis by principal component analysis (PCA) of all variables in water (using Spearman Ranks) suggested that most of the data variability was accounted for in the first two components generated, as together, they explained 72% of the variance. The first principal component (PC1) explained 60% of the variability, and factor loadings of approximately 0.9 suggested it was

strongly associated with the human-associated PCR markers and steroid ratios (Table 16). For the second component (PC2) of the PCA, the highest correlation was with the three human steroid ratios (H3-H5). However, these three ratios were not cleanly loaded onto either of the first two principal components as they had similar moderate correlations (approximately 0.6) in PC1 and PC2. This made it difficult to interpret the relevant associations between variables contributing to the second component.

The avian-associated steroid ratios that indicate wildfowl and/or plant runoff (Av1, Av2 and P1) had strong to moderate, significant ($p < 0.001$) but negative correlations with other steroid ratios and human-associated PCR markers. This was represented in the PC analysis where the two avian-associated steroid ratios had strong negative factor loadings on PC1. In general, the avian steroid ratios also had weak to moderate negative correlations with microbial indicators ($p < 0.012$), protozoa ($p < 0.025$) and no significant correlations with *Campylobacter*.

The pathogen, *Campylobacter*, had the least significant correlations with all FST variables compared with other pathogens and indicators and had only a significant but weak, positive correlation with the HumM3 PCR marker (r_s 0.40, $p \leq 0.012$), and as noted in the section on microbial relationships, similar weak correlations with only *E. coli* and *Giardia*. The third component of the PCA contributed 7% of the data variability with only *Campylobacter* having a strong association (factor loading of 0.77) with PC3. PC4 accounted for 5% of the data variability with the wildfowl PCR marker having the only high factor loading (0.82) with this component. The Wildfowl PCR marker also reported non-significant correlations with *E. coli* and most FST markers, including the three avian-associated steroid ratios, and no correlations with pathogens or microbial indicators. From comparisons of the correlation and PCA data, *Campylobacter* and the wildfowl PCR marker would both appear to be unrelated to each other or any of the other variables.

In contrast to *Campylobacter*, both protozoa, *Giardia* and *Cryptosporidium* had moderate, positive correlations (range, r_s 0.53 to 0.65, $p \leq 0.0003$) with all PCR markers (except the wildfowl), and weak to moderate, positive correlations with human-associated steroid ratios (range, r_s 0.43 to 0.74, $p \leq 0.0018$). The protozoa also had negative, weak to moderate correlations with the avian-associated steroid ratios (range, r_s -0.41 to -0.74, $p \leq 0.024$). In general, these correlations between FST markers and protozoan pathogens were stronger than those between *Giardia* and the microbial indicators (range, r_s 0.40 to 0.47, $p \leq 0.032$) and similar to those between *Cryptosporidium* and microbial indicators (range, r_s 0.68 to 0.70, $p < 0.001$), excluding the non-correlation between *Cryptosporidium* and *C. perfringens* (Table 13).

Table 16: Factor loadings identified for each variable in water by Principal Component Analysis. Shading indicates those variables with the highest factor loading contributing to a particular principal component (PC).

Variable in water	Factor loadings for water			
	PC1	PC2	PC3	PC4
<i>E. coli</i>	0.719	-0.265	0.356	-0.042
General PCR marker	0.836	-0.276	0.133	0.249
B. adol PCR marker	0.907	0.048	0.065	0.150
HumBac PCR marker	0.929	-0.005	0.023	0.075
HumM3 PCR marker	0.894	-0.123	0.254	0.070
<i>C. perfringens</i>	0.611	0.238	0.365	-0.190
F-RNA Phage	0.812	0.028	0.236	0.068
<i>Campylobacter</i>	0.194	-0.409	0.769	-0.256
<i>Cryptosporidium</i>	0.730	-0.424	-0.022	-0.049
<i>Giardia</i>	0.608	-0.433	0.120	0.077
Wildfowl PCR marker	-0.160	-0.432	-0.067	0.816
Dog PCR marker	0.606	-0.476	0.060	0.018
AC/TC	-0.528	0.225	-0.411	-0.008
Steroid ratios				
F1	0.947	0.202	-0.157	0.075
F2	0.893	-0.186	-0.277	-0.188
H1	0.946	0.139	-0.223	-0.024
H2	0.947	0.202	-0.157	0.075
H3	0.690	0.669	0.140	0.086
H4	0.690	0.669	0.140	0.086
H5	0.705	0.653	0.149	0.043
H6	0.949	0.083	-0.099	0.073
R1	0.778	-0.416	-0.403	-0.085
P1	-0.788	0.253	0.369	0.294
Av1	-0.890	0.190	0.281	0.195
Av2	-0.943	-0.208	0.160	-0.075

*-, variable not tested

Figure 11 plots the factor scores for each sample against the first two components of the PCA and divides the samples into active and post-discharge events (including 2013 data). The figure shows a clear division between the two discharge phases of the study, in that all samples collected during the active discharge phase occur on the right-hand side of the dotted line. In addition, the active discharge samples from KR and OT cluster along the positive axis of the first component (PC1), whose variability was explained by the human-associated FST markers and high correlations with the potentially pathogenic protozoa. In comparison, the samples collected post-discharge are more closely associated with the positive axis of PC2, which had some relationship with the steroid markers that discriminated between human and herbivore pollution,

although the contributing variables to this component were not clearly defined by the factor loadings.

Based on the strong and significant correlations between human FST markers in water samples, logistic regression of these variables was investigated. The human PCR marker and human steroid ratio data were converted to binary values of one and zero to compare the concordance between the detection of human faecal inputs using these two types of FST marker (refer to statistical methods section for detail). Table 17 presents the binary data for the PCR and steroid markers as a contingency table and represents an 89% agreement between the two FST methods. This concordance was assessed by applying Cohen's kappa to produce a kappa statistic of 0.78, which provided sufficient evidence to infer that there was substantial agreement (kappa >0.6) between the two FST methods ($p = 0.023$) when detecting human faecal pollution.

Logistic regression was also performed between Log transformed *E. coli* and the dependent binary variable for human PCR markers, and between *E. coli* and human steroid binary data. Both logistic regressions showed that *E. coli* provided predictive value of human pollution in water only when concentrations exceeded approximately Log 3.75 *E. coli*/100 mL, and therefore, was a less useful parameter compared to the FST methods. The lack of differentiation between *E. coli* concentrations in water samples where human pollution was present and where it was absent (as identified by FST markers) can be viewed in the boxplots of Figure 12.

Figure 13 presents the logistic regression of the percentage of coprostanol (H1) as the independent variable and the Human PCR markers displayed as binary data for the non-detection/detection of human pollution ($n = 47$). There were only two occasions where the %coprostanol was >6% (6.8 and 8.6%) and human PCR markers were not detected and this occurred during the post-discharge phase in late 2011 and early 2012. There were no occasions where %cop was <5% and human PCR markers were detected. These results alongside the concordance between PCR and steroid markers presented in Table 17 support the threshold of 5-6% coprostanol (Table 3) as indicating a human faecal input when sewage is discharged into a river.

Table 17: Contingency tables for concordance between steroid and human PCR markers in water. Refer to methods for details of criteria for assigning binary values to steroid and human PCR markers for detection of human faecal inputs.

Human inputs detected by steroids and PCR markers; Water samples, n = 47			
		Detection by PCR	
Detection by steroids		No human inputs	Human inputs
No human inputs		17	1
Human inputs		4	25

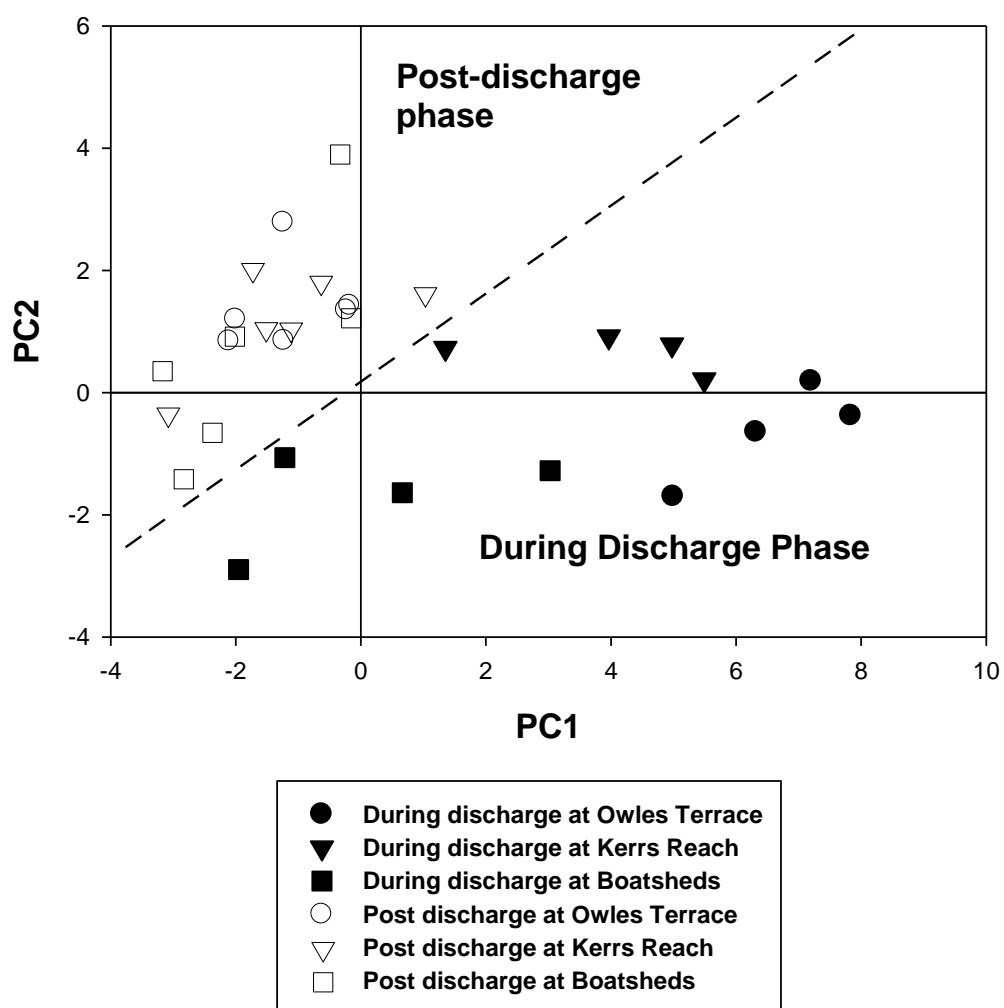


Figure 11: PCA of observations plotted as site location and discharge status against the two dominant components that accounted for 72% of the variability of the data. The dotted line delineates between the observations that occurred during the active discharge phase and post-discharge (September, 2011-2013 data).

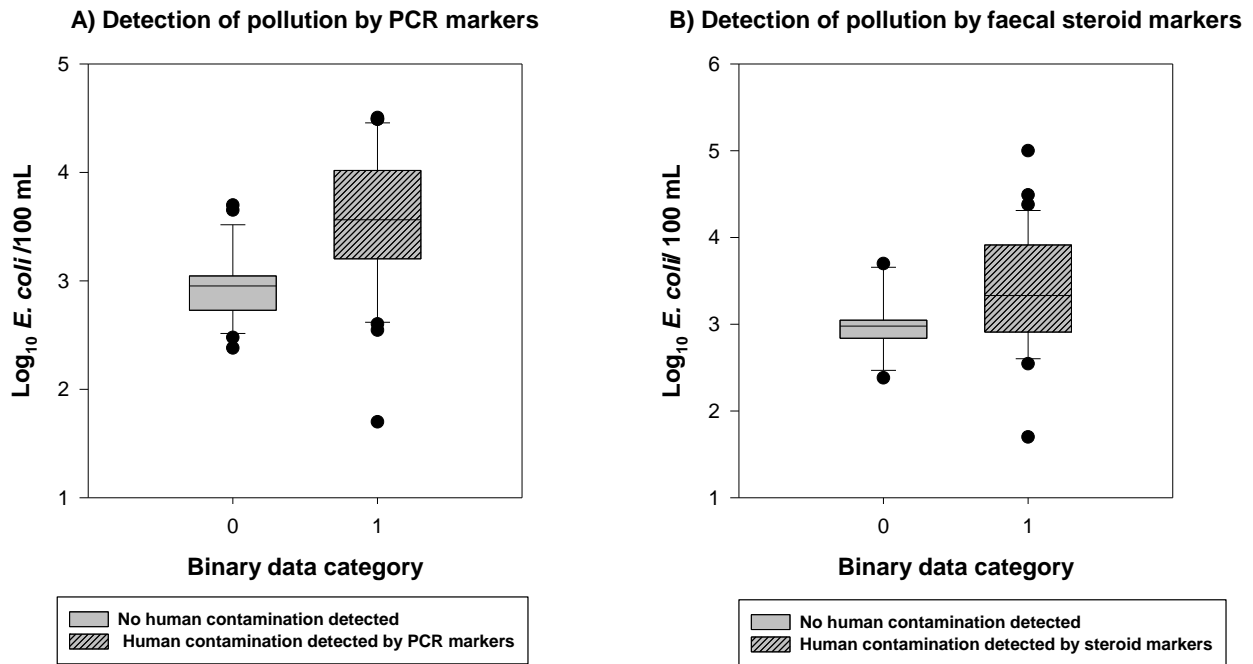


Figure 12: *E. coli* concentration in water in the presence/absence of human contamination as detected by A) Human PCR markers, B) steroid markers. Conversion of human faecal pollution markers in water to binary data plotted against *E. coli* concentrations shows that there is a notable overlap between *E. coli* concentrations when FST markers identify/do not identify human pollution. The boundary of the box closest to the x-axis indicates the 25th percentile, the line within the box represents the median, and the boundary of the box farthest from the x-axis indicates the 75th percentile. Whiskers below and above the box indicate the 10th and 90th percentiles, respectively; ●, outlier measurements.

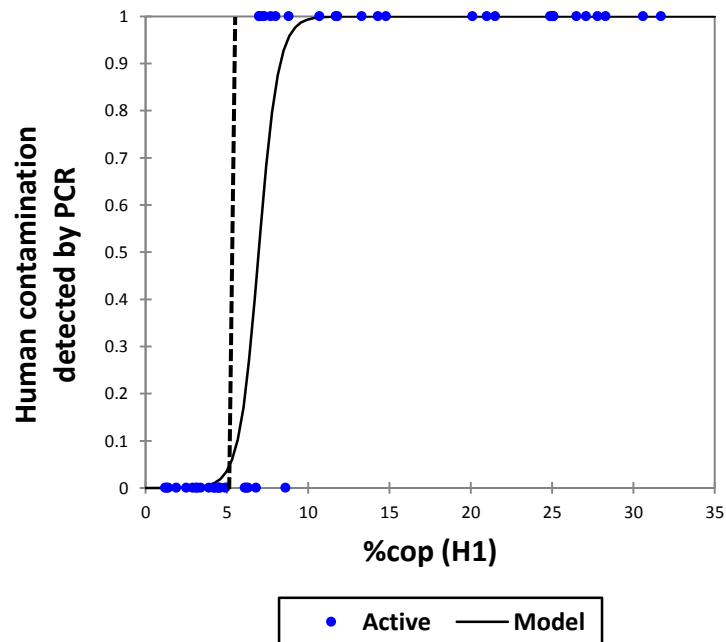


Figure 13: Logistic regression of the %coprostanol (H1) (with other steroid ratios confirming human pollution) and the Human PCR marker (two of three PCR markers positive) binary data for detection and non-detection of human faecal contamination in water samples. Active points measure actual water data as compared with the logistic regression model. Dotted line represents the 5% coprostanol threshold for identifying human faecal pollution in a waterway.

3.3.6 Water: the potential faecal ageing ratio of AC/TC

Figure 14 presents the bacterial faecal ageing ratio of AC/TC plotted against the concentration of *E. coli* detected in water at the three river locations (2011-2013 data). In general, AC/TC ratios were low (indicating fresh faecal inputs) when associated with *E. coli* concentrations above water quality guidelines. Throughout the study, AC/TC values at BS varied between 0.3 to 5.8 with a mean of 1,303 CFU/100 mL *E. coli*. Less variability in AC/TC was seen at KR with all values <3.0 and a higher mean of 4,469 CFU/100 mL *E. coli*. During discharge at OT, *E. coli* concentrations were approximately 10-100 fold higher compared with post-discharge, with AC/TC ratios below 0.92 during discharge. After discharge, the AC/TC ratio at OT ranged between 1.2 and 15.8, except for the first sampling post-discharge (September, 2011) when *E. coli* was still very high and strong human FST signals were detected (Table 10). AC/TC values during active discharge were statistically, significantly different to the AC/TC ratio after discharges ceased ($p < 0.001$), but only at OT.

Correlation analysis between *E. coli* and the AC/TC ratio, using Spearman Ranks at the two discharge sites, KR and OT ($n = 38$), showed the expected negative correlations, which were moderate ($r_s -0.675$, $p < 0.001$). For combined data from all three sites ($n = 57$), there was a lower moderate but still significant ($p < 0.001$) negative correlation. The AC/TC faecal ageing ratio had weak to moderate, negative correlations ($p \leq 0.036$), with human steroid and PCR markers and no correlation with the Wildfowl PCR marker ($p \leq 0.858$). Weak, negative correlations ($p \leq 0.03$) were noted between AC/TC and all pathogens and F-RNA phage, and not *C. perfringens*.

3.3.7 Water: a steroid ratio indicative of untreated human faecal inputs

The coprostanol/epicoprostanol (cop/epicop) ratio was investigated in water as providing discrimination between untreated and treated sewage. During the discharges at KR and OT, the sterol ratio of cop/epicop (H6, Table 9 and Table 10) in water had median ratios of approximately 95, reducing to 21 and 15 post-discharge, respectively. Median values are reported for H6 due to the wide variability of the data. By 2013, the median ratio had decreased to 4 at OT but was 13 at KR. At BS, median H6 ratios were 10 during discharge, 19 post-discharge when human pollution was often detected, decreasing to 6 during the 2013 sampling (Table 8). Throughout the urban river study, at all sites the percentage of epicoprostanol in water was <1% of total steroids (mean 0.4%, SD 0.2).

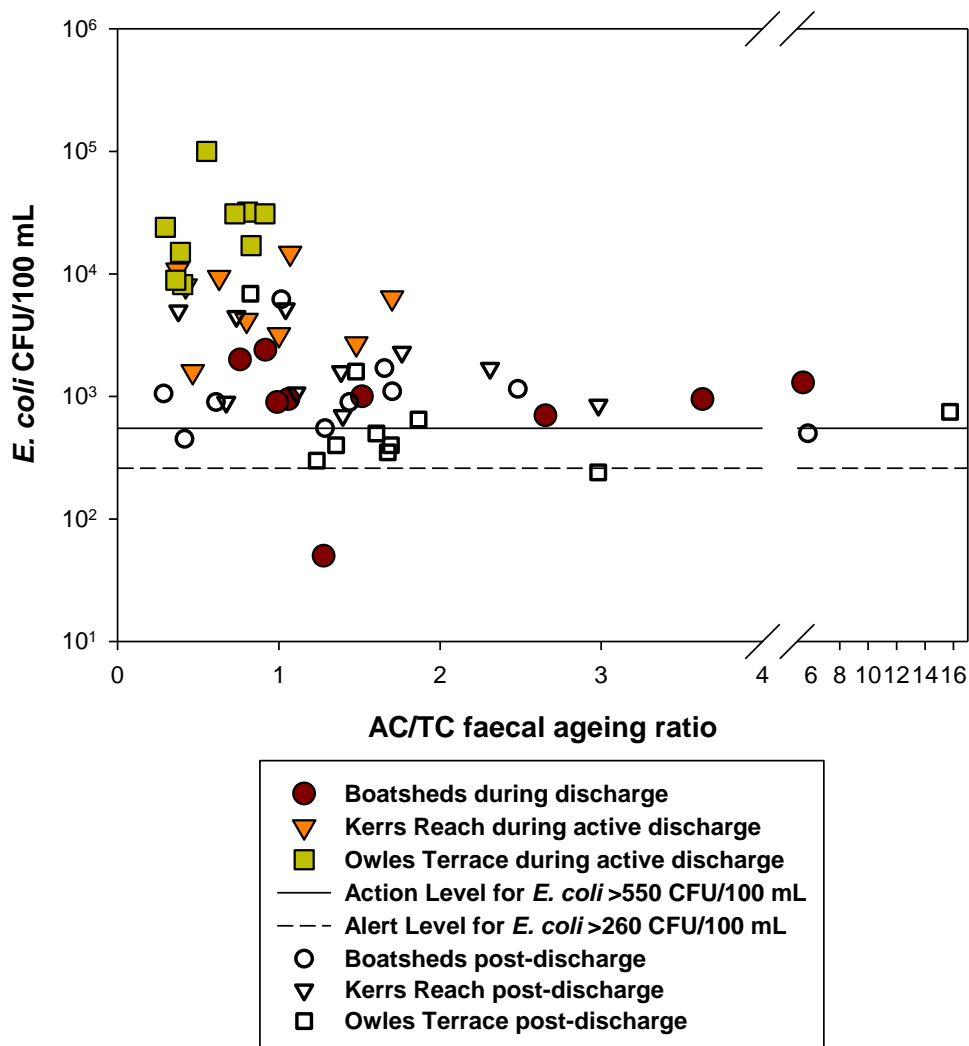


Figure 14: Faecal aging ratio AC/TC versus *E. coli* concentrations in river water during 2011-2013.

3.3.8 Sediments: Chemical FST markers

Chemical FST markers in sediments

Statistical comparisons of sediment data were performed only on 2011-2012 data as presented in Devane et al. (2014) because intermittent data was collected for FST analysis from the three sites during the 2013 sampling. In addition, at KR, the 2nd and 3rd sampling of 2013 were taken approximately 50 m downstream of the samples collected in 2011 and 2012.

FWA levels in sediments were tested from April 2011 to March 2012 (n = 11 per site) but not during 2013. In April and May 2011, levels of FWA at BS (Table 18) and KR (Table 19) were below the limit of quantification of 2.0 µg/kg but >5.1 µg/kg at OT (Table 20). Over the

course of the study, FWA appeared to be stored in the sediments, with maximum levels of 131 and 273 µg/kg at KR and OT, respectively, after the active discharges ceased in mid-September, 2011. In comparison, levels of FWA in sediments at BS remained below 17.5 µg/kg. Median values of FWA at KR (79 µg/kg) and OT (155 µg/kg) were much higher post-discharge compared with during active discharges (16 and 15 µg/kg respectively), which indicated that FWA was stored in the sediments at these two locations.

Analysis of faecal steroid ratios and FWA levels indicated that it was predominantly wildfowl contamination that was detected in the sediments at BS from March till May, 2011, similar to what was observed in the water (Table 8). From June, 2011 until March, 2012, pollution sources of steroids were unclear in sediments at BS, as the %coprostanol (mean 4.4%, SD 2.4) was borderline for human, while FWA were generally, indicative of human sources. During this period at BS, intermittent human pollution was observed in the water. In comparison, according to steroid analysis, human contamination dominated the sediments at KR and OT from March 2011 till February 2012. During this period, human sources were also supported by FWA analysis at OT, but only at KR after discharges ceased in September, 2011.

In March and April, 2013 sediment samples taken from BS and KR showed an avian steroid signature at both sites; OT sediments were not sampled for FST markers in 2013. There were no significant correlations between the percentage of coprostanol identified in the sediment and the overlying water at any of the river sites. For example at BS, with the exception of the October 2011 sampling, all sediment samples contained less than 5% coprostanol (the definitive threshold for human sources) even when overlying water had up to 25% coprostanol.

Total steroids were detected in all river sediments at levels ranging from 4,000 to 223,000 ng/g dry weight (Table 18 to Table 20). There were no significant patterns of accumulation of steroids in sediments at BS or OT. There was a significant difference, however, between the levels of total steroids in sediments at KR during and post-discharge ($p = 0.018$). The highest levels of steroids at KR occurred post-discharge (mean 147,100 ng/g, SD 79,000) compared with the during discharge phase (mean 28,400 ng/g, SD 17,000). However, by the two samplings in 2013, levels had decreased to less than 7,300 ng/g at KR. In comparison, mean levels of steroids in OT sediments were 25,000 ng/g (SD 13,000) and 42,000 ng/g (SD 21,000) during active and post-discharge (2011 - 2012), respectively.

Table 18: Chemical FST markers in sediment at the Boatsheds

SEDIMENT		Human-associated steroid ratios								Herbivore	Avian-associated steroid ratios			FWA	**Summary of all chemical FST markers
BS	Total Steroids	F1	F2	H1	H2	H3	H4	H5	H6	R1	‡P1	Av1	Av2	(µg/kg)	
DATE	(ng/g)	>0.5	>0.5	>5%	>0.7	>1.0	>0.37	%	>1.5	>5%	>4.0	>0.4	>0.5	≥2.0	
8-Mar-11	23,074	1.0	0.8	1.7	0.50	1.1	0.53	42	5.5	1.5	17.7	0.52	0.46	*NT	Wildfowl
23-Mar-11	25,878	0.6	0.9	2.3	0.36	0.7	0.43	14	4.3	3.1	12.7	0.50	0.59	NT	Wildfowl
26-Apr-11	29,460	0.3	1.4	1.2	0.25	0.6	0.38	0	3.4	1.9	20.8	0.38	0.70	0.5	Wildfowl
16-May-11	24,251	0.9	1.5	2.6	0.46	0.7	0.42	12	8.8	3.5	14.4	0.38	0.51	0.5	Wildfowl
28-Jun-11	26,895	0.8	1.1	2.7	0.43	0.8	0.43	15	7.5	3.5	11.3	0.45	0.53	2.3	Wildfowl, and low level human according to FWA
8-Sep-11	25,185	0.7	1.4	2.9	0.43	0.8	0.43	15	8.1	3.9	7.7	0.39	0.55	10.4	Wildfowl, and human according to FWA
27-Sep-11	30,938	1.3	1.9	4.7	0.56	1.1	0.51	38	11.7	4.5	9.0	0.32	0.42	14.2	Human
11-Oct-11	27,057	5.8	2.1	11.0	0.85	3.4	0.77	100	18.8	3.2	9.8	0.31	0.14	17.4	100% human
8-Nov-11	30,654	2.5	2.1	3.9	0.71	1.3	0.56	51	9.8	3.1	8.8	0.31	0.27	8.7	Borderline human
22-Nov-11	8,576	1.2	2.3	1.9	0.55	1.2	0.54	45	5.3	1.6	7.6	0.30	0.41	1.9	Unknown source, FWA suggestive of human
6-Dec-11	66,258	1.3	1.1	3.4	0.56	1.7	0.63	72	2.4	2.0	16.7	0.45	0.36	12.9	Human and plant runoff/ Wildfowl
20-Feb-12	68,537	2.5	1.4	3.6	0.71	1.8	0.65	76	6.0	1.9	24.2	0.39	0.26	2.2	Borderline human and plant runoff
6-Mar-12	42,639	3.0	1.7	3.9	0.75	1.8	0.65	77	7.5	2.1	14.0	0.35	0.23	5.6	Human and plant runoff
8-Apr-13	3,963	0.6	0.7	1.6	0.37	1.3	0.56	52	3.3	1.3	24.3	0.56	0.57	NT	Wildfowl

‡P1 ≥ 7.0 is supportive of avian pollution (Devane et al., 2015); *NT, Not tested;

**Colour code for type of faecal pollution detected:

faecal pollution detected human herbivore avian

Table 19: Chemical FST markers in sediments at Kerrs Reach. Detection of herbivore steroid ratio (R1) in the presence of human steroid ratios H1 >5% and H3 >1.0 indicates that human pollution is the source of mammalian stanols, coprostanol and 24-ethylcoprostanol

SEDIMENT		Human-associated sterol ratios									Herbivore	Avian-associated sterol ratios			FWA	**Summary of all chemical FST markers
KR	Total Steroids (ng/g)	F1	F2	H1	H2	H3	H4	H5	H6	R1	¥P1	Av1	Av2	µg/kg		
DATE		>0.5	>0.5	>5%	>0.7	>1.0	>0.37	%	>1.5	>5%	>4.0	>0.4	>0.5	≥2.0		
8-Mar-11	32,636	5.0	1.5	12.1	0.83	2.0	0.67	82	3000	6.1	6.7	0.39	0.17	*NT	Human	
23-Mar-11	10,481	1.1	1.1	4.9	0.52	1.8	0.64	75	14.4	2.7	14.5	0.46	0.47	NT	Plant runoff/ wildfowl and borderline human	
26-Apr-11	33,866	3.8	3.8	14.8	0.79	2.6	0.72	97	27.3	5.8	6.3	0.20	0.20	0.5	Human by sterols only	
16-May-11	13,347	1.5	1.2	2.8	0.60	1.3	0.57	53	14.8	2.1	20.0	0.44	0.38	0.5	Unknown source of <i>E. coli</i>	
28-Jun-11	16,434	1.6	1.0	4.5	0.61	1.6	0.62	68	9.6	2.8	16.0	0.49	0.36	1.6	Plant runoff/ wildfowl and borderline human	
8-Sep-11	49,911	6.2	3.9	22.6	0.86	3.3	0.77	100	20.6	6.8	3.2	0.19	0.13	64.2	Human	
27-Sep-11	62,857	1.7	1.8	9.2	0.62	1.4	0.59	60	17.5	6.3	4.2	0.34	0.36	38.4	Human	
11-Oct-11	179,800	1.1	0.9	10.4	0.53	1.6	0.62	68	7.4	6.5	4.7	0.51	0.44	109.0	Human	
8-Nov-11	199,133	3.0	2.7	13.2	0.75	1.7	0.64	73	19.4	7.6	4.1	0.27	0.24	94.5	Human	
22-Nov-11	165,495	2.4	3.5	15.9	0.70	1.7	0.63	71	11.7	9.5	2.9	0.22	0.28	131.3	Human	
6-Dec-11	222,951	1.2	0.8	7.3	0.54	1.2	0.55	49	8.6	5.9	7.8	0.53	0.44	69.4	Human and plant runoff/ wildfowl	
20-Feb-12	189,244	2.0	1.8	14.5	0.67	2.3	0.70	90	9.3	6.3	4.7	0.34	0.31	106.4	Human	
6-Mar-12	10,223	0.5	1.1	0.5	0.33	1.3	0.56	50	9.9	0.4	30.2	0.46	0.65	1.7	Wildfowl	
25-Mar-13	7,281	0.3	1.0	0.5	0.22	0.5	0.35	0	4.8	1.0	47.1	0.49	0.74	NT	Wildfowl	
8-Apr-13	6,786	0.2	0.3	0.5	0.18	0.6	0.38	1	4.2	0.7	66.1	0.70	0.78	NT	Wildfowl	

¥P1 ≥ 7.0 is supportive of avian pollution (Devane et al., 2015); *NT, Not tested;

**Colour code for type of faecal pollution detected:

faecal pollution detected human herbivore avian

Table 20: Chemical FST markers in sediments at Owles Terrace. Detection of herbivore steroid ratio (R1) in the presence of human steroid ratios H1 >5% and H3 >1.0 indicates that human pollution is the source of mammalian stanols, coprostanol and 24-ethylcoprostanol

SEDIMENT		Human-associated steroid ratios								Herbivore	Avian-associated steroid ratios			FWA	**Summary of all chemical FST markers
OT	Total Steroids	F1	F2	H1	H2	H3	H4	H5	H6	R1	‡P1	Av1	Av2	FWA (µg/kg)	
DATE	(ng/g)	>0.5	>0.5	>5%	>0.7	>1.0	>0.37	%	>1.5	>5%	>4.0	>0.4	>0.5	≥2.0	
8-Mar-11	7,385	6.8	3.4	24.9	0.87	2.3	0.70	92	22.6	10.6	1.9	0.23	0.12	*NT	Human
23-Mar-11	24,428	7.3	8.0	22.1	0.88	2.5	0.71	94	36.2	9.0	1.0	0.11	0.12	NT	Human
26-Apr-11	38,719	6.6	9.8	24.4	0.87	2.6	0.73	99	31.7	9.2	1.4	0.09	0.13	5.2	Human
16-May-11	30,997	5.3	6.4	25.1	0.84	1.9	0.65	78	24.1	13.4	1.5	0.13	0.15	24.7	Human
28-Jun-11	13,009	4.1	7.5	19.9	0.80	1.5	0.60	62	15.9	13.5	0.6	0.11	0.19	5.6	Human
8-Sep-11	36,894	4.1	7.9	19.6	0.80	1.4	0.59	59	29.3	13.8	0.6	0.11	0.19	159.1	Human
27-Sep-11	70,875	7.2	14.6	41.3	0.88	2.3	0.70	91	30.2	17.8	0.5	0.06	0.12	226.5	Human
11-Oct-11	25,816	5.6	8.3	28.2	0.85	2.5	0.71	95	24.7	11.4	1.1	0.11	0.15	110.9	Human
8-Nov-11	60,608	6.9	6.5	25.2	0.87	2.6	0.72	97	22.5	9.8	2.3	0.13	0.12	218.1	Human
22-Nov-11	30,854	5.1	5.8	25.5	0.84	2.1	0.67	84	17.5	12.4	1.8	0.14	0.16	154.6	Human
6-Dec-11	55,270	5.4	4.5	27.2	0.84	2.3	0.70	91	18.9	11.8	2.5	0.18	0.15	273.4	Human
20-Feb-12	14,326	2.4	3.0	13.8	0.71	1.6	0.62	68	7.1	8.5	4.0	0.24	0.27	132.4	Human
6-Mar-12	33,583	2.8	3.0	18.4	0.74	1.7	0.64	73	8.0	10.5	3.0	0.24	0.24	128.0	Human

‡P1 ≥ 7.0 is supportive of avian pollution (Devane et al., 2015); *NT, Not tested;

**Colour code for type of faecal pollution detected:

 faecal pollution detected  human  herbivore  avian

3.3.9 Sediments: Microorganisms

Microbial indicator concentrations in sediments are presented in Figure 15 and pathogen concentrations in Figure 16. In addition, mean (\pm standard deviation) levels of all microbes in sediment during discharge, post-discharge and the 2013 sampling are presented in Table 21. Tables of all data for microbes in sediments can be found in the Appendix, Table 33 for BS and KR and Table 34 for OT.

E. coli levels were quite variable in sediments, with no clear pattern of accumulation. The highest concentrations of *E. coli* during 2011-2012 were 45,000 and 34,000 CFU/g of dry sediment at OT during March 2011 and at KR just after the sewage discharges ceased (respectively). In general, on cessation of active discharges (September, 2011- March, 2012) the levels of *E. coli* in sediment were, at all three sites, less than during the discharges, although at BS and KR this difference was not statistically significant. At OT there was a statistically significant difference between *E. coli* in sediment during and post-discharge ($p = 0.018$). KR and OT had positive correlations between the concentration of *E. coli* in the water and the sediment (r_s 0.67 and 0.65; $p = 0.0234$ and 0.0290, respectively).

Low levels of *E. coli* continued to be identified in the sediments during the 2013 sampling at BS and OT. In contrast, in 2013, the maximum concentration of *E. coli* for the entire study was observed at KR with a level of 92,000 CFU/g and a mean of $>43,000$, whereas post-discharge (2011-2012), mean *E. coli* levels were $<5,200$ CFU/g. Due to problems with access to the river at KR due to demolition of buildings, the second and third samplings of 2013 were taken approximately 50 m downstream of the original sampling site, therefore, caution is required when comparing results with previous events. The highest *E. coli* level in sediment for the study was identified at KR during 2013, in conjunction with the study's maximum level of 11.1 MPN/g for *Campylobacter* in sediment (Appendix, Table 33), and when FST markers were identifying avian sources (Table 19). Overall, *Campylobacter* species were detected in 12 of the 39 river sediment samples with the initial detections of *Campylobacter* reflecting recent human sewage discharges. *Campylobacter* species were not identified in the sediments at BS and OT during 2013 and did not appear to be stored in sediments.

Variable levels of *Cryptosporidium* were detected in river sediment samples. In contrast to the reduced levels of *Giardia* in water post-discharge (Figure 8), the river sediments had the highest concentrations of *Giardia* once discharges ceased. In general, the mean concentration of protozoa, post-discharge, was higher in the river sediments than those seen previously and may reflect a build-up in the sediment (Table 21). During this post-discharge phase, FST analysis of sediments at

KR and OT were still dominated by human sources, whereas a mix of human and avian was identified at BS. Differences in sediment concentration of *Cryptosporidium* and *Giardia* during active and post-discharge, however, were not statistically significant at any river sites ($p > 0.05$). *Cryptosporidium* was not detected in any sediment samples during 2013, while *Giardia* was detected in one sample at KR, but at low levels (0.8 cysts/g).

The highest concentrations of both protozoa in sediments were seen at BS compared to the two sites receiving active discharges. During the active discharges, the highest levels of *Cryptosporidium* (2.8 oocysts/g) and *Giardia* (70 cysts/g) in sediment occurred at BS when chemical FST analyses were identifying avian sources. Post-discharge, the highest protozoan concentrations also occurred at BS in February 2012, with *Cryptosporidium* (113 oocysts/g) and *Giardia* (2254 cysts/g sediment) detected in sediment when steroid and FWA analyses were suggesting plant runoff and borderline human sources. In the overlying water sample, *Cryptosporidium* was not identified and *Giardia* levels were 45 cysts/100 L, with PCR and steroid markers identifying avian, dog and recent human faecal sources.

C. perfringens was present at much higher concentrations in the sediment than *E. coli* (Table 21). *C. perfringens* was observed in high concentrations in the river sediment throughout the study, and there were no significant differences between the concentrations during active discharge and post-discharge when all sites were analysed ($p = 0.167$) and when only the two sites receiving active discharges were analysed ($p = 0.171$).

F-RNA phage were detected in all sediments on the first sampling occasion, but thereafter, their presence was intermittent. OT had the most consistent but still low levels of F-RNA phage in sediment (compared with other microbial indicators) during the active discharge period. There was infrequent detection of F-RNA phage in the sediments post-discharge, with no detections at any location during the 2013 samplings.

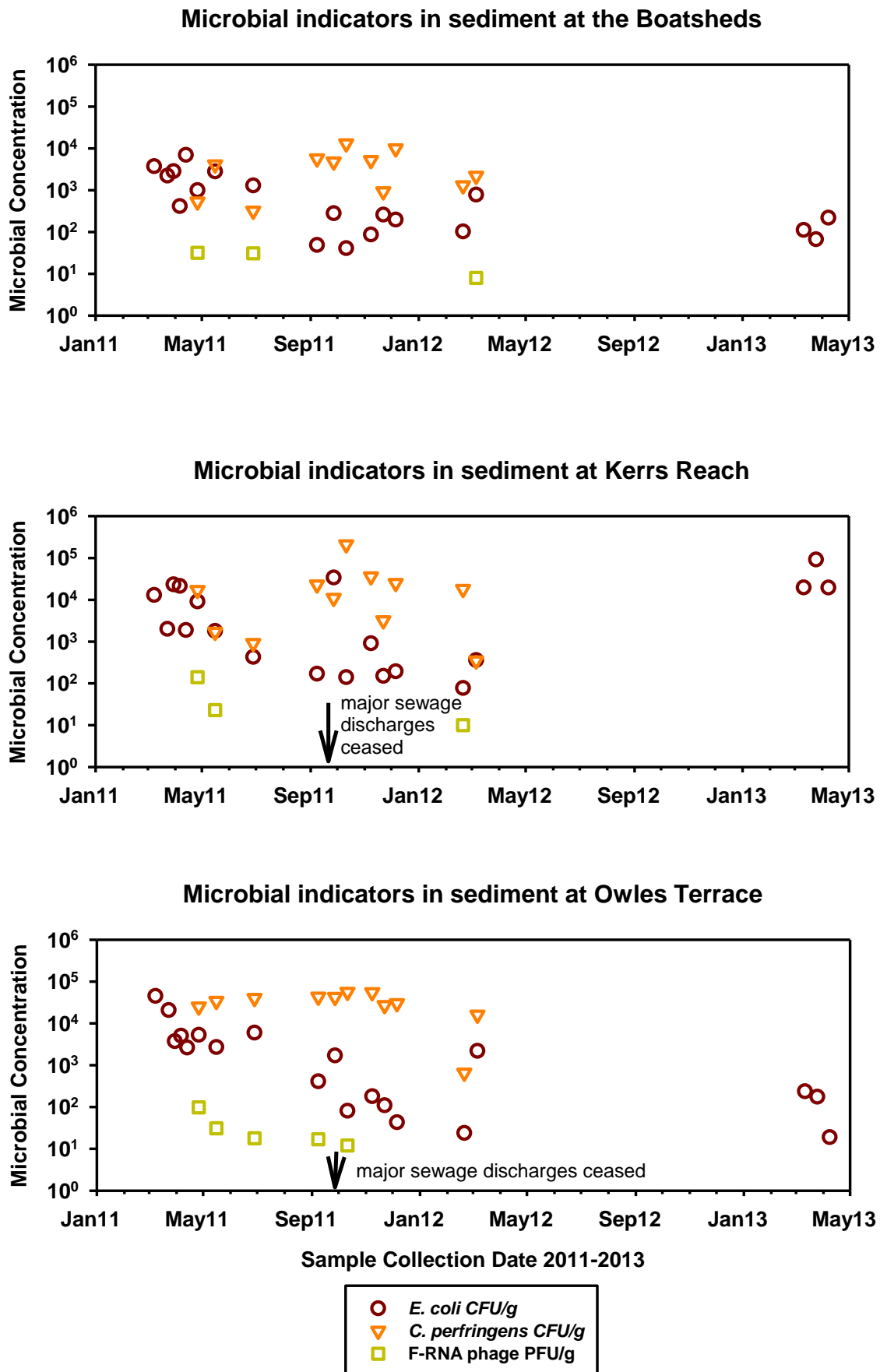


Figure 15: Microbial indicators detected in river sediments. *C. perfringens* was not tested in sediments during 2013.

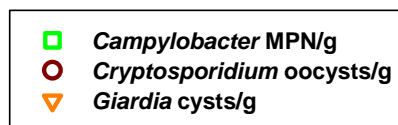
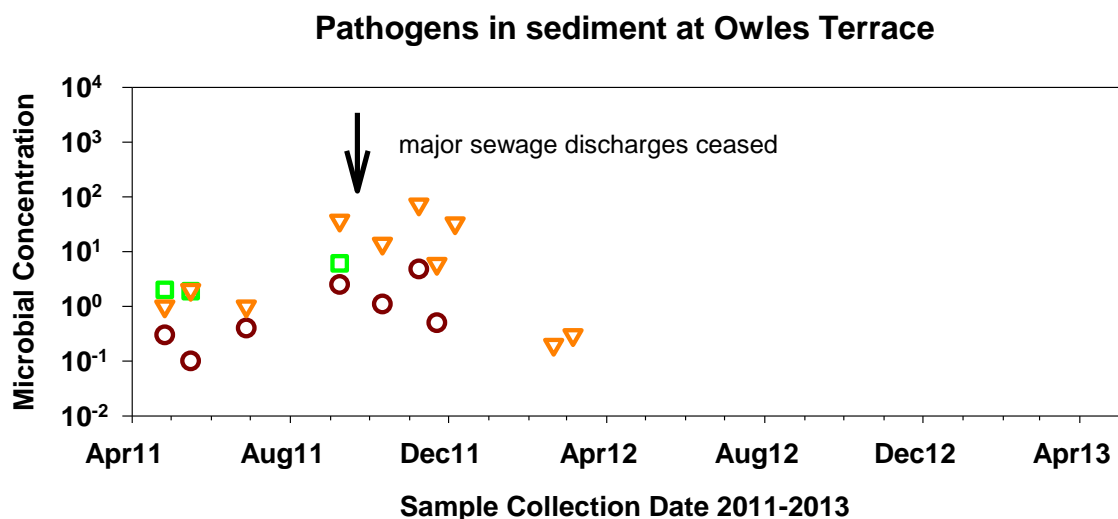
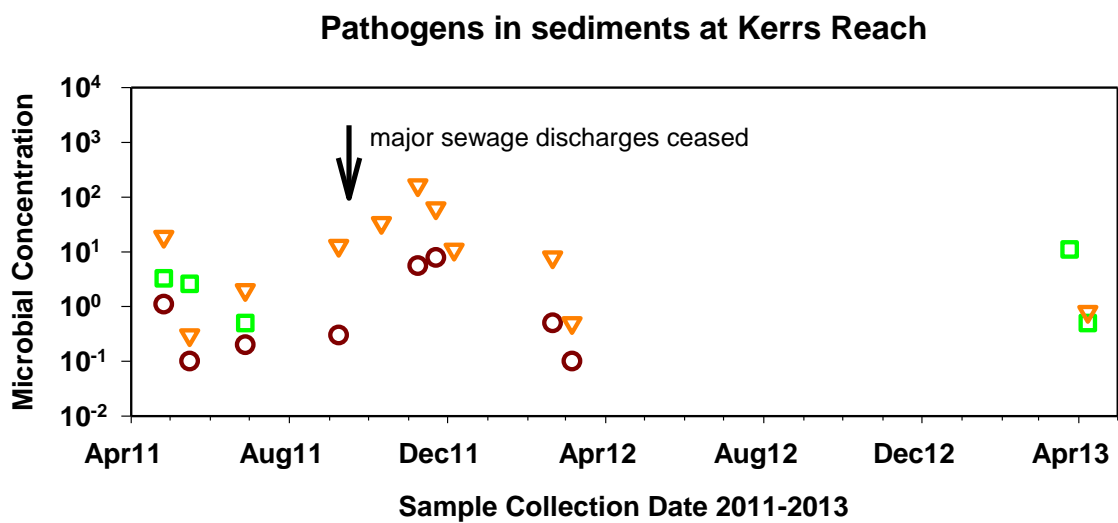
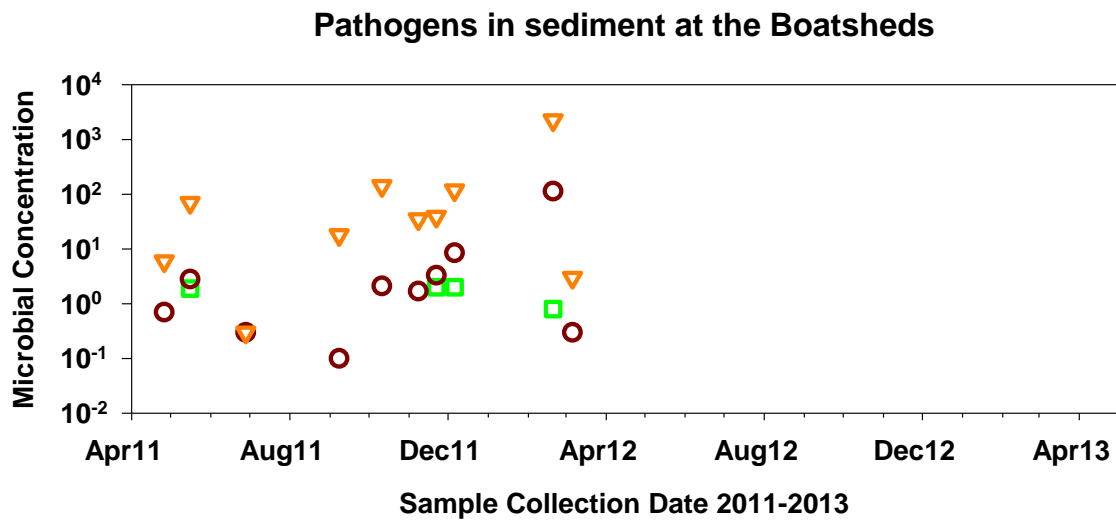


Figure 16: Pathogens detected in river sediments. All pathogens were tested during 2013

Table 21: Mean levels (\pm standard deviation) of microorganisms in sediment during active discharges (April – 8 September) and post-discharge (27 September 2011 - March 2012) and 2013 sampling (March - April 2013).

Discharge Phase	Samples /site	Boatsheds	Kerrs Reach	Owles Terrace
<i>E. coli</i> CFU/g				
Active discharge	9	2,372 (\pm 2,113)	8,138 (\pm 9,179)	10,124 (\pm 14,421)
Post-discharge	7	250 (\pm 252)	5,118 (\pm 12,738)	619 (\pm 921)
2013	3	133 (\pm 78)	43,661 (\pm 41,546)	144 (\pm 113)
Overall <i>E. coli</i>	19	1,237 (\pm 1,798)	12,613 (\pm 21,792)	5,046 (\pm 10,827)
F-RNA phage PFU/g				
Active discharge	4	16 (\pm 18)	41 (\pm 67)	41 (\pm 38)
Post-discharge	6	1.3 (\pm 3.3)	1.7 (\pm 4.1)	2.0 (\pm 4.9)
2013	3	0 (\pm 0)	0 (\pm 0)	0 (\pm 0)
Overall F-RNA phage	13	5.5 (\pm 11.8)	13.3 (\pm 38.7)	13.5 (\pm 27.3)
<i>C. perfringens</i> CFU/g				
Active discharge	4	2,662 (\pm 2,666)	10,655 (\pm 11,070)	35,500 (\pm 7,937)
Post-discharge	7	5,332 (\pm 4572)	43,364 (\pm 74,513)	32,450 (\pm 20,336)
Overall <i>C. perfringens</i>	11	4,362 (\pm 4,061)	31,470 (\pm 60,336)	33,560 (\pm 16,413)
<i>Campylobacter</i> MPN/g				
Active discharge	4	0.48 (\pm 0.95)	1.60 (\pm 1.60)	2.50 (\pm 2.57)
Post-discharge	6	0.80 (\pm 0.98)	0 (\pm 0.0)	0 (\pm 0.0)
2013	3	0 (\pm 0.0)	3.88 (\pm 6.26)	0 (\pm 0.0)
Overall <i>Campylobacter</i>	13	0.52 (\pm 0.86)	1.39 (\pm 3.11)	0.77 (\pm 1.76)
<i>Giardia</i> cysts/g				
Active discharge	4	23.6 (\pm 31.8)	8.6 (\pm 8.9)	10.3 (\pm 17.8)
Post-discharge	6	432.0 (\pm 894)	47.0 (\pm 63)	21.0 (\pm 28)
2013	3	0 (\pm 0)	0.27 (\pm 0.46)	0 (\pm 0)
Overall <i>Giardia</i>	13	207 (\pm 617)	24 (\pm 46)	13 (\pm 22)
<i>Cryptosporidium</i> oocysts/g				
Active discharge	4	0.98 (\pm 1.24)	0.43 (\pm 0.46)	0.83 (\pm 1.12)
Post-discharge	6	21.5 (\pm 44.9)	2.4 (\pm 3.5)	1.1 (\pm 1.9)
2013	3	0 (\pm 0)	0 (\pm 0)	0 (\pm 0)
Overall <i>Cryptosporidium</i>	13	10.2 (\pm 31.0)	1.2 (\pm 2.5)	0.7 (\pm 1.4)

3.3.10 Sediments: relationships between indicators and pathogens

There were few statistically significant correlations between microbes and FST markers in sediments using all data (2011-2013). For the steroid ratio markers there were significant ($p < 0.001$) strong-moderate, positive correlations between steroid ratios, with the strongest being between human-associated steroid ratios ($r_s \geq 0.79$, $p < 0.001$). The avian-associated steroid markers had strong, negative correlations with human steroid markers ($p = 0.001$). However, the total steroids had only weak, positive correlations with any steroid markers. PCA in sediment confirmed the lack of correlation between steroids and microbes (Table 22). The first three components of the PCA explained 78.5% of the variance in the data. PC1 explained 54.5% of the variability, and factor loadings suggested it had strong positive associations with all of the human/herbivore (F1, F2, H1-H6 and R1) steroid ratios, but only moderate and weak, positive associations with FWA and total steroids, respectively, and negative correlations with avian-associated steroid ratios.

There were few significant relationships between *E. coli* and other microbes or chemical FST markers in sediments. *E. coli* did have a moderate correlation with *Campylobacter* (r_s 0.51, $p = 0.001$), and a weak correlation with F-RNA phage (r_s 0.40, $p = 0.012$). There was an unexpected negative correlation between *E. coli* and FWA (r_s -0.42, $p = 0.015$). These relationships with *E. coli* were supported by PCA, with the same variables having the highest factor loadings in the second principle component which was explaining 14% of the variance (Table 22). The lack of correlation of steroid ratios with microbes is also evident in the factor loadings for PC1, where all chemical FST markers were highly associated. Logistic regression analyses did not identify a relationship between *E. coli* and either human steroid or FWA markers in the sediment. There was a lack of differentiation by *E. coli* concentration when chemical FST markers detected human pollution (scored as 1); compared with the *E. coli* concentrations when FST markers did not identify human pollution (scored as 0) (Figure 17). This figure supports the negative correlation noted between FWA and *E. coli* concentrations.

FWA had its highest positive correlations with %cop and %24-ethylcop (r_s 0.743 and 0.765; $p < 0.001$). In contrast, similar to human steroids, FWA had moderate, negative correlations with the two avian-associated steroid ratios ($p \leq 0.003$). *C. perfringens* had strong to moderate, positive correlations ($p < 0.002$) with human steroid ratios and FWA.

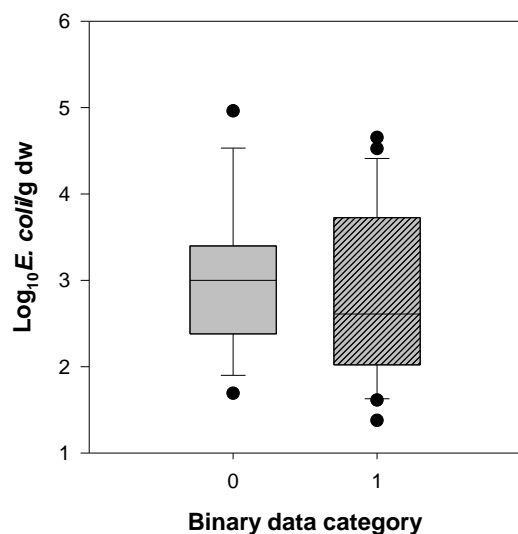
The third component of the PCA explaining 10% of the variance was associated with levels of *Giardia* and *Cryptosporidium* as the only two contributors, which in the correlation

analysis were observed to have a strong positive correlation of r_s 0.815 ($p < 0.001$) in sediment. From the scatter plots, however, this correlation was skewed by a single high concentration of *Giardia*.

Table 22: Factor loadings identified for each variable in sediment by Principal Component Analysis. Shading indicates those variables with the highest factor loading contributing to a particular principal component (PC).

Variable in sediment	Steroid ratios in sediment				Steroid ratios in sediment		
	PC1	PC2	PC3		PC1	PC2	PC3
<i>E. coli</i>	-0.106	0.765	-0.222	F1	0.967	0.063	0.028
<i>Cryptosporidium</i>	0.303	0.274	0.811	F2	0.900	0.140	-0.077
<i>Campylobacter</i>	-0.130	0.679	0.241	H1	0.965	-0.122	-0.166
<i>C. perfringens</i>	0.739	-0.177	-0.147	H2	0.967	0.063	0.028
F-RNA Phage	0.239	0.704	-0.092	H3	0.868	-0.032	0.133
<i>Giardia</i>	0.348	-0.026	0.858	H4	0.868	-0.032	0.133
AC/TC	-0.457	-0.738	0.144	H5	0.868	-0.032	0.129
FWA	0.617	-0.647	-0.188	H6	0.842	0.277	-0.104
				R1	0.857	-0.122	-0.295
				P1	-0.889	0.052	0.248
				Av1	-0.887	-0.138	0.089
				Av2	-0.962	-0.058	-0.044
				Total steroids	0.461	-0.405	0.450

A) Detection of pollution by faecal steroid markers



B) Detection of pollution by FWA

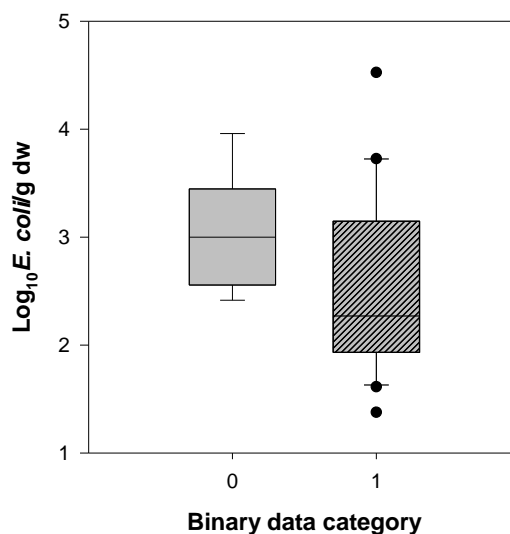


Figure 17: Conversion of human pollution markers in sediment to binary data plotted against *E. coli* concentrations and showing there is a lack of discrimination by *E. coli* concentrations when FST markers A) steroid markers and B) FWA markers identify/do not identify human pollution. The boundary of the box closest to the x-axis indicates the 25th percentile, the line within the box represents the median, and the boundary of the box farthest from the x-axis indicates the 75th percentile. Whiskers below and above the box indicate the 10th and 90th percentiles, respectively; ●, outlier measurements

3.3.11 Sediments: potential faecal ageing ratios

Figure 18 plots the AC/TC ratio against Log_{10} *E. coli* concentrations for all sites showing the differences between the active discharge and the post-discharge phase, which included 2013 data. In general, the active discharge phase was associated with higher *E. coli* in the sediments and low AC/TC values below 5.0 with the converse true during the post-discharge phase. During active discharge at KR and OT, the faecal ageing ratio of AC/TC in sediment had a median of 1.2, while post-discharge the median was 3.4, increasing to 10.6 during the 2013 sampling. BS observed similar increases in AC/TC in sediment with a median of 2.7 during discharge, 3.0 post-discharge and increasing to 12.7 during 2013. Comparison of the AC/TC ageing ratio during the discharge phases of the study using Mann-Whitney test revealed a significant difference between the active discharge and post-discharge phase (which included 2013 data) for the AC/TC ratio ($p = 0.000$) at all sites.

The other potential faecal ageing ratio of cop/epicop (H6) at KR (Table 19) and OT (Table 20) had a mean of $28.9 \pm \text{SD } 23.7$ (median 23.4) during discharge and $15.2 \pm \text{SD } 7.4$ (median 14.6) post-discharge (2011-2012) in sediments. At these two sites, there were small but consistent increases in percentage of epicoprostanol in sediment post-discharge with concurrent decreases of %coprostanol. At BS the mean values for cop/epicop were similar during both discharge phases, mean 6.2 (SD 2.2), during discharge, and 8.1 (SD 5.3) post-discharge (Table 18). The steroid ageing ratio is only relevant when human contamination is detected, and therefore, the Mann-Whitney for the cop/epicop ratio was applied at KR and OT for the 2011-2012 data only. At these two discharge sites, comparison of the cop/epicop ageing ratio during the discharge phases of the study using Mann-Whitney test revealed a significant difference between the active discharge and post-discharge with $p = 0.020$, and was not significant, as expected, when all sites were tested.

There were significant moderate, negative correlations ($p \leq 0.0002$) between the AC/TC faecal ageing ratio and *E. coli* ($r_s -0.52$), F-RNA phage (-0.57) and the steroid ageing ratio cop/epicop (-0.62). There were also significant but weak correlations with other steroid markers. Cop/epicop was correlated with human steroid markers (range, $r_s 0.76$ to 0.82 , $p < 0.001$) and negatively correlated with avian-associated steroid markers (range, $r_s -0.73$ to -0.79 , $p < 0.001$). In contrast to the AC/TC ageing ratio, cop/epicop was not correlated with *E. coli*, but had significant correlations with *C. perfringens* ($r_s 0.64$) and F-RNA phage ($r_s 0.36$). Both faecal ageing ratios had no significant correlations with either pathogens or FWA in sediments.

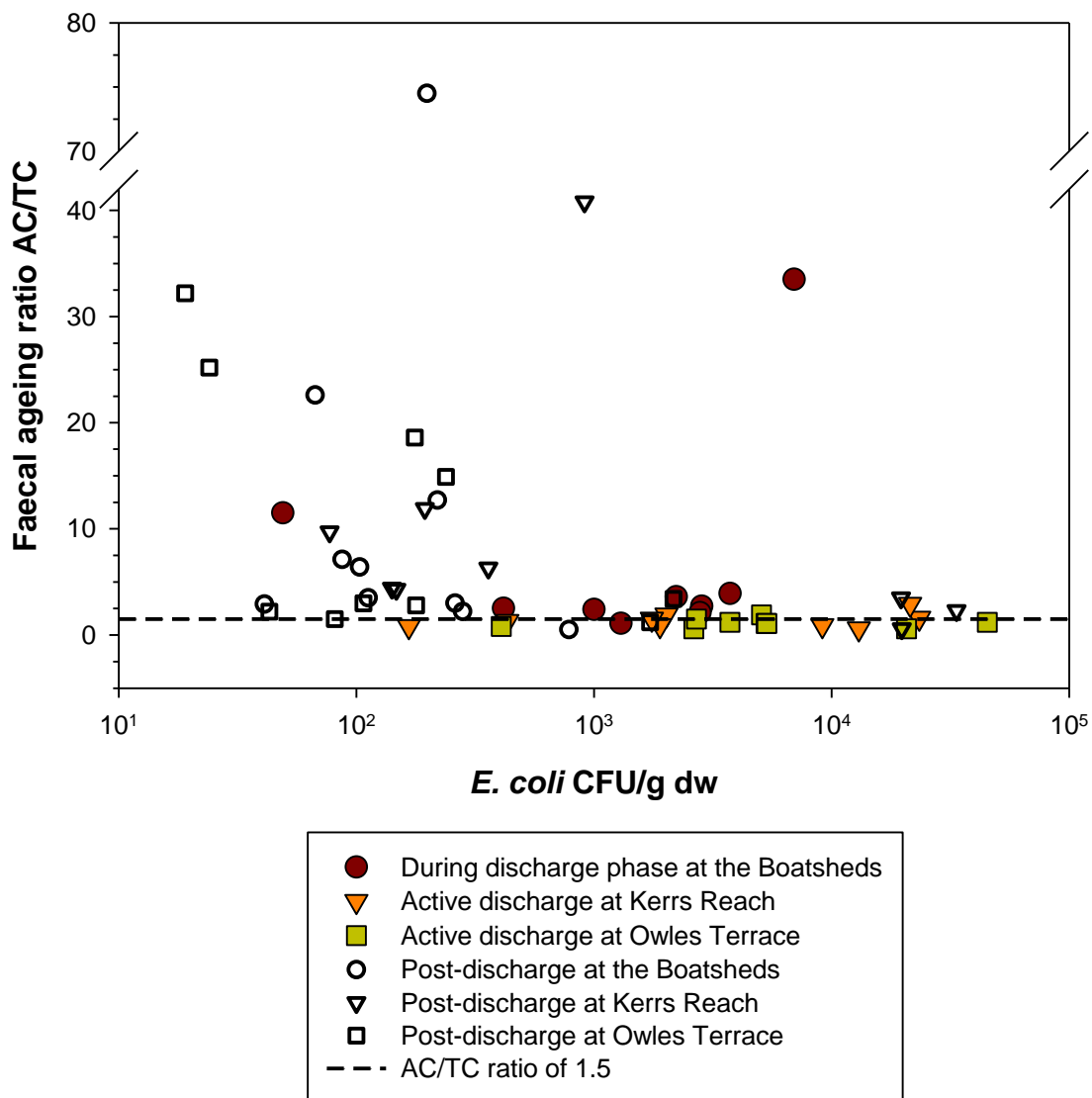


Figure 18: Faecal ageing ratio AC/TC plotted against concentrations of *E. coli* in river sediments

3.4 Discussion

Prior to the 2010/2011 Canterbury and Christchurch earthquakes, microbial water quality measurements of the Avon/Otākaro River, intermittently exceeded recreational water quality guideline values. A pre-earthquake FST study of the Avon/Otākaro River indicated this was typically due to wildfowl and dog faecal inputs, although after heavy rain some human sewage contamination was possible (Moriarty and Gilpin, 2009). Major infrastructure damage to the sewerage system as a result of the 2011 earthquakes led to the direct discharge of raw human sewage into the lower reaches of the Avon/Otākaro River over an eight month period (February to September, 2011), followed by intermittent, low volume discharges in the ensuing months (post-discharge phase).

Analysis of FST markers and microorganisms in the Avon/Otākaro River identified human faecal pollution in the water at two sites, Kerrs Reach and Owles Terrace, which was congruent with their locations downstream of major sewage inputs. The situation at the uppermost location, the Boatsheds, however, was more complex because there were no known direct sewage discharges upstream of the central business district, where BS was sited. Prior to May 2011, the elevated numbers of *E. coli* (maximum 1,300 CFU/100 mL) detected in water at BS (Figure 5) were attributed by FST methods to wildfowl and dog contamination (Figure 7 and Table 8). Elevated levels of *E. coli* (maximum 6,000 CFU/100 mL) attributed to human sewage were detected in the water from May till October 2011. Investigations by City Council staff identified a blocked sewer pipe upstream of the site and subsequent remedial work on the pipe resulted in lowered levels of *E. coli* and human FST markers at BS.

The intermittent nature of the minor discharges to the river at all sites after September 2011 was due to ongoing aftershocks and breakages by repair contractors. These intermittent discharges impacted on the ability of the study to compare the fate of microbes and FST markers during active discharge and post-discharge. However, the study still provided the ability to assess the relationship between all variables and showed a clear delineation between the two phases of discharge when all parameters were assessed by PCA.

High variability of one to two orders of magnitude was noted for microbial counts in water at all sites (for example *E. coli*) within a discharge phase (Table 11). The question arose as to whether this variability was associated with sampling and analytical errors or due to the nature of the sewage discharges. The uncertainty associated with microbial analysis has been estimated as an overall error of 12.5% for the enumeration of microbes by plate counting methods (Jarvis, 2008). These errors include those associated with sampling procedures and take into account the inherent heterogeneity of microbial distribution in a water sample due to factors such as

clumping of bacterial cells. Dilution errors associated with pipetting techniques are also accounted for in this error calculation, which provide estimations for dilutions down to six orders of magnitude. Other researchers have suggested that counts of aerobic colonies have expected 95% confidence limits of ± 0.5 log cycles (Jarvis et al., 1977; Kramer and Gilbert, 1978).

At Owles Terrace, during the active discharge phase the *E. coli* levels varied between 8,200 and 100,000 CFU/100 mL (Appendix, Table 32). As noted above sampling errors can account for up to 12.5% of variability within replicates of a single sample and are unlikely to account for the larger variations between sampling events observed in Table 11. The variability between sampling events within a discharge phase were more likely due to the nature of the discharges, which were decreasing over time at the active discharge sites accounting for reductions in microbial concentrations, as evidenced at Owles Terrace. In addition, when sites were impacted by intermittent sewage discharges as observed at the Boatsheds, human faecal markers were generally detected in water samples in association with *E. coli* concentrations that were an order of magnitude higher compared with samples where only dog and avian markers were detected (Appendix, Table 30).

3.4.1 Microbial indicators for assessing pathogen presence

E. coli as an indicator of health risk

Significant weak to moderate correlations in water samples ($p < 0.05$) were identified between the indicator bacteria *E. coli* and all other microorganisms tested in this urban river study (Table 13). These correlations included statistically significant relationships between *E. coli* and the potential pathogens, *Campylobacter*, *Giardia* and *Cryptosporidium*. Therefore, after an event where a waterway was impacted by a high volume of untreated sewage, the identification of *E. coli* in the waterway was shown to be a suitable indicator for establishing a public health risk.

The identification of *E. coli* levels above 550 CFU/100 mL in the river water was associated with an increased likelihood of detection of potential pathogens, although these findings were not statistically significant (Table 14). The lack of significance may have been impacted by the variability in the measures and the low number of data points. It is also well documented that *E. coli* occurs as an inhabitant of soil, submerged sediments and macrophytes (Byappanahalli et al., 2003a; Byappanahalli and Fujioka, 2004; Byappanahalli et al., 2003b). Environmental sources of *E. coli*, therefore, and *E. coli* from animals (Bettelheim et al., 1976), may have increased the numbers of total *E. coli* load in the waterways.

E. coli also had moderate to strong positive correlations with all PCR markers except the wildfowl marker, and with the human-associated steroid ratios but not the avian-associated steroid ratios. Study results, therefore, suggested good concordance between *E. coli* and the identification of human pollution, but not wildfowl pollution. In this urban river study, *E. coli* was an adequate indicator of potential infection from pathogens associated with a known point source of untreated sewage contamination discharged into a river environment. This finding is supported by other research in urban and rural catchments. A strong correlation was observed between %cop and *E. coli* concentrations in water impacted by human effluent in regions with temperate and tropical climates (Isobe et al., 2004; Isobe et al., 2002) and in an agricultural catchment where *E. coli* (closely followed by faecal coliforms) was identified as the most appropriate bacterial indicator for predicting the presence/absence of the potential pathogens *Cryptosporidium*, *Giardia* and *Salmonella* in surface waters (Wilkes et al., 2009). Wilkes et al. (2009), however, stressed that their study supported previous suggestions that no single indicator is sufficient to predict contamination from all bacterial and protozoan pathogens.

Equations from the current study have been generated for predicting pathogen concentrations based on *E. coli* levels attributed to human sewage. These equations will allow incorporation into models for prediction of pathogen concentrations (Table 15). This is, however, not a simple linear relationship and in some situations, elevated *E. coli* levels may overestimate health risk, as has been observed in previous studies (Harwood et al., 2005; Korajkic et al., 2011). It has been suggested that routine monitoring of faecal indicator bacteria (FIB) is not sufficient for prediction of pathogen presence due to poor correlations between FIB and pathogens as outlined in the review by Field and Samadpour (2007). Routine testing for pathogens directly, however, is not a good use of resources as current testing methodologies are expensive and time consuming (Brookes et al., 2005). Furthermore, specific pathogens will not be detected if levels of infection in the community are very low or non-existent at the time of sewage overflow. This non-detection may give a false sense of safety, resulting in an underestimation of the risk of infection. The potential indicators, *C. perfringens* and F-RNA phage, therefore, were investigated as additional indicators of pathogens.

C. perfringens as an indicator of health risk

C. perfringens forms spores that confer survival characteristics similar to protozoa, and is much cheaper to assay than *Cryptosporidium* and *Giardia*. The larger size of *C. perfringens* compared with vegetative bacteria, may mimic the settling characteristics of protozoa, which are known to settle out of the water column into sediments (Medema et al., 1998). The results of the current

study confirmed the findings of previous studies which have observed longer persistence of clostridia spores in wastewater (Vierheilig et al., 2013), in river water (Lucena et al., 2003) and sediment (Mueller-Spitz et al., 2010). In comparison to *E. coli* in water, *C. perfringens* was identified in lower levels in water during active discharge, and in higher levels in sediment throughout the study, though the concentrations were higher post-discharge compared with during discharge. In addition, it was the only microorganism, in the current study, identified as having no significant difference in concentration in water before and after the active discharges ceased. *C. perfringens* was also noted to have significant but weaker correlations with human FST markers in water compared with other microbial indicators, and only a weak correlation with one pathogen (*Giardia*).

The widespread presence of *C. perfringens* in this study reflects its ability to survive in the environment and its numerous sources, including humans, pets and decaying vegetation (Pons et al., 1994). Low levels of *C. perfringens* have been identified in feral animals (Cox et al., 2005), and in particular, herbivorous wildlife (Vierheilig et al., 2013). The lack of differentiation of *C. perfringens* concentrations between discharge phases may have reduced the ability of this urban river study to identify a significant relationship between *C. perfringens* and protozoa in the water column. *C. perfringens* has been identified as a useful indicator of sewage contamination in tropical environments, where environmental populations of *E. coli* in soil and beach sand environments have been observed to contribute to concentrations of waterborne *E. coli* confounding its use as a faecal indicator in warmer climates (Fujioka, 2001; Fung et al., 2007). In the temperate environment of the current study, *E. coli* was superior to *C. perfringens* as a microbial water quality indicator of sewage discharge, and a better predictor of the presence of *Giardia* and *Cryptosporidium* supporting the findings of Wilkes et al. (2009) in a rural, temperate environment. In contrast, a study of Hawaiian streams identified enterococci as the indicator with the highest association with pathogens targeted in that study, which included *Campylobacter*, *Salmonella* and *Vibrio* species (Viau et al., 2011). *C. perfringens* was also identified as a better predictor of pathogens compared with *E. coli* in that study of a tropical environment.

F-RNA phage as an indicator of health risk

The F-RNA phage have been suggested as useful models for the aquatic behaviour of human pathogenic viruses released into the environment (Vergara et al., 2015; Wolf et al., 2008). Sinton et al. (2002) suggested that F-RNA phages are likely to be better indicators of enteric viruses than somatic coliphages in freshwater due to increased survival characteristics, whereas in

marine water, the converse is true. In contrast, the study of Lucena et al. (2003) showed that somatic coliphages have higher concentrations in freshwater than F-RNA phage, and Moriñigo et al. (1992) identified a better correlation between somatic coliphages and faecal coliforms in freshwater compared with F-RNA phage. Researchers observed that F-RNA phage had a greater reduction in concentration compared with somatic coliphages during treatment of wastewater, but both phages were resistant to chlorination (Mandilara et al., 2006a; Mandilara et al., 2006b). They also noted strong correlations between F-RNA phage and *E. coli* in raw and treated wastewater.

There was a significant correlation between F-RNA phage and *E. coli* in this urban study of river water and a weaker but still significant correlation between F-RNA phage and *Campylobacter* (Devane et al., 2014). This latter correlation, however, became statistically insignificant, when 2013 data (n = 9) was incorporated into the analysis (Table 13). In contrast to the indicator *C. perfringens*, F-RNA phage were not stored in river sediments, and levels were much lower in river water post-discharge. *Campylobacter* was also present in low concentrations in sediment throughout the study. This lack of accumulation in the river environment may suggest F-RNA phage have potential as indicators of sewage inputs. There were, however, a number of samples that registered non-detects for F-RNA phage, but which contained pathogens, including *Campylobacter*, with the converse also occurring. The additional 2013 water data in which *Campylobacter* was identified on every occasion at all sites, was in conjunction with FST markers indicating predominantly avian sources and intermittent detection of F-RNA phage. NZ avian species are known to be carriers of *Campylobacter* (Moriarty et al., 2011b). Therefore, the identification of *Campylobacter* during 2013, in conjunction with avian sources but not F-RNA phage does not negate F-RNA phage as an indicator of human faecal sources and associated *Campylobacter*.

Months after active sewage discharges ceased in the Avon/Otākaro River, levels of *E. coli* in water were still above the alert and action boundaries, compared with the lower levels of F-RNA phage (Figure 5). F-RNA phage, therefore, may have value as indicators of fresh sewage when detected in conjunction with elevated *E. coli* in water. The evidence supporting F-RNA phage as an indicator of recent human faecal contamination in water included significant positive correlations with all FST markers. In particular, there were strong correlations with human PCR markers including HumM3, and significant negative correlations with the bacterial faecal ageing ratio of AC/TC. Furthermore, molecular methods have been developed for the detection of the four genotypes of F-RNA phage that allow differentiation between F-RNA phage derived from animal and human faecal sources (Friedman et al., 2011; Wolf et al., 2008).

The genogroups G1 and GIV dominate in animal faeces compared with GII and GIII in human sewage and faeces. A tropical study of an urban freshwater catchment observed significant correlations between F-RNA phage GII and four human enteric virus groups (Vergara et al., 2015), with researchers concluding that F-RNA phage have validity as indicators of human enteric viruses.

3.4.2 Pathogen concentrations from direct sewage discharge

Potentially pathogenic *Campylobacter*

Throughout the study, *Campylobacter* were detected in low concentrations in the river water (≤ 110 *Campylobacter*/100 mL). In sediments, low levels of *Campylobacter* were detected at KR and OT (< 7.0 MPN/g) during the active discharge of sewage, and during both discharge phases at BS. The low level of *Campylobacter* in water and short residence time in sediments after wastewater discharges ceased, reflected the low survival rate known for *Campylobacter* after voiding into the environment (Moriarty et al., 2011a; Obiri-Danso et al., 2001). The highest level of *Campylobacter* in sediment was observed at KR (11.1 MPN/g) during the 2013 sampling but was not detected during this period at the other two sites. Some of these *Campylobacter* may have been derived from wildfowl as FST data was identifying wildfowl as the dominant source in water and sediments.

The pathogen, *Campylobacter*, had the least significant correlations with all variables in water, but did have significant positive correlations with *E. coli*, and the HumM3 PCR marker, and a negative correlation with the AC/TC ratio. HumM3 had a lower concentration in all river water samples compared with the other two human PCR markers (Figure 6). The lower levels of HumM3 was probably due to its target gene, a putative sigma factor, (Shanks et al., 2007; Shanks et al., 2009) being present in lower copy number in the bacterial genome compared with the 16S rDNA gene target (approximately five copies/cell) of the other PCR markers (Klappenbach et al., 2001). HumM3 may be an indicator of recent human pollution, as due to its intrinsic lower levels, it decreases in concentration more rapidly than the other human markers. In the event of a human sewage discharge into a waterbody, the shorter term persistence of HumM3, *Campylobacter* and F-RNA phage may explain why detection of elevated *E. coli*, HumM3 PCR marker, F-RNA phage and a low AC/TC ratio < 1.5 (suggesting a fresh faecal event) appeared to be indicative of a health risk associated with *Campylobacter*. The requirement for these indicators to be detected in conjunction, in order to specify a likely health risk from *Campylobacter*, is an example of the suite of indicators proposed by Harwood et al. (2005).

Potentially pathogenic protozoa

Concentrations of *Cryptosporidium* were less than 20 oocysts per 100 L in water during discharges into the Avon/Otākaro River and were undetectable once discharges ceased. This led to significant differences ($p < 0.001$) between the two discharge phases for *Cryptosporidium* at all three sites (Table 12). Low levels of *Cryptosporidium* were detected in all river sediments during discharge and on many occasions post-discharge. In contrast, *Giardia* was detected on most occasions in the water column as well as the sediments and at much higher levels than *Cryptosporidium*. In contrast to *Campylobacter*, the two pathogenic protozoa, which have longer survival times (Olson et al., 1999), had moderate, positive correlations with all FST human-associated PCR and steroid markers in water samples. This supports the detection of the human PCR and steroid FST markers in water as indicators of a potential public health risk.

Harwood et al. (2005) reported that 40% of *Cryptosporidium* oocysts detected in untreated wastewater samples were infective. The median infectious dose of protozoa is low. After assessing 6 clinical trials, McBride et al. (2012) determined the median infectious dose (ID₅₀) for *Cryptosporidium* to be ≈ 35 oocysts which is similar to the ID₅₀ for *Giardia*. Recovery of protozoa from sediments by current methods is also low, (typically $< 10\%$), therefore the true concentration of protozoa in sediment may actually be twenty times higher. Identification of protozoa in sediments highlights that despite non-detection in the water column there may be a health risk associated with re-suspension of sediments. Both protozoa were identified in higher concentrations in the river sediments after active sewage discharges had ceased.

Overall, levels of potential pathogens in the Avon/Otākaro River were lower than expected for a waterway receiving large inputs of raw sewage. One factor contributing to lower pathogen levels may have been the high levels of groundwater infiltration into damaged sewer pipes acting to dilute microorganism concentrations (personal communication, Mike Bourke, Christchurch City Council). There was also no reported increase in community levels of infection, which will have contributed to the lower than expected levels of pathogens in the sewage entering the Avon/Otākaro River. The number of cases of reported gastrointestinal illness for the period of June 2010 to June 2012 was lower than the two previous years (Institute of Environmental Science and Research, sourced from EpiSurv). Fortunately, the detrimental health outcomes were, therefore, less than may have been expected.

3.4.3 Wildfowl and canine markers

Waterfowl populations inhabiting the areas along the river may have been a source of pathogens, in particular at BS, which received only intermittent human discharges. There were, however, no

significant associations between the wildfowl PCR marker and pathogens, microbial indicators or human FST markers in water (Table 16), and this supported the pathogens being derived from human sources. *Campylobacter* species have been identified in NZ avian species (Moriarty et al., 2011b) and while protozoa have been detected in the faeces of wildfowl such as Canada Geese (Graczyk et al., 1996; Graczyk et al., 1998; Moriarty et al., 2011b) and sandhill cranes (Vogel et al. (2013), levels have often been low with variable prevalence. *Cryptosporidium* and *Giardia* species have also been identified in the faeces of non-human species including agricultural animals and wildlife species (Moriarty et al., 2008; Wilkes et al., 2013). There were, however, no known major agricultural activities in this catchment as the Avon/Otākaro River arises from an underground spring in the western suburbs of the city and flows through an urban environment. Subtyping of *Giardia* and *Cryptosporidium* (oo)cysts was beyond the scope of this study, therefore, caution is required in assigning protozoa to specific faecal sources.

The wildfowl PCR marker was also not correlated with the three avian-associated steroid ratios, which did have significant negative correlations with protozoa. When human contamination is identified, the high levels of coprostanol will, however, limit the use of the avian steroid ratios as noted in Devane et al. (2015), which may explain the lack of correlation between the avian PCR and steroid markers. This factor also suggests caution is required in interpreting the significance of correlation analyses for the avian steroids where human pollution is the dominant source.

The moderate positive correlations between the dog-associated PCR marker and human FST markers in water was exemplified by its detection at KR and OT on almost every occasion during the discharge phase, but not post-discharge. There were few occasions of significant rainfall above 5 mm in the 48 hours preceding the sampling events throughout the current study. The dog PCR marker was identified in water samples on a total of five occasions out of 9 rainfall-associated samples, and all five occurred during the discharge phase. Overall, 22 of 25 observations of the dog PCR marker occurred during the discharge phase of the study, and therefore rainfall is unlikely to account for the close association between the dog PCR marker and human FST markers noted during the correlation studies. These findings suggest dog faecal inputs were associated with domestic sewage, implicating disposal via the sewerage system as noted in another study (Caldwell and Levine, 2009). These findings are in contrast to the pre-earthquake Avon/Otākaro River study of Moriarty and Gilpin (2009) where dog faeces were identified in river water as a secondary source to wildfowl contributions during baseflow evaluations, and in the absence of human sources. However, in that same study, during high flow

events, dog faeces were the dominant faecal input, suggestive of overland rainfall runoff from dog faecal scats on land.

3.4.4 Relationships between FST markers and microbes

Wu et al. (2011) investigated indicator-pathogen correlations from 540 studies over 40 years of research from many different water types including human wastewater and freshwater. The majority of the studies were based on conventional assays (using culture methods) of microbial (FIB) indicators (57%), but also 17% molecular assays and 4% of combined assays (which included molecular and conventional), with the remainder immunoassays. The conventional assays were more likely to show significant correlations between pathogens and indicators. Important findings included that correlations were more likely where numbers of samples tested were greater than 30 and where positive pathogens were detected in more than 13 of those samples. In general, the urban river study fitted this sample criterion and showed significant correlations between protozoa, *E. coli*, faecal sterols and PCR markers.

Moderate to strong correlations were identified in water between human FST markers (except for FWA), protozoa and microbial indicators. These correlations suggested that PCA analysis of the combined data set of all variables may be useful in identifying a reduced number of FST markers for discrimination of faecal sources when sewage discharges into a river system. On the strength of evidence provided by the correlations and PCA analysis it would appear that the (three) human PCR markers, and the steroid ratio analysis (of ten steroids), as individual tools, are able to provide consistent discrimination of human pollution in waterbodies. The active discharge samples, from the two sites (KR and OT) impacted by continuous upstream inputs, clustered around the positive axis of the first component of the PCA (Figure 11), which was explained by the human-associated FST markers and high correlations with protozoan pathogen detection. Water managers, therefore, could be confident that FST methods are providing information on health risk associated with sewage contaminated water. In particular, when human FST markers (PCR or faecal steroids) are detected in a waterway there is a high probability of the presence of pathogenic protozoa.

Logistic regression analysis also revealed significant relationships between human PCR markers and human-associated steroid ratios as shown by the 89% agreement between the two methods in water (Table 17). This concordance was tested using Cohen's kappa approach for dichotomous variables to show a significant, substantial agreement between the two FST methods. These predictions were premised on two of the three human PCR markers reporting detection and the %coprostanol being supported by other ratios H3 and H2; indicating that the

coprostanol detected was derived from primarily human not herbivore sources (H3) and human not environmental sources (H2). These findings highlight the importance of not relying on the identification of a single steroid as a biomarker but as observed by other researchers, relating it to the concentrations of other faecal steroids assayed concurrently (Furtula et al., 2012a; Shah et al., 2007). Furthermore, the discharge of raw human sewage directly into a river allowed validation of the ratio threshold of >5-6% coprostanol used to evaluate a human pollution event. There were only two occasions (n = 47) where non-detection of human PCR markers was not congruent with %coprostanol as an indicator of human faecal inputs (Figure 13). These occasions occurred at the two active discharge sites but after cessation of active discharges. This data supports the 5-6% coprostanol suggested by Reeves and Patton (2001) as indicative of human pollution.

FWA in water

The levels of FWA in this river system were very low, and therefore, this variable was excluded from correlation analyses. Even during the period of major discharges of human sewage, levels of FWA indicative of human faecal pollution (0.2 µg/L) were detected on only two occasions. The mean concentration of DAS1 (the FWA used in NZ) reported in raw sewage in a Swiss study was 10.5 ± 2.8 µg/L (range 6.6 to 12.9) (Poiger et al., 1998). Levels in Christchurch sewage compared with the Swiss study, were on average, five to tenfold lower but more similar to the low end of the range observed in a Japanese study of raw sewage (range 2.9 to 8.2 µg/L) (Hayashi et al., 2002). In river water, levels of DAS1 derived from sewage were 0.4 to 0.6 µg/L (Poiger et al., 1999) and around 1.0 µg/L with a range of approximately 0.1 to <8.0 µg/L (Hayashi et al., 2002). Dilution into a large river is likely to have played an important role in the low levels of FWA detected, as is sedimentation due to strong absorption to sewage particles and the degradation effects of photolysis (Poiger et al., 1999). In addition, low FWA may have been due to a number of earthquake associated factors including less washing of clothes due to water restrictions, diversion of laundry waste into backyards, and infiltration of groundwater and stormwater into cracked sewer pipes diluting sewage and FWA levels (personal communication, Mike Bourke, Christchurch City Council). Together these factors suggest that FWAs may be a less useful tool for detection of raw human sewage in river water.

3.4.5 Sediments as a reservoir of microorganisms

Microorganisms in the Avon/Otākaro River sediment were detected using a method that re-suspended a known amount of sediment into sterile diluent. This method targeted the microorganisms in sediment that would be available for re-suspension into the overlying water

column during recreational and flood events, and therefore, potentially pose a public health risk to recreational users. Many researchers have concluded that the *E. coli* available for re-suspension is derived from the top layers of the sediment and that *E. coli* levels decrease by orders of magnitude with increasing depth. Pachepsky et al. (2009) and Pachepsky and Shelton (2011) suggested that approximately the top one centimetre of sediment is typically impacted by re-suspension during heavy flow events. Some studies have used an estimation of re-suspension of sediment into the water column in the order of 100 mg L^{-1} (Haller et al., 2009; Lee et al., 2006). Using this figure for re-suspension, sediment concentrations would need to be $>2.6 \times 10^4$ *E. coli*/g for *E. coli* re-suspension from sediment to exceed the recommended water quality guidelines of 260 CFU/100 mL under moderate-high flow conditions. This figure for *E. coli* in sediment was exceeded on several occasions at KR and OT during the study.

There are no water quality guidelines for microbial indicator concentrations in sediment in NZ. Concentrations of *E. coli* were variable in the river sediments as exemplified by the KR site, but there was no pattern of accumulation at BS and OT where levels of *E. coli* decreased by one to two orders of magnitude after discharges ceased (Figure 15). High variability of *E. coli* concentrations in sediment samples taken from the same locale have been noted in previous studies (Cho et al., 2010; Yakirevich et al., 2013). However, the heterogeneous distribution of *E. coli* in stream beds is relatively unimportant as the natural mixing processes during high flows will even out the spatial effects of entrainment from sediments (Wilkinson et al., 2006). Korajkic et al. (2011) found comparable levels of FIB in water and sediment. Other studies, however, have identified higher levels of FIB in the sediments compared with the overlying water column (Korajkic et al., 2009; Solo-Gabriele et al., 2000). Differences between study findings is likely due to variations in topography and hydrology between sites (Korajkic et al., 2011), and differences in sediment type (Cantwell and Burgess, 2004). These findings have led researchers to suggest that conclusions about sediments need to be location specific.

Transport of microbes between the sediment and water column may be a dynamic process that research suggests can occur during base flow as well as high flow events (Litton et al., 2010; Piorkowski et al., 2014a; Yakirevich et al., 2013). The hyporheic exchange of water flowing across the sediment-water interface has been proposed as a mechanism for the transfer of microbes from the sediment into the water column during base flow conditions (Grant et al., 2011). Piorkowski et al. (2014a) observed sediment-associated *E. coli* subtypes in the overlying water column which were not correlated to normal sediment transport processes of resuspension, suggesting hyporheic exchange was occurring. For example, they noted that in a downstream site characterised as a low energy section, the waterborne *E. coli* subtypes were more related to

the *E. coli* subtypes from upstream sediments in higher energy areas than those from the underlying sediments.

Campylobacter and F-RNA phage did not appear to be accumulating in river sediments after discharges had ceased. In comparison, elevated levels of *C. perfringens*, and low levels of the protozoa (*Giardia* and *Cryptosporidium*) were identified in river sediments, months after the major sewage discharges ceased. On-going intermittent discharges of sewage from a fragile sewer system may have impacted on these conclusions. In addition, prior to the earthquakes, levels of these microorganisms in the sediments were not evaluated, so there were no background data for comparison. The concentrations post-discharge of these indicators and potential pathogens in sediments, however, suggest that the riverbed sediments can be a reservoir for *C. perfringens* and protozoa. Although, by the 2013 sampling, approximately two years after the February 2011 earthquake, *Giardia* was detected on one occasion at less than 1 cyst/g and *Cryptosporidium* was not detected. Due to their size, protozoa can settle out of the water column into the sediment and remain undisturbed for long periods of time and *Cryptosporidium* has been shown to preferentially attach to organic particles in sewage effluent increasing their settling velocity (Medema et al., 1998). The high volumes of sewage discharge, therefore, may have increased deposition of protozoa in the Avon/Otākaro River sediments.

The highest concentrations of *Cryptosporidium* and *Giardia* in sediment were observed at BS in the urban river study during both discharge phases with mixed faecal sources identified on both occasions. BS was the only sampling location not influenced by tides. At the other two tidal sites, re-suspension and deposition of sediment may not have allowed for the significant build-up of protozoa as seen at BS. In recognition of the role of sediments and sand as reservoirs for harmful microbes, a workshop of water quality experts convened in Lisbon, Portugal, recommended the routine monitoring of beach sands for pathogens as part of a health risk assessment for recreational waters (Sabino et al., 2014).

3.4.6 Sediments as a reservoir of chemical FST markers

FWA appeared to be stored in river sediments at both sites of continuous sewage discharge, while storage of faecal steroids was only identified in KR sediments but not OT. KR sediments had a 70% component of fine gravel, whereas a similar percentage of silt and clay dominated at OT (data not shown). Although Froehner et al. (2010) identified a higher association between steroids in sediments containing higher concentrations of silt and clay, the greater influence of the tides at OT, the site closest to the estuary, may have lowered steroid accumulation.

In this urban study, the FST markers identified in the water column embodied a snapshot of contamination at the time of sampling. In contrast, the contamination signature in the underlying sediment represented a historical picture of the impact of pollution inputs to a river system and did not appear to be correlated with real-time events. This was shown by the lack of significant correlations between the percentage of coprostanol identified in the sediment and the overlying water at any of the river sites. As an example, at BS, in general, sediment samples contained less than 5% coprostanol (the definitive threshold for human sources) even when overlying water had up to 25% coprostanol. Other examples of this disconnect was observed at KR, where levels of FWA in sediments dropped from >100 µg/kg in February 2012 to <2 µg/kg in March 2012 (Table 19). This reduction in the sediment concentration in March 2012 occurred concurrently with the highest levels of FWA observed in the overlying water (0.4 µg/L) for any sampling event or site (Table 9). Re-suspension may have occurred at KR prior to the last sampling event in March 2012, as dredging of the river had taken place in this area to remove sediment build-up due to the earthquakes. However, in general, in the absence of re-suspension events and with the proper sampling techniques, reservoirs of steroid and FWA markers in the sediments did not appear to be impacting on water quality testing. Sampling technique must, therefore, avoid re-suspending sediments.

Further illustrating the disconnect between chemical FST markers in sediment and recent faecal inputs, there was no relationship between *E. coli* and any of the FST markers tested in the sediment, except for a weak negative correlation with FWA. The lack of association between *E. coli* and chemical FST markers could be seen in Figure 17 where *E. coli* concentrations had a wide distribution with no clear delineation between sediment samples containing human FST signals and those that did not. Similarly, there were few significant correlations between chemical FST markers and F-RNA phage, and pathogens. The high number of positive correlations of chemical FST markers with *C. perfringens* may be more a factor of the ubiquitous and high concentrations of *C. perfringens* identified in the sediments throughout the study, as noted in other freshwater systems (Mueller-Spitz et al., 2010). In the current study, there was a strong positive relationship between FWA and the human-associated steroids in sediments. The lack of correlation of chemical FST markers with microbial indicators or pathogens, however, suggests that FWA and steroid ratios were indicative of historical faecal sources in the sediments, and restricted their predictive value for health risks.

3.4.7 Potential faecal ageing ratios

AC/TC faecal ageing ratio in water

Two potential faecal ageing ratios were investigated, which have been used in previous studies of waterways to ascertain the relative freshness of a faecal input and/or the level of sewage treatment. The lack of a definitive ending to intermittent discharges, however, affected the interpretation of FST in regards to validating faecal ageing tools. The first ratio (AC/TC) was bacterial, and compared the high numbers of Total Coliforms (TC) found in fresh sewage with the background microflora of the river as indicated by atypical colonies (AC) on the same media (Brion, 2005; Nieman and Brion, 2003). The low ratios of AC/TC less than 1.0 and often <0.5 observed in water during continuous discharges (Figure 14), were consistent with the ratios identified in fresh human sewage (≤ 1.5) by Brion (2005). AC/TC ratios of <5.0 suggest the input of fresh faecal material (Brion, 2005), whereas higher AC/TC ratios (>15-20) indicate the passage of time as the river system returns to a healthier environment (Black et al., 2007), as was observed during the 2013 sampling at OT. The mean AC/TC ratio for all sites during 2011-2013 was 1.6 (range of 0.29–15.8) confirming the FST results that this river system was impacted by on-going faecal inputs, from human, dog and avian sources.

There were significant negative correlations in river water between the faecal ageing ratio AC/TC and all human FST markers, pathogens and microbial indicators excluding the ubiquitous *C. perfringens*. These findings confirm that low AC/TC values <1.5 may be a useful indicator of recent human faecal inputs into water indicating potential pathogens supporting the studies of Black et al. (2007) and Chandramouli et al. (2008) who developed models for predicting viral presence. Their models were based on the three parameters of faecal source, faecal age and faecal load represented by epicoprostanol, AC/TC ratio, and faecal coliform concentration (respectively) to provide the best fit for correct classification of viral presence. As a frontline addition to the water quality microbial indicator toolbox, alongside *E. coli* in this urban river study, the AC/TC ratio proved to be a quick and cost-efficient test.

Significant correlations between the AC/TC ratio and the two avian-associated steroid ratios were weakly positive, with no correlation between the AC/TC ratio and the wildfowl PCR marker. The lack of correlation may be a factor of the wildfowl PCR marker being prevalent in ducks (76%) (Devane et al., 2007), with lower prevalence for other wildfowl such as Canada Geese (15%), which are commonly present in the Avon/Otākaro River area. Additional avian PCR markers specific to other bird species may be useful in testing the efficacy of the AC/TC ratio in cases of avian pollution. As noted above, the high levels of coprostanol from human

sources confound the avian steroid ratios (Devane et al., 2015). Further investigations of waterways where avian faecal pollution is suspected as the dominant faecal input would need to be carried out to test if the AC/TC ratio is valid for determining the faecal age of avian inputs.

Coprostanol/epicoprostanol ratio in water as an indicator of untreated sewage

In urban environments, estimating the prevalence and abundance of pathogens in human sewage is complex and dependent on whether the sewage is raw or treated effluent (Soller et al., 2010). The differential decay between FIB and pathogens (Ottoson et al., 2006; Shannon et al., 2007) means that FIB concentrations may be within water quality guidelines but there is potential for infection by pathogens. The differentiation between treated and untreated sewage is, therefore, imperative if water managers are to understand the potential for health risks associated with the detected human contamination event.

Levels of epicoprostanol in human sewage increase in an anaerobic environment such as during sewage treatment when cholesterol and/or coprostanol are converted to epicoprostanol (McCalley et al., 1981). Furtula et al. (2012a) investigated changes in steroid ratios between the influents and effluents of six sewage treatment plants with either secondary or tertiary treatment regimes. Mean ratio of cop/epicop in STP influent (n = 8) was 37.3 (SD 9.3) and decreased to 11.6 (SD 5.7) for the effluent (n = 10).

In this urban study, median ratios of cop/epicop in river water were approximately 95 during active discharge at KR and OT, reducing to 21 and 15, respectively, post-discharge and remaining below 13, a year later (H6, Table 9 for KR and Table 10 for OT). The values during active discharge were indicative of untreated sewage and much higher than those identified by Furtula et al. (2012a) in influent. However, post-discharge, the cop/epicop ratio was similar to the treated ratio values noted by Furtula et al. (2012a), which could be influenced by the intermittent nature of the low level discharges that occurred post-discharge. Throughout the urban river study at all sites, the %epicoprostanol/total steroids was <1% in water, which is indicative of the low levels identified in fresh human faeces (Férézou et al., 1978).

It has been shown that coprostanol derived from sewage and diluted into water undergoes aerobic degradation (>95% reduction) within one week as a result of microbial degradation by the natural microflora of waterbodies (Switzer-Howse and Dukta, 1978). Bartlett (1987) and Marty et al. (1996) concluded that without continuous sewage inputs into a waterbody, the persistence of coprostanol in a water column would be short-lived. Furthermore, detection of coprostanol would be negligible after 20 days due to aerobic degradation processes, dilution and transport processes. The reductions in the cop/epicop ratio in the urban river water post-

discharge at <19 were in the presence of FST markers indicating borderline/low level human sources (Table 9 and Table 10). This ratio, therefore, was reflecting aerobic degradation of coprostanol and dilution processes, rather than a concomitant increase in epicoprostanol concentration in river water. A cop/epicop ratio of ≥ 20 with %epicop/total steroids of <1% in a river is likely to indicate untreated discharges. Further investigation would be required to validate this ratio and establish the ratio thresholds of cop/epicop once treated sewage is discharged into a river system. This ratio, however, has shown potential in identifying an untreated human sewage discharge allowing water managers to assess its potential health risk.

Coprostanol/epicoprostanol ratio in sediment as a faecal ageing indicator

The particulate fraction of the water was shown by Marty et al. (1996) to contain the majority (99%) of the steroids and deposition of these steroids could lead to their persistence in sediments, particularly if anoxic conditions were prevalent. The ratio of cop/epicop has been used as an indicator of human faecal contamination in sediment with a ratio >1.5 indicative of human inputs (Fattore et al., 1996; Patton and Reeves, 1999). This ratio has also been used to assess the treatment status/age of detected sewage inputs in sediment (Frena et al., 2015; Gomes et al., 2015; Mudge et al., 1999; Mudge and Duce, 2005).

In this urban river study, the cop/epicop ratio in sediment was investigated as an indicator of faecal ageing in an event where raw sewage was being discharged into a waterbody. It is suspected that a similar conversion process of coprostanol and/or cholesterol to epicoprostanol occurs in sediments as in treated sewage (Gomes et al., 2015). However, there are no clear guidelines for assessing aged, untreated sewage inputs to sediment. In this study, it was noted that the reduction of the cop/epicop ratio in the sediments over time (mean of 29 in sediment during discharge and 15 post-discharge at KR and OT) was similar to that identified by Furtula et al. (2012a) in the influent and effluent (respectively) of the sewage treatment process. However, in the Avon/Otākaro River, where discharge was raw sewage, the decline in cop/epicop, in sediment may have signified a change from a recent sewage input to an ageing environment after cessation of active discharges. This decline in ratio was accompanied by a small increase in the epicoprostanol concentration in the sediment. The ageing of faecal inputs was supported by the accumulation of FWA in sediments post-discharge (medians >79 ng/g) at the two discharge sites compared with active discharge (medians <16 ng/g). This accumulation of FWA post-discharge signalled a historical faecal signature. Further clarification of cop/epicop ratio criteria in sediments is required, including comparisons in sediments with indicators of recent faecal inputs such as the human PCR marker, HumM3.

The lack of a relationship between cop/epicop and pathogen detection in sediment may illustrate a disconnect between an ageing faecal environment and low pathogen concentrations. In addition, there are differential persistence rates in sediment for pathogens, as noted for *Campylobacter* and the protozoa in this urban river study. Interpretation of faecal ageing indicators, therefore, would require caution to mitigate an underestimation of the health risk when aged or historical faecal inputs are detected in sediments. This is supported by the lack of correlation in sediment between all chemical FST markers and pathogens in this study.

3.4.8 Conclusions

- In this study where large volumes of raw sewage were discharging continuously into an urban river, *E. coli* was a better predictor of pathogen presence than *C. perfringens*.
- F-RNA phage is a potential indicator of recent inputs of untreated human sewage.
- There was a significant correlation between *Campylobacter* and F-RNA phage, however, only when FST markers identified human pollution.
- A bacterial ageing ratio AC/TC discriminated fresh from aged faecal inputs in water.
- In association with elevated *E. coli* levels, detection of the following combination of markers: the human PCR marker, HumM3; a low AC/TC ratio <1.5, and F-RNA phage suggested recent human faecal inputs and increased health risk from *Campylobacter*.
- There was substantial agreement between the two FST methods of human-associated PCR and steroid ratio markers for identifying faecal sources.
- In addition to identifying faecal sources, human-associated PCR and steroid FST markers were useful indicators of potential protozoan pathogens in water.
- Human-associated PCR and steroid FST markers were better predictors of human pollution compared with microbial indicators.
- F-RNA phage and *Campylobacter* did not accumulate in sediments.
- The protozoa, *Giardia* and *Cryptosporidium* persisted in river sediments after cessation of sewage discharges.
- Sediment re-suspension increases health risk from re-mobilisation of potential pathogens.
- Water samples represented a snapshot of recent contamination compared with sediment, where chemical FST markers in sediment represented historical faecal signals, unrelated to the overlying water.
- A steroid ratio (cop/epicop) of ≥ 20 in association with low levels of epicoprostanol (1% of total steroids) identified untreated human sewage as the predominant faecal source in river

water. In sediment, cop/epicop showed potential in discriminating between fresh and aged faecal inputs when derived from untreated human sewage. Further assessments are required to establish/validate ratio thresholds for cop/epicop in these two matrices.

4 Chapter Four:

Impacts on FST markers as cowpats decompose under field conditions

4.1 Introduction

The intensification of land use has been a feature of the NZ agricultural environment over the last four decades (MacLeod and Moller, 2006). NZ has almost 5 million dairy cows and approximately 11,900 herds with herd sizes on the increase (NZ Dairy Statistics, 2013 - 2014). Concomitant with the increase in dairying, the numbers of beef cattle and sheep have both been decreasing, with 2-4% fewer over the last season (<http://www.beeflambnz.com>). The conversion of many lowland farms from sheep and beef cattle to dairying, has seen an increase in animal feed and fertiliser inputs and the associated increases in animal excreta on pasture land and at the milking shed (Monaghan et al., 2007). Consequently, agricultural practices can be a significant source of non-point pollution, particularly in regard to surface water and near surface groundwater quality (Clapham et al., 1999).

Dairy cows can be carriers of zoonotic pathogens such as pathogenic *E. coli*, *Campylobacter* and *Cryptosporidium* (Fish et al., 2009; Moriarty et al., 2008; Stott et al., 2011). Dairy cow/beef cattle excrete high volumes of faeces per day. The number of defecations per dairy cow/day have been recorded as 11-16 with a range of 1.5-2.7 kg deposited in a single defecation event (Haynes and Williams, 1993). The overall loading from one dairy cow per day has been recorded as ranging between 17.8 to 29.7 kg wet weight of faeces per day.

Identification of the sources of faecal contamination

Overland flow after rainfall is a major transport route for faecal contamination and hence pathogens into waterways (USDA, 2012). Rainfall is one of the main ways that faecal microbes can be transported overland and deposited into waterbodies, but another important contributor is the washing down of dairy milking sheds (Oliver et al., 2009). *E. coli* and other FIB are powerful sentinels of potential faecal contamination in waterways. FIB, however, do not identify the animal source of contamination, because they are generally present in the faeces of all mammals and avian species (Bettelheim et al., 1976). Faecal source tracking (FST) tools such as PCR and faecal sterols provide information about the sources of faecal contamination when elevated levels of *E. coli* are encountered (Sinton et al., 1998). PCR markers target the microbes that are unique to the intestinal environment of a particular animal species. Many PCR markers amplify 16S rDNA from members

of the Bacteroidetes Phylum such as the general faecal marker GenBac3, which has been shown to provide evidence of faecal contamination but is not source specific (Dick and Field, 2004). In a multi-laboratory comparison of host-specific PCR markers, Raith et al. (2013) concluded that BacR and CowM2 are suitable microbial source tracking (MST) markers for bovine-associated populations and that CowM2 was a more sensitive marker compared with CowM3.

Faecal sterols such as cholesterol, are identified in the faeces of all animals and have been used as chemical markers of faecal pollution (Leeming et al., 1996). Identification of an animal species' unique fingerprint is reliant on the differences in steroid concentration between each species, which is dependent on factors such as diet, microbial gut composition and whether the animal synthesises any of the steroids to supplement diet. The microflora of the gut have an important role to play in the final steroid composition of faeces due to their biodegradation of steroids, for example, conversion of unsaturated sterols to hydrogenated stanols, which is mediated by the anaerobic microflora of the intestine (MacDonald et al., 1983). These sterol transformations occur in the homeostatic environment of the intestine, and once voided into the terrestrial or aquatic environment, it is assumed that conditions will no longer support the microbial conversion of steroids. It is, however, known that sterols are degraded in aerobic environments, but that in some environments, such as anoxic sediments, the sterol signature remains as a stable indicator of historical faecal inputs (Bartlett, 1987; Nishimura and Koyama, 1977).

Monitoring changes in the microbial community of the decomposing cowpat

The question of stability of the FST signature arises in dairy excrement because of the bulk of individual faecal deposits (1-2 kg), which may support persistence/growth of microbial populations within the cowpat. The cowpat provides a sheltered environment including protection from sunlight inactivation (Haynes and Williams, 1993). Physical changes to the ageing cowpat include water and nutrient loss due to leaching, encrustation of the cowpat surface and temperature fluctuations (Kress and Gifford, 1984; Thelin and Gifford, 1983). These physical changes were hypothesised to challenge the initial cowpat microbial community, influencing modifications to dominant biological species. It is important, therefore, to examine the trends for the cow faecal microbes that are the target of FST PCR markers, and the anaerobic microbial community that metabolises the steroids in the host intestine. The changes in the microbial community could alter the ratio between sterols and their biodegradation products, the stanols.

The advent of next generation sequencing has allowed the study of the microbial composition of environmental samples by procedures that allow amplification of the genome of most of the microbes within a sample/environment leading to the term metagenome (Staley and

Sadowsky, 2015). A 16S rRNA gene amplicon-based study targeting the metagenome of the cowpat allows the characterisation of the total microbial community of the cowpat. There are nine hypervariable regions (V1 to V9) associated with the 16S rRNA gene which have been targeted by amplicon-based metagenomic studies because of their ability to discriminate between bacterial species, for example, regions V1 to V3 (Unno et al., 2010), V6 (Staley et al., 2013), V4 to V6 (Staley et al., 2015) and V6 to V9 (Degnan et al., 2012).

Degradation/decay of microbial and FST indicators post-defecation

Studies have identified the long-term maintenance and even growth of FIB in cowpats over time (Moriarty et al., 2008; Muirhead, 2009; Muirhead et al., 2005; Sinton et al., 2007b; Texier et al., 2008). Sinton et al. (2007b) determined the decay rates of FIB and bacterial pathogens in simulated cowpats under temperate field conditions over a six month period. They noted that growth and die-off patterns of FIB were predominantly related to moisture content of the cowpat and its internal temperature also played a role. They suggested that compared with enterococci, *E. coli* could be the preferred FIB for bovine faeces due to its higher levels in cowpats. Muirhead et al. (2005) and Muirhead et al. (2006) determined the decay rates of *E. coli* in runoff after simulated rainfall events on fresh and aged (up to 30 days) cowpats and demonstrated that *E. coli* are mobilised from cowpats as individual cells rather than in clusters. Moriarty and Gilpin (2014) showed that substantial *E. coli* can be mobilised from sheep faeces by simulated rainfall up to 21 days post-defecation.

The understanding of the degradation and decay of FST markers in the environment is recognised as an important part of the interpretation of faecal contamination in aquatic environments (Brown and Boehm, 2015). The decay of FIB and host-specific PCR markers has been well studied in simulated freshwater and seawater environments with, in general, differential decay rates noted between PCR markers and culturable FIB (Gilpin et al., 2013; Walters and Field, 2009; Walters et al., 2009). In general, PCR markers have been observed to have greater persistence compared with culturable FIB. An increasing number of studies on the decay of PCR markers have consistently shown that reduced temperature, higher salinity, lower sunlight inactivation and reduced predation are factors that contribute to the persistence of PCR markers in the aquatic environment (Bell et al., 2009; Dick et al., 2010; Gilpin et al., 2013; Green et al., 2011; Kreader, 1998; Okabe and Shimazu, 2007; Schulz and Childers, 2011; Silkie and Nelson, 2009).

Studies on the decay of PCR FST markers in cowpats include the ageing of naturally deposited cowpats under shaded and non-shaded conditions for 57 days (Oladeinde et al., 2014). Decay rates of PCR markers were similar in the two shading treatments with persistence of bovine-

associated PCR markers (CowM3 and Rum-2-Bac) noted for at least one month in cowpats. There have also been studies of the decay rates of PCR FST markers from studies of bovine manure and slurries applied to soil/land and monitored for 72 and 120 days, respectively (Piorkowski et al., 2014b; Rogers et al., 2011). Short term persistence (maximum Day 6) was noted for the CowM2 PCR in manure-amended soil compared with BacR PCR marker (Piorkowski et al., 2014b). Rogers et al. (2011) observed higher rates of decay for host-associated PCR markers (including CowM2 and CowM3) compared with FIB and the bacterial pathogens *Salmonella* and *E. coli* O157:H7.

Studies of the degradation of faecal sterols alongside other FST markers have been conducted in various matrices including seawater and freshwater for human sources (Jeanneau et al., 2012) and pig faecal sources (Solecki et al., 2011) and rainfall runoff from pig and cattle manure-amended soils (Jaffrezic et al., 2011). Derrien et al. (2011) recognised that in addition to animal diet, the storage and treatment of pig slurries and cow manure could also affect the interpretation of the faecal sterol signature. However, there has been little research on the impact of ageing on the cowpat in regards to mobilisation of the FST markers from cowpats under flood conditions and rainfall. In particular, there are questions about whether the individual faecal steroids have equivalent reductions in mobilisation rates from cowpats. This factor is important to understand because it impacts on the maintenance of consistent ratios between steroids during the ageing process. Fluctuating steroid concentrations could lead to changes in the interpretation of the FST signature, which is based on ratios between steroids rather than the absolute concentration of individual steroids (Chapter One, Table 3).

This rural study investigated the effects of ageing on bovine faecal indicators (microbial indicators and FST markers: PCR markers and steroids) by monitoring the decomposition of simulated cowpats under field conditions over a five and a half month period. Two separate spring/summer trials were conducted to evaluate mobilisation of the faecal indicators in cowpat runoff. In Trial 1, mobilisation of cowpat runoff was generated by the simulation of a flood event by re-suspension of single 2 kg cowpats, which were divided into two treatments of irrigated and non-irrigated cowpats. At each sampling interval, triplicate subsamples were taken from individual cowpat re-suspensions. In addition, amplicon-based metagenomic analyses of the 16S rRNA gene were conducted on the microbial community of faecal extracts from Trial 1 irrigated cowpats to monitor taxonomic changes in decomposing cowpats.

In Trial 2, mobilisation of cowpat runoff was generated by both a simulated flood and a simulated rainfall event on 1 kg non-irrigated cowpats. In Trial 2 at each sampling interval, three individual cowpats were used as replicates for each treatment (flood versus rainfall).

The microbial community changes within the decomposing cowpat were expected to impact on the bacterial targets of the PCR markers and alter the ratio between sterols and their biodegradation products, the stanols. It was hypothesised, therefore, that changes in the microbial composition of the decomposing cowpats (as illustrated by the amplicon-based metagenomic analysis) would:

- i) change the concentration of *E. coli* and the PCR markers mobilised into cowpat runoff
- ii) change the FST signature of faecal steroid ratios from bovine to human/avian/plant steroid signatures
- iii) effect a difference in the mobilisation decline rates of all analytes within a treatment regime and between treatments.

In addition, amplicon-based metagenomic analyses, PCR and faecal steroid markers were monitored for novel signatures that would signal a change to an aged faecal environment and have the potential for discriminating between recent and historical faecal inputs to aquatic environments.

4.2 Methods

4.2.1 Collection of cow faeces for making simulated cowpats

For the first cowpat field experiment in 2011-2012 (Trial 1), composite cowpats were prepared from a mix of fresh cowpats collected in the field immediately after deposition or from warm cowpats with a moist sheen indicating recent defecation. Care was taken to avoid soil and grass during all collections. For Trial 2 conducted in 2013-2014, the cow faeces were collected from the concrete pad leading into the milking shed, which had been washed down prior to the cows entering for milking time. Cows were pasture-fed and free-range and faeces were collected from the same farm for both trials. A total of 60 L of cow faeces was collected for Trial 1 and returned to the laboratory in sterile plastic buckets with lids and stored on ice. On arrival in the laboratory, faeces were stored overnight at 4°C in the dark, and simulated cowpats were made the next day within 20 hours of collection (Day 0), and sampled and processed the same day within 24 hours of collection. For Trial 2, 80 L of cow faeces was collected and cowpats were made on the same day as collection (Day 0) but the first sampling took place on Day 1, otherwise protocols for cow faecal collection were the same as Trial 1.

4.2.2 Making simulated cowpats

The shared University of Canterbury-ESR Lysimeter outdoor facility was used as the setting for both Trials 1 and 2. Cowpats were placed on grass which was composed of ryegrass and clover. Composite cowpats were mixed in a large sterilised plastic container (100 L) with sterilised broom handles and large spatulas. Individual simulated cowpats were prepared by weighing a mean of 2.12 kg (standard deviation (SD) ± 0.079) for Trial 1 and 1.0 kg (SD ± 0.013) for Trial 2.

On Day 1 of Trial 1, nine cowpats were formed by pouring homogenised cowpat faeces into a sterile plastic ring (22 cm diameter) that was placed on grass. The rings were immediately removed after pouring and a sterile spatula was used to remove residual cow faeces from the ring and added to the cowpat. An irrigation system was set up over these cowpats as per Figure 19. An equal number of additional cowpats were prepared on the ground alongside the first set of cowpats to be used as non-irrigated control cowpats sampled at the same time as the irrigated cowpats.

For Trial 2, on the same day (Day 0) the cow faeces were collected, seventy 1 kg cowpats were poured into a sterile plastic ring (22 cm diameter) sitting on autoclaved 800 μm nybolt mesh (#40792, Clear Edge Filtration, Avondale, Auckland, NZ) cut into squares of $\sim 26 \times 26$ cm and placed on grass (rye grass and clover mix) in the lysimeter site. To measure the internal temperature

of five cowpats, five temperature probes (T107 sensors, Campbell Scientific, Inc., Logan, USA) were placed on the nybolt mesh squares and cow faeces poured on top within the plastic ring to form a cowpat on top of each probe. All cowpats in Trials 1 and 2 were covered with protective wire mesh to prevent disturbance by birds and allow entry of rain and sunlight (Figure 19 and Figure 20). Grass around the cowpats was trimmed by hand-held clippers on a regular basis to prevent shading of the cowpats.



Figure 19: Irrigated cowpat set up used during Trial 1. Photo credit: Brent Gilpin, ESR Ltd.

4.2.3 Trial 1: Sampling of cowpats

Cowpats were irrigated every week for the first six weeks with 10.8 L delivered over two hours. Subsequently, the irrigation regime switched to fortnightly watering with the same volumes. The irrigation regime was similar to that followed by local dairy farmers. Following irrigation the cowpats were sampled on Day 0 (*i.e.* the day the cowpats were placed on the lysimeters), Days 7, 14, 21, 28, 42, 77, 105, 133 and 161. On each of the ten sampling occasions, one irrigated and one non-irrigated cowpat was sampled in its entirety.

Following the completion of irrigation, the single cowpat was removed. Cowpats were weighed and half of the cowpat was used for dry weight analysis and the other half (1 kg equivalent) re-suspended in sterile distilled water (MilliQ, Millipore) to a final weight of 5 kg. The cowpat was homogenised for 10 min by stirring and in the latter stages with minimal breaking up of the cowpat with a sterile broom handle to mimic the tumbling action that could impact a cowpat during a flood event. Re-suspended cowpat supernatant was allowed to settle for 5 min before decanting 4 L for faecal steroid analysis and remainder for microbial and PCR assays. On each sampling occasion, the same procedure was followed for a single non-irrigated cowpat as for the irrigated cowpat. All supernatant samples were stored in the dark at 4°C and homogenised before

analysis which occurred within 6 hours, except for faecal steroids, which were filtered through GF/F glass microfiber filters and filters frozen at -20°C until further extraction.

Following each irrigation event during Trial 1, three samples were taken from each cowpat supernatant (irrigated and non-irrigated) and analysed for *E. coli*, and FST markers (faecal steroids and PCR markers: general faecal, GenBac3; ruminant, BacR, and bovine-associated CowM2) as outlined in Chapter 2. All triplicate samples from each matrix were analysed in duplicate, except for steroids, which were analysed once for each of the three replicate samples.

4.2.4 Trial 2: Rainfall simulation experiment

Trial 2 investigated the mobilisation of cowpat microbial and FST markers after a rainfall event and it also included an investigation of the fate of the markers in cowpats as measured in the re-suspended cowpat supernatant. During Trial 2, seven cowpats were sampled on Days 1, 8, 15, 22, 29, 50, 71, 105, 134, 162 with three cowpat replicates for cowpat rainfall runoff, and three cowpat replicates for the re-suspended cowpat supernatant plus one cowpat for dry weight analysis (100 g x 3 replicates). Differences between the two trials were that the cowpats in Trial 2 were 1 kg as opposed to the 2 kg cowpats of Trial 1. In addition, the 1 kg entire cowpat was re-suspended in final weight of 2 kg of supernatant with sterile distilled MilliQ water in comparison to Trial 1's re-suspension of 1 kg equivalent (half of the 2 kg cowpat) in 5 kg final weight.

A rainfall simulator was constructed which contained 25 needles of 20 gauge size. These were evenly placed in a circular drip tray at a distance of 92 cm above the faecal samples and the simulator was wrapped in plastic to prevent wind disturbance (Figure 20). Water bubbles were removed from needles using a manual sterile syringe before and between rainfall simulations and all needles were monitored for consistent raindrops to ensure even distribution over the cowpat. Sterile water (1146 ml) was added to the tray and water was gravity fed through the needles over 20 mins. This produced a rainfall event of 20 mm/hr, with the formation of <2 mm raindrops at terminal velocity, and therefore represented light rainfall (Moriarty and Gilpin, 2014). Three cowpats were individually collected by lifting up the nybolt mesh plus cowpat, and transferring to a pre-weighed 450 x 300 mm length drip tray and weighing the cowpat. The drip tray had an approximate 10% slope to facilitate collection with four 10 mm holes and nine 3 mm holes drilled at one end to allow the runoff to flow through a sterile funnel into a sterile 500 mL polypropylene bottle. The bottles were placed into holes in the ground to directly capture the rainfall runoff (Figure 20). The funnel contained an additional filter of the autoclaved nybolt mesh to prevent collection of insects, grass and leaves etc. The volume of runoff was recorded and analysed for *E. coli*, the faecal ageing ratio AC/TC, and faecal steroids and the same PCR markers as for Trial 1. A blank of sterile MilliQ

water (1146 mL) was run through the rainfall simulator prior to each sampling event and monitored for contamination by analysing for *E. coli* and FST markers.

In addition, during Trial 2, three cowpats were weighed (1 kg equivalent) and re-suspended into sterile distilled water (MilliQ) to a final weight of 2 kg. The cowpat was homogenised and supernatant collected as outlined in Trial 1. The Trial 2 re-suspended cowpat supernatant was analysed for the same microbial and FST markers as the rainfall runoff. This Trial 2 supernatant represented a similar experiment to the analysis of the supernatant from non-irrigated cowpats performed in Trial 1 except that 1 kg simulated cowpats were used.

4.2.5 Analytical Methods

Details of analytical methods for *E. coli*, AC/TC faecal ageing ratio, FST PCR markers and faecal steroids, and the amplicon-based metagenomic analysis are provided in Chapter Two.

In Trial 2 of the rural cowpat studies, the AC/TC faecal ageing ratio was determined for the cowpat supernatant and rainfall runoff. In order to provide a background river microflora for the AC counts, the cowpat supernatant and rainfall runoff were diluted 1:10 into freshly collected water from a local stream to simulate overland flow of cowpat runoff into a waterway.

4.2.6 Physical Data

Global radiation (mejjoules (MJ)/m²/month) was measured at NIWA, Kyle Street and sunshine hours at the Christchurch airport for both Trial 1 and 2 (www.cliflo.niwa.co.nz). Ambient air temperature was measured as maximum and minimum daily temperatures at NIWA, Kyle Street during Trial 1. In comparison, during Trial 2, air temperature and cowpat internal temperatures were recorded hourly on site at the University of Canterbury-ESR Lysimeter facility. For Trial 2, five cowpats had temperature probes inserted as the dairy faecal slurry was poured into the cake tin. All temperatures were monitored by a Campbell Scientific (CS1000) datalogger. For Trial 2, ambient air and cowpat temperatures were presented as the 12 hourly means of hourly temperatures collected between 0900 to 2000 and 2100 to 0800 to give an indication of the fluctuations in temperatures between day and overnight. Rain data was collected on-site for both trials using a tipping bucket, which was connected to the Campbell Scientific datalogger.



Rainfall simulator with aged cowpat on tray



Drip tray with 25 needles of 20 gauge size



Making simulated cowpats for rainfall runoff experiment



Pouring weighed 1kg of cow faeces into sterile ring for simulated cowpats

Figure 20: Trial 2: the making of cowpats; and the rainfall simulator with drip trays for collection of rainfall runoff from cowpats. Photo credit: Brent Gilpin, ESR Ltd.

4.2.7 Statistical analyses

Statistical analysis was undertaken using SigmaPlot version 11.0 (Systat Software, San Jose, California, USA, 2008) and XLSTAT (2007.6) to calculate inferential statistics. Values below the limit of quantification for analytes were assumed to be zero. All counts were expressed as arithmetic means. Significance was characterised at the α -level of 0.05 for all statistical analyses. Non-parametric statistical analyses were performed because the distribution of much of the data failed the Shapiro-Wilkes normality tests. Spearman Ranks (Spearman rho, r_s) was used to test if there was a relationship between the FST variables and microbes, with correlation values $r_s \geq 0.75$ reported as strong; r_s 0.50-0.74 as moderate; and below r_s 0.50 as weak.

Calculation of inactivation parameters for PCR markers and steroids in cowpat runoff

In order to derive mobilisation decline rate coefficients for markers in Log_{10} units per day, a linear regression line was fitted to Log_{10} transformed *E. coli*, (CFU/100 mL), PCR markers (GC/100 mL) and steroids (ng/mL for supernatants and ng/L for rainfall runoff) for all cowpat supernatants from Trial 1 and 2 and cowpat rainfall runoff in Trial 2. The mobilisation decline curves were calculated as monophasic (Lee et al., 2009; Rogers et al., 2011; Solecki et al., 2011) based on the Chick model (Chick, 1908) using Equation 1. Where the curves were not monophasic as was the case for the faecal steroids, then the change in slope between the two regression lines was based on expert judgement rather than regression analysis. This procedure was used because combining the two equations as biphasic produced residuals that were no longer random. The linear regression analysis of the second stage (k_2) of the mobilisation curves for steroids in both trials, revealed that in general, the slopes of the regression were not significantly different from zero. Therefore the concentration of each steroid was not changing significantly over time but was still above the limit of quantification.

The second phase k_2 mobilisation curves for *E. coli* in rainfall runoff (Days 22-162); GenBac3 and BacR (Days 29 to 162 and Days 29 to 134, respectively) were tested to see if their slopes were significantly different from zero or if marker degradation had become negligible. In the cowpat supernatant, k_2 values were significantly different from zero (GenBac3, $p = 0.003$; BacR, $p = 0.002$). In contrast in the cowpat rainfall runoff, only *E. coli* and the GenBac3 marker slope were significantly different from zero ($p = 0.030$). BacR in the runoff, therefore, was represented by a monophasic decay curve up to Day 29 after which the BacR was approaching the detection limit and was just above the LOQ (20 copies per PCR reaction). The biphasic (two stage) die-off model (Crane and Moore, 1986) was applied (Equation 2) to simulate mobilisation decline from the

cowpat runoff for GenBac3 and BacR in Trial 2 supernatant and for GenBac3 and *E. coli* in rainfall runoff.

$$N_{(t)} = N_0 * 10^{-kt} \text{ when } t < t_1 \quad \text{Monophasic Equation (1)}$$

$$N_{(t)} = N_0 * 10^{(-k_1 t)} * 10^{(-k_2 * t - t_1)} \text{ when } t > t_1 \quad \text{Biphasic Equation (2)}$$

Where N_0 is the mean concentration of the target marker at Day 0 (Trial 1) or Day 1 (Trial 2). $N_{(t)}$ is the mean target marker concentration at time t , and t is the time representing the number of days since the start of the experiment and t_1 is the time (days) when the first decline phase ends. The mobilisation decline constant is k expressed as mobilisation decline rate (Log_{10} units) per day. To enable comparison with other studies the T_{90} was calculated. The T_{90} represents the time required for decline in marker concentration to 90% of its original concentration (1 log reduction) using the Chick model when the 90% reduction in concentration occurred within the first phase ($t < t_1$).

The slopes of the linear regression of Log_{10} transformed variables were compared using the method of Zar (2010) to test for significant differences (α -level 0.05) between the regression coefficients (slopes) of two populations. Each of the variables was compared for differences in mobilisation rates between the two irrigation regimes in Trial 1, and between Trial 2 supernatant and rainfall runoff. In addition, the three PCR markers were compared with each other, within the same treatment regime (either IRR or NIR) in Trial 1; and within the Trial 2 supernatant and the rainfall runoff using the procedure of Zar (2010) to perform a multiple comparison of more than two slopes by analysis of covariance (ANCOVA). The same procedure was applied to the ten faecal steroids and the total steroids within the same treatment.

4.3 Results

4.3.1 Weather conditions

Trial 1 took place between 1 November 2011 and 10 April 2012, which covered the period of early summer and mid-autumn in NZ's temperate climate. Trial 1 incorporated an irrigation treatment regime on one set of cowpats that followed irrigation volumes having a delivery frequency similar to those employed by dairy farmers. Weather parameters including rainfall, sunshine hours and global radiation and temperature for Trial 1 are presented in Table 23 and all of the daily data for temperatures is shown in Figure 21. The mean temperatures for all months ranged between 18 to 22°C. December had the highest rainfall recorded and February was the driest month for rainfall, and surprisingly, had the lowest sunshine hours. January, which marks the middle of summer, recorded the highest sunshine hours, Global Radiation (GR) and maximum temperatures and the second lowest rainfall. Cumulative rainfall over the experimental period was 258 mm.

Trial 2 took place between 1 October 2013 and 11 March 2014, which covered the period of late spring, summer and early autumn in NZ. Table 24 shows the physical weather-related parameters recorded during this period. As seen in Trial 1, January recorded the highest sunshine hours and GR. The driest month was January recording 23 mm of total rain. Cumulative rainfall was 420 mm over the five and a half month period. Although this amount was notably higher compared with Trial 1 (258 mm), it was heavily impacted by a deluge of 158 mm recorded over 36 hours in March 2014. This deluge, however, only impacted the last sampling day (Figure 22b).

Air temperatures were measured on site for Trial 2 and daytime temperatures had a mean of 18°C and range of 6 to 28°C. In comparison, overnight air temperatures had a mean of 13°C and range of 2 to 22°C (Figure 22a). The internal daytime temperature of the five cowpats had a mean of 24°C and range from 9 to 37°C (Figure 22b). The overnight temperatures of the cowpats had a mean of 13°C and range from 1 to 19°C. Using the Student *t*-test to compare between the ambient air and internal cowpat temperatures, it was determined that the air temperature was significantly different to the internal cowpat temperature during day light hours ($p < 0.0001$) but not during the night. The maximum daytime temperatures, in individual cowpats, reached 45 to 52°C during late November, December, January and February. The maximum temperature of 52°C occurred in individual cowpats on two occasions in mid-February (when mean temperatures of cowpats were 35 and 36°C).

4.3.2 Total solids in cowpats

The percentage of total solids in the cowpats over the course of both trials is presented in Figure 23. The %total solids was calculated for irrigated (IRR) and non-irrigated (NIR) cowpats in Trial 1. The %total solids was analysed in triplicate, except where there was not enough material during the latter part of the experiment when the cowpat was dried out. In Trial 2, a single cowpat (with triplicate samples) was tested for total solids at each sampling interval because there were no different treatments applied to the cowpats prior to processing for either supernatant or rainfall runoff.

In general, for both trials, the %total solids increased during the experiment as the cowpats lost moisture and dried out forming a hard crust on the surface. It was noted in both Trials that after Day 42 and 22 (Trials 1 and 2, respectively) the cowpats were no longer completely re-suspended in the supernatant and were minimally broken up before stirring for 10 minutes to reflect the tumbling that would occur during a flood event.

Initial total solids in cowpats for both trials ranged between 8.8 to 10.7%. By sampling Day 42 in Trial 1, the NIR cowpat had dried out faster and was above 60% total solids compared with the IRR cowpat at <30%, showing the effect of irrigation on moisture retention. By Day 77 in Trial 1, the converse was true, however, the temporary increase in moisture noted in the NIR cowpats was probably due to a leaking irrigation hose in the property adjacent to the experimental site causing water seepage around the NIR cowpats but not the IRR cowpats. After this episode both cowpat treatments fluctuated between 48 to 61% total solids till the end of the experiment. In Trial 2, Day 71 showed a decline in the % total solids of cowpats before returning to >75% total solids for the remainder of the experiment.

Based on the total solids, the moisture content of initial cowpats were around 90% and by the end of the experiments moisture content ranged between 39 and 42% in IRR and NIR 2 kg cowpats, respectively, whereas it was lower (24%) in the 1 kg cowpats of Trial 2.

Table 23: Weather parameters recorded during Trial 1

Month, year	Sampling Days that occurred in each month	Rainfall total (mm)/month	Total sunshine hours/month	Total Global radiation (MJ/m ²)/month	Mean maximum temperature/month (°C, SD)	Mean minimum temperature/month (°C, SD)
November, 2011	Days 0, 7, 14, 21, 28	61.6	232	481	19 (4)	9 (3)
December, 2011	Day 42	67.4	185	472	19 (4)	12 (3)
January, 2012	Day 77	43.0	233	494	22 (4)	11 (3)
February, 2012	Day 105	25.2	150	350	20 (3)	12 (2)
March, 2012	Day 133	52.4	189	333	19 (4)	9 (3)
April, 2012 (10 days)	Day 161	8.0	66	89	18 (1)	9 (2)
TOTAL	-	257.6	1055	2219	-	-

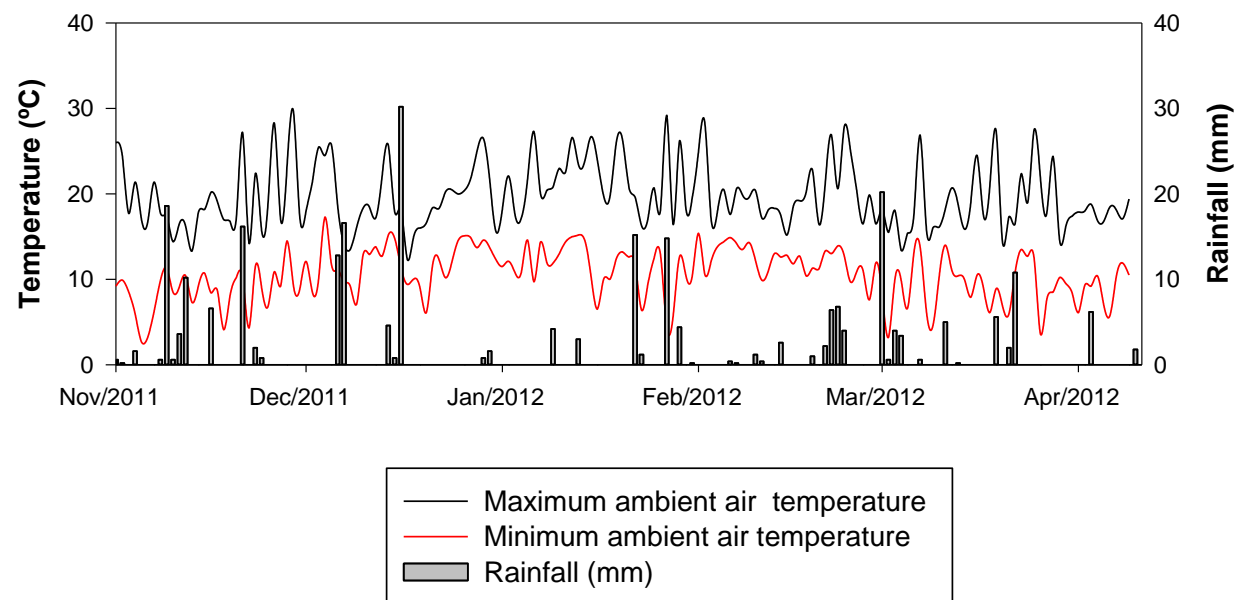
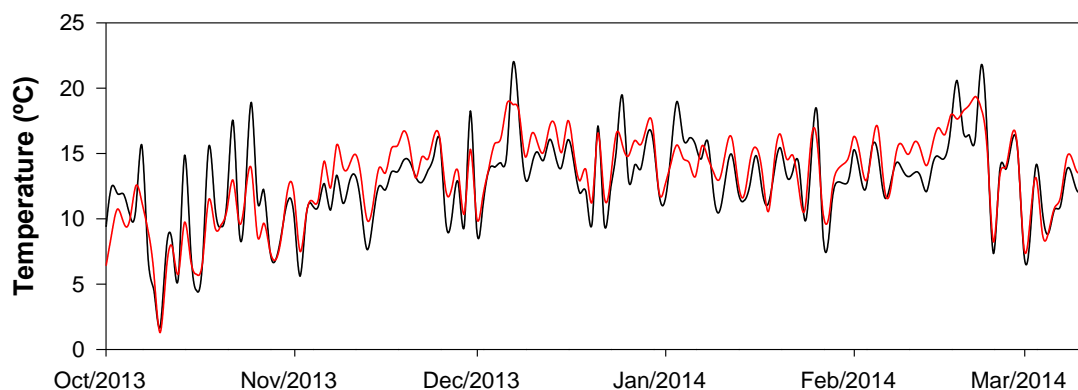


Figure 21: Trial 1- Maximum and minimum daily ambient air temperatures and rainfall

a) Ambient air and internal cowpat temperatures overnight



b) Rainfall, and ambient air and internal cowpat temperatures during the day

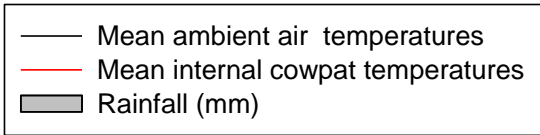
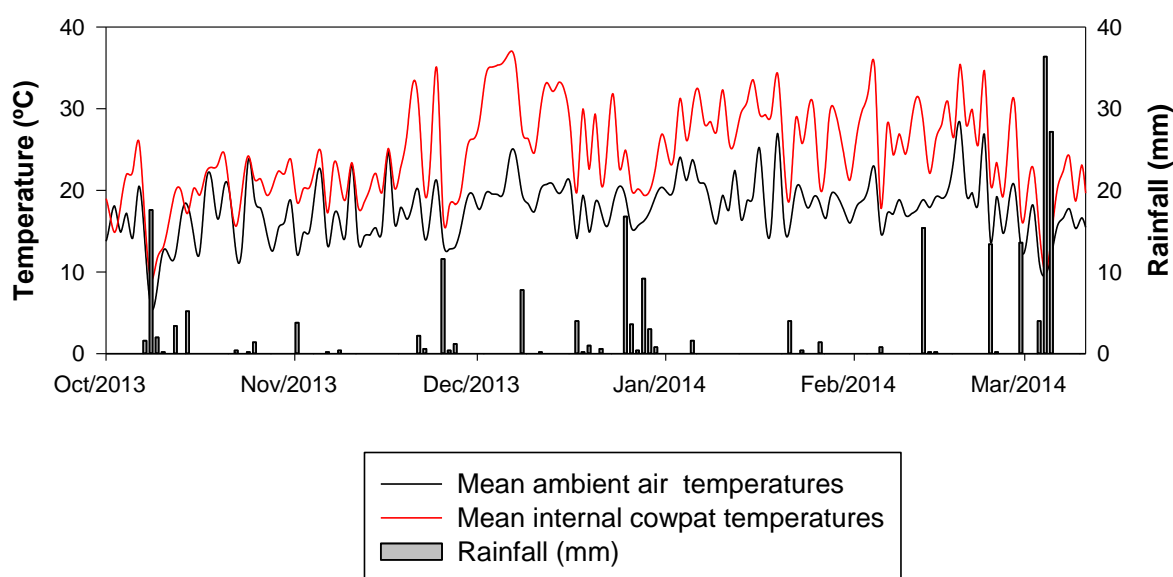


Figure 22: Trial 2 - Rainfall, ambient air and internal cowpat temperature

Table 24: Monthly rainfall, sunshine hours and Global Radiation for Trial 2

Month, year	Sampling Days that occurred in each month	Rainfall total (mm)/month	Total sunshine hours/month	Total Global radiation (MJ/m ²)/month
October, 2013	Days 1, 8, 15, 22, 29	59.2	214	525
November, 2013	Day 50	36.4	169	568
December, 2013	Day 71	74.4	188	610
January, 2014	Day 105	22.8	247	690
February, 2014	Day 134	53.8	195	538
March, 2014 (11 days)	Day 162	173.0	61	149
TOTAL	-	419.6	1074	2554

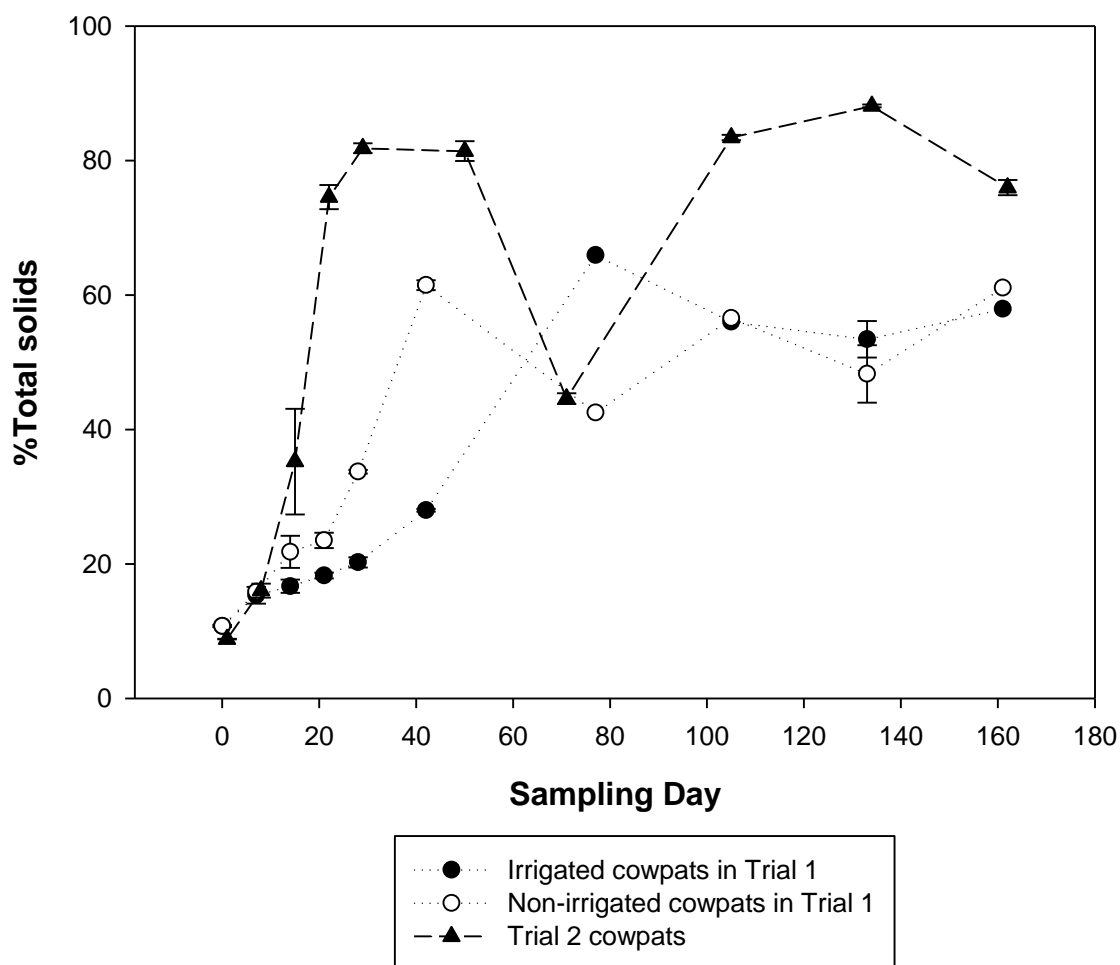


Figure 23: Percentage of total solids in cowpats from Trials 1 and 2

4.3.3 *E. coli* mobilised from cowpats

Trial 1: *E. coli* concentrations in re-suspended cowpat supernatant

Over the five and a half month period, there were ten sampling events for all analytes during Trials 1 and 2. Tables of all data for *E. coli* in Trials 1 and 2 can be found in Appendix, Table 35 to Table 37. The initial mean concentration of *E. coli* in fresh cowpat supernatant on Day 0 was 3.8×10^7 (SD 7.7×10^6) CFU/100 mL (Figure 24). This was the same starting concentration for both IRR and NIR cowpats, as no irrigation occurred on Day 0 of the experiment. The same order of magnitude of *E. coli* was observed in the IRR supernatant until there was an increase of one order of magnitude in *E. coli* on Day 21. This increase was followed by a decline in numbers to a plateau of 10^5 CFU/100 mL from Day 77 to 133. At the end of the experiment on Day 161, levels in the IRR supernatant were 7.7×10^4 (SD 6.1×10^3) *E. coli*.

In the NIR cowpat supernatant, the *E. coli* increased from initial concentrations, one order of magnitude on Days 14 to 21, before decreasing to 10^6 CFU/100 mL by Day 42, and

stabilising on 10^5 CFU/100 mL during Days 105-161. At the end of the experiment, *E. coli* numbers were 1.1×10^5 (SD 1.9×10^4), which was an order of magnitude higher compared with the IRR cowpat supernatant.

The mobilisation rates (k) and coefficient of determination (r^2) values for *E. coli* in Trial 1 are presented in Table 25. The mobilisation rate for *E. coli* in IRR cowpat supernatant was calculated as a first order monophasic decline curve, although Figure 24 suggests some increases in concentration, particularly on Day 21. Comparison of the slopes for the mobilisation curve calculated using all ten sampling events versus only the data points from Day 28 onwards, showed that there was not a significant difference between the slopes of the two curves for the IRR supernatant. Therefore, it was probable that the increase in concentration on Day 21 was likely due to variability in measurements rather than an increase in mobilisable *E. coli* from the cowpat into the supernatant. In comparison, for the NIR supernatant, there was a significant increase in *E. coli* counts in the supernatant ($p = 0.047$) during the initial phases, and then *E. coli* counts showed a monophasic decline rate after Day 21 as represented by the k_1 in Table 25. The slopes of the linear regression of *E. coli* in the two irrigation treatments were compared using the method of Zar (2010) to test for significant differences (α -level 0.05) between the regression coefficients (slopes) of two populations. The decline rate for *E. coli* was not significantly different between the two irrigation treatments.

Trial 2: *E. coli* concentrations in re-suspended cowpat supernatant and rainfall runoff

In Trial 2, there was greater variability in concentrations (Figure 24) compared with Trial 1 as evidenced by the higher coefficients of variation (CV) seen for *E. coli* from Trial 2. The CV for *E. coli* in Trial 2 supernatants ranged from 15 to 81% and in runoff 24 to 148% compared with a range of 3 to 20% for all supernatants in Trial 1. These differences are likely due to the differences in methodology, in that Trial 1 triplicates were derived from the same cowpat supernatant whereas the triplicate samples in Trial 2 for both supernatant and rainfall runoff were from three individual cowpats, resulting in higher variability between replicates. Further evidence of this variability in Trial 2 was seen in the rainfall runoff triplicates for Day 29 and for Day 71, which both spanned three orders of magnitude, 10^2 to 10^4 *E. coli*/100 mL and subsequently the standard deviation was larger than the mean and could not be accommodated by the error bars on the graph (Figure 24).

The initial mean concentration of *E. coli* in re-suspended cowpat supernatant on Day 1 was 1.6×10^7 (SD 2.4×10^6) CFU/100 mL, which was a similar order of magnitude as the supernatants on Day 0 of Trial 1. *E. coli* concentrations decreased an order of magnitude in the

supernatant on Day 8 and plateaued at 10^6 CFU/100 mL until Day 105 when there were incremental reductions till the final concentration on Day 162 of 8.2×10^3 (SD 4.8×10^3) *E. coli*.

E. coli was not detected in the blank of sterile MilliQ water which was run through the rainfall simulator prior to each sampling event. Mean rainfall runoff collected from cowpats was 1.09 L (SD 0.19). The initial mean concentration on Day 1 of *E. coli* in fresh cowpat runoff collected after a rainfall impact was 1.1×10^7 (SD 2.6×10^6) CFU/100 mL, which was the same order of magnitude as the *E. coli* in the supernatants for Trial 2. On Day 8 there was a notable decrease in *E. coli* concentration in the cowpat runoff down to 3.0×10^4 (SD 2.4×10^4). This level was followed by fluctuations in concentration within two orders of magnitude, 10^3 - 10^4 CFU/100 mL, up to Day 105 when the *E. coli* concentration reduced to a mean of 27 CFU/100 mL and remained below this level but still detectable till the end of the trial. The *E. coli* concentrations in the supernatant and rainfall runoff were moderately correlated (0.64, $p < 0.0001$).

As for Trial 1, the mobilisation rate of *E. coli* in the Trial 2 supernatant was calculated as a monophasic decline curve, producing a similar rate and T_{90} value as Trial 1 IRR cowpats (Table 25). The slopes for the linear regression of *E. coli* between the supernatant and the rainfall showed that the mobilisation rates for *E. coli* were not significantly different ($p > 0.05$) between the two treatments in Trial 2 when the comparison included a log-linear regression of Days 1-162 for both treatments. This did not reflect, however, the steep decline in concentration between Days 1 and 8, therefore, the rainfall runoff mobilisation rate for *E. coli* was calculated from a biphasic curve. When the mobilisation rates were compared between *E. coli* in the supernatant and the rainfall runoff, there was a significant difference ($p < 0.05$) in mobilisation reflecting the decrease of three orders of magnitude between Day 1 and 8 in the rainfall runoff. The T_{90} for *E. coli* in rainfall runoff of 5 days compared with 44 to 52 days observed in the supernatants of both trials also reflected the marked decrease in mobilisation of *E. coli* after the first sampling day.

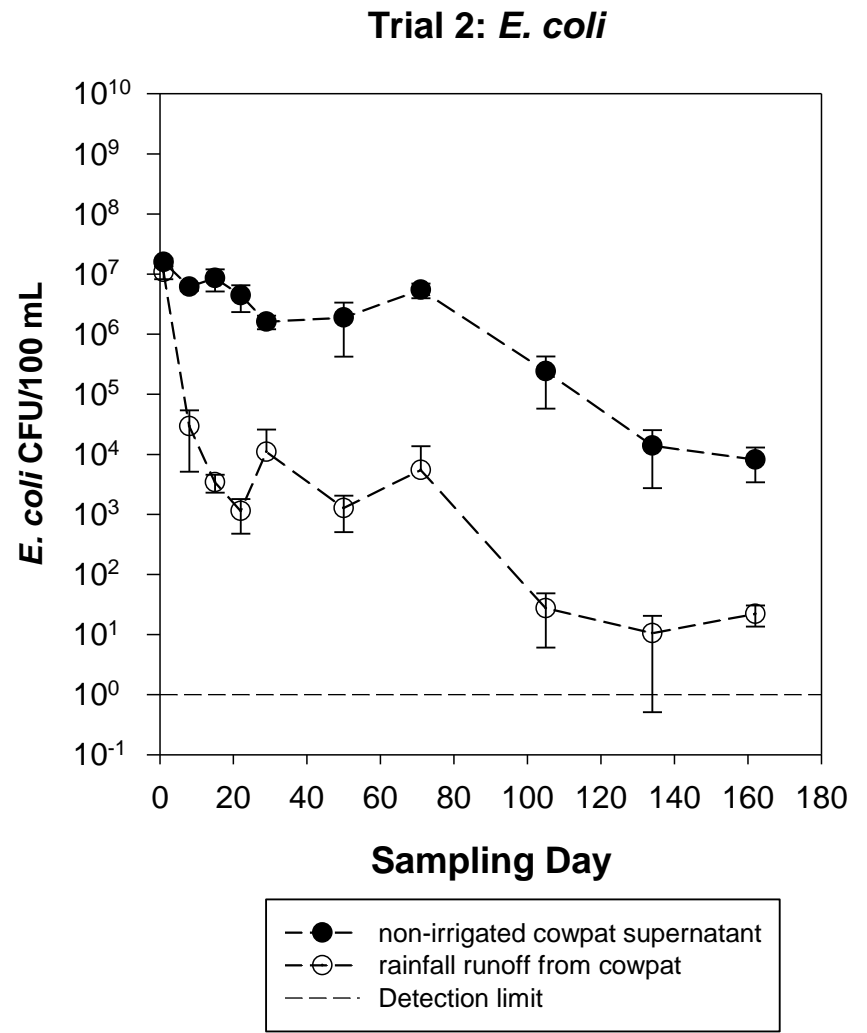
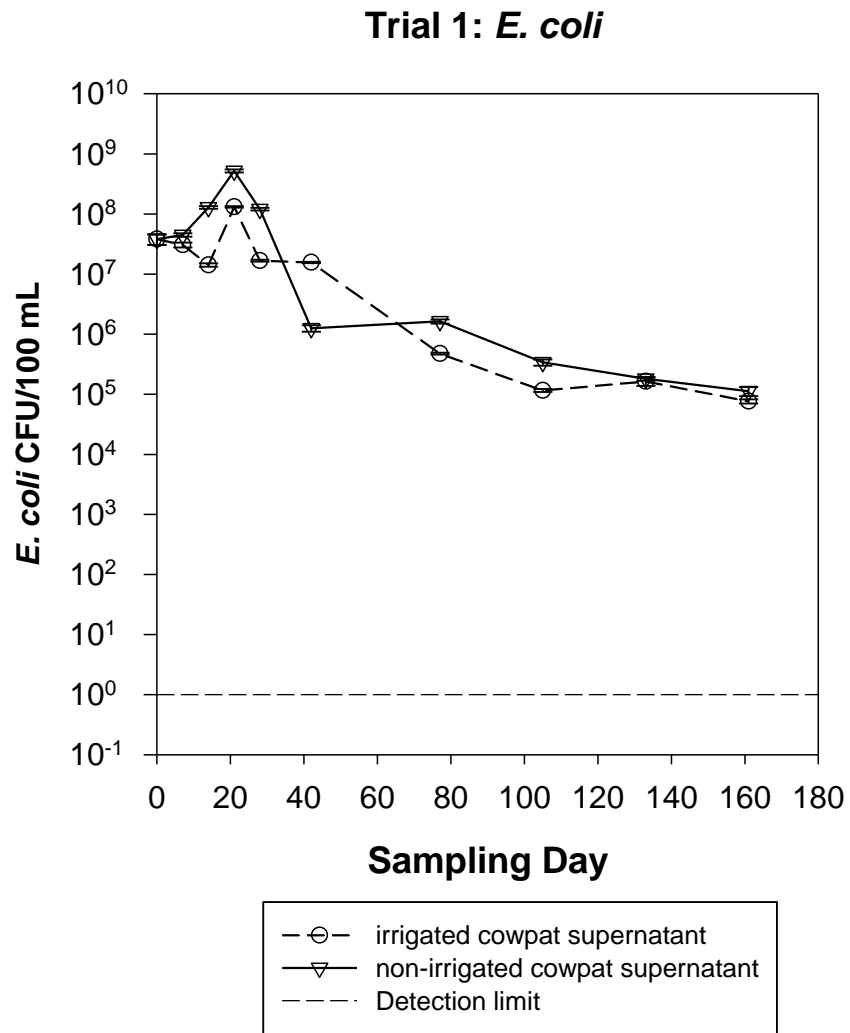


Figure 24: Mobilisation of mean *E. coli* concentrations (\pm SD) in matrices for Trials 1 and 2.

Table 25: Mobilisation rates (k) from cowpats for *E. coli* and PCR marker decay rates in Trials 1 and 2 with coefficient of determination (r^2) and time taken in days for reduction in 90% of concentration (T_{90}) for each analyte. Trial 1 produced monophasic mobilisation decline curves for all analytes. In Trial 2, *E. coli* (supernatant) and CowM2 PCR marker (both matrices) produced monophasic curves. In contrast, GenBac3 and BacR, and *E. coli* (in rainfall runoff only) had two stage mobilisation decline curves and mobilisation rates (k_1 and k_2) were measured over different days as outlined in the footnotes.

	Trial 1						Trial 2					
	IRR			NIR			supernatant			Rainfall runoff		
	k	r^2	T_{90}	k	r^2	T_{90}	k	r^2	T_{90}	k	r^2	T_{90}
<i>E. coli</i>	-0.020	0.88	50.3	+0.055*	0.91	N/A	-0.019	0.89	52	k_1 -0.184**	0.88	5.4
				k_1 -0.023	0.76	43.7				k_2 -0.02	0.74	
GenBac3	-0.054	0.93	18.5	-0.054	0.99	18.5	k_1 -0.140 [†]	0.87	7.2	k_1 -0.228 [†]	0.92	4.4
							k_2 -0.017 [‡]	0.92		k_2 -0.009 [‡]	0.73	
BacR	-0.069	0.97	14.4	-0.060	0.97	16.8	k_1 -0.158 [†]	0.90	6.3	k_1 -0.220 [†]	0.92	4.5
							k_2 -0.016 [‡]	0.92		k_2 -0.008 [¥]	0.53	
CowM2	-0.058	0.96	17.4	-0.057	0.83	17.4	-0.110	0.87	9.1	-0.223	0.91	4.5

*represents *E. coli* **increase in mobilisation** from Day 0 – 21, therefore, k_1 measured from Day 21 onwards for *E. coli* in NIR;

** k_1 in rainfall runoff for *E. coli* measured over Days 1-22, and k_2 over Days 22-162

[†] k_1 in supernatant and rainfall runoff measured over Days 1-29 in GenBac3 and BacR

[‡] k_2 in supernatant and rainfall runoff measured over Days 29-162 and 29-134 in GenBac3 and BacR (respectively)

[¥] k_2 slope does not vary significantly from zero ($p = 0.17$) in BacR rainfall runoff

4.3.4 PCR markers mobilised from cowpats

Trial 1: PCR markers

Comparable concentrations were obtained for the detection of PCR markers in both irrigated and non-irrigated re-suspended cowpat supernatants (Appendix, Table 35). Mean gene copies (GC) for PCR markers on Day 0 in IRR and NIR cowpat supernatant were initially 7.0×10^{10} (SD 2.8×10^{10}) GC/100 mL for the general faecal marker GenBac3 (Figure 25); 1.1×10^{10} (SD 3.8×10^9) for the ruminant marker BacR and 2.4×10^8 (SD 7.1×10^7) for the bovine-associated marker CowM2 (Figure 26).

Concentrations of GenBac3 were still 10^8 GC/100 mL by Day 42 in both IRR and NIR cowpat supernatants but declined more steeply to 10^4 GC/100 mL at Day 77 for the IRR supernatant and similar concentrations by Day 105 for the NIR cowpats. By the end of the experiment on Day 161, concentrations of GenBac3 were 1,200 GC/100 mL but still detectable in the IRR cowpat supernatant only. A similar trend of decreasing concentration was noted for the BacR marker and by Day 77 both irrigation treatments had approximately 10^4 GC/100 mL in the supernatant, with the marker undetected from Day 133 onwards. Concentrations of the bovine marker, CowM2 on Day 42 were 10^5 and 10^6 GC/100 mL, in IRR and NIR supernatants respectively. Thereafter this bovine PCR marker was not detected in either irrigation treatment regime.

Trial 2: PCR markers

Mean GC for PCR markers on Day 1 in re-suspended cowpat supernatant of Trial 2 (Appendix, Table 36) were similar to Day 0 concentrations in Trial 1. Concentrations for GenBac3 were 3.5×10^{10} (SD 3.0×10^9) GC/100 mL (Figure 25); 8.0×10^9 (SD 1.1×10^9) for the ruminant marker BacR, and 1.3×10^8 (SD 1.3×10^7) for the bovine-associated marker Cow M2 (Figure 26). In general, GenBac3 showed a steady decline in copy number from Day 15 (10^9 GC/100 mL) till Day 29 (10^6 GC/100 mL) and then the decline slowed until there were 1.6×10^4 GC/100 mL in the supernatant by the last day of sampling.

Up to Day 15, BacR had supernatant concentrations in the same order of magnitude (10^9 GC/100 mL) as Day 1 but by Day 22 the concentration had decreased by three orders of magnitude. From Days 29 to 71, BacR was stabilised on 10^5 GC/100 mL before decreasing to 10^3 where it remained until the end of the experiment. CowM2 declined by one order of magnitude between Days 1 and 8. On Day 22, the CowM2 marker notably decreased by three orders of magnitude and was no longer detected in the supernatant from Day 71 onwards. There was no result for Day 134 in the CowM2 assay (only) due to contamination of the faecal

extraction control blank (1.2×10^3 GC/PCR reaction of CowM2 marker) but this marker was again below the detection limit in the samples on Day 162.

The blank of sterile MilliQ water which was run through the rainfall simulator prior to each sampling event was monitored for all three PCR markers. The GenBac3 was present in the rainfall runoff blanks only on Day 22 (33 GC/100 mL), BacR on Day 1 (23 GC/100 mL), and CowM2 (122 GC/100 mL) on Day 162 when all other samples were not detected for this marker. Blanks positive for the respective PCR marker were subtracted from the concentration of the runoff sample.

The cowpat rainfall runoff samples showed similar concentrations to the supernatant on Day 1 (Appendix, Table 37). Mean GC for PCR markers on Day 1 were initially 3.6×10^{10} (SD 9.8×10^9) GC/100 mL for the general faecal marker GenBac3 (Figure 25); 7.9×10^9 (SD 3.3×10^9) for the ruminant marker BacR and 9.4×10^7 (SD 5.3×10^7) for the bovine-associated marker CowM2 (Figure 26). As occurred for *E. coli* concentrations in the rainfall runoff, all of the PCR markers exhibited a concentration reduction of three to four orders of magnitude from Day 1 to Day 8. Increasing volumes of rainfall runoff sample were filtered as the experiment progressed to maintain detection of FST markers. By the end of the experiment 600 mL of runoff was filtered for each sample. GenBac3 and BacR showed sequential decline in copy number until Day 29, when both markers had 10^3 GC/100 mL after which they fluctuated within an order of magnitude (10^2 GC/100 mL) until Day 162 when they were not detected. CowM2 was no longer detected in the runoff after Day 22. The standard deviation for CowM2 on Day 22 was large at 1,340 GC/100 mL due to the differences in concentration between the three cowpat replicates. On the same day the cowpat supernatant contained 5.6×10^4 GC/100 mL (SD 7.7×10^4) of CowM2 with a similar variability between replicate cowpats but was still detectable up to Day 50.

4.3.5 Inactivation coefficients for PCR markers

The mobilisation curves of PCR markers after both irrigation treatments in Trial 1 showed monophasic decline curves. The regression coefficients (slopes) between the two irrigation treatments for each of the PCR markers: GenBac3 (Figure 25), BacR and CowM2 (Figure 26) were not significantly different between the two irrigation treatments. The same insignificance was true when the slopes of all three PCR markers were compared within the same treatment regime (either IRR or NIR).

The mobilisation curves for Trial 2 supernatant and rainfall runoff showed two stage mobilisation decline curves for the GenBac3 (Figure 25) and BacR markers (Figure 26) and

monophasic for the CowM2 (Figure 26). The regression coefficients for the initial mobilisation curves of GenBac3, BacR and CowM2 PCR markers were tested within each matrix and showed that they were not significantly different ($p > 0.05$) from each other. In the case of the cowpat rainfall runoff samples, again the initial slopes of the three PCR markers were not significantly different ($p > 0.05$) from each other. The results suggest similar initial mobilisation rates for all three PCR markers within the same treatment, however, the CowM2 marker is below the detection limit after Day 22 in the rainfall runoff, whereas the other two PCR markers show a decrease in rate (k_2) after Day 29 but are still detectable. A comparison of the regression coefficients between the supernatant and rainfall runoff for each of the PCR markers also revealed that the mobilisation rates between the two treatments for individual markers were not statistically different ($p > 0.05$).

T_{90} values calculated for each of the PCR markers are shown in Table 25 for Trial 1 and 2. The time taken for a one log reduction in all PCR marker concentrations in IRR and NIR supernatants was similar with a range of 14.4 to 18.5 days. In Trial 2 supernatant, the PCR marker T_{90} values (< 9.2 days) were lower compared with Trial 1, and T_{90} values in the rainfall runoff (< 5.0 days) were similar to *E. coli* in the same matrix.

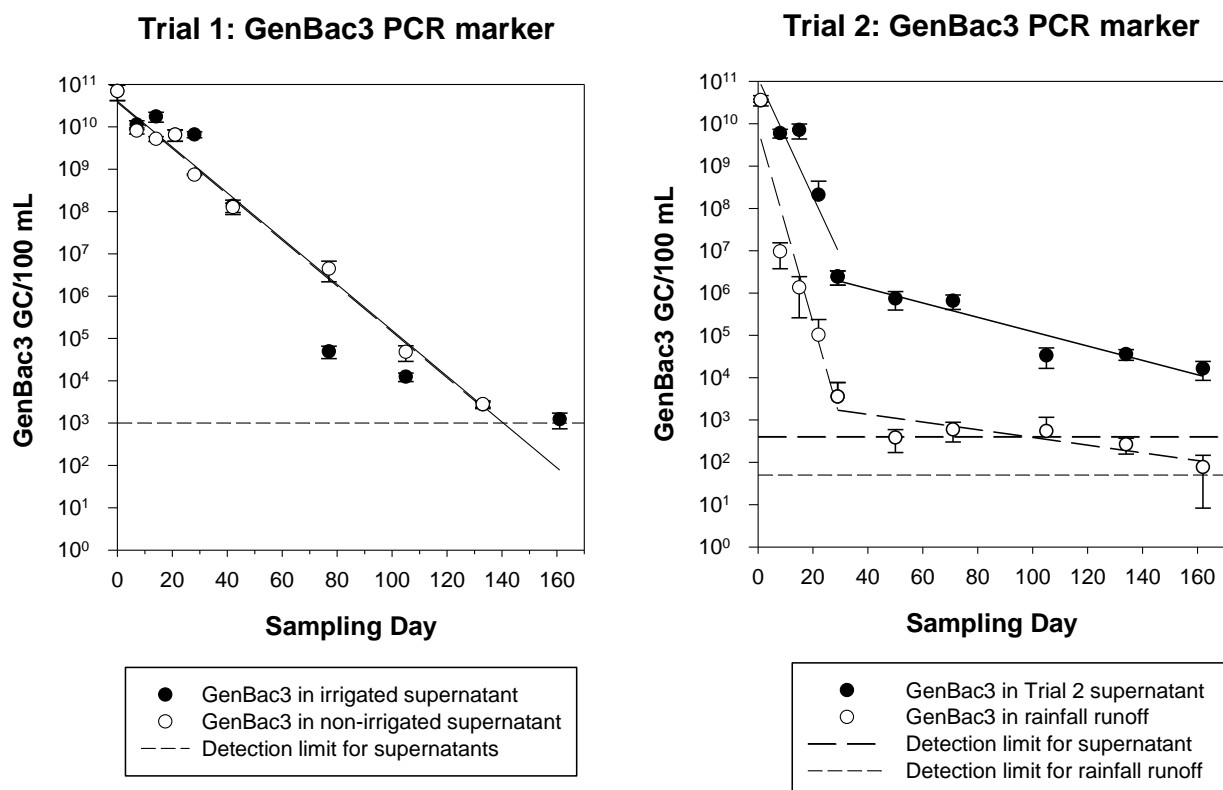


Figure 25: Mobilisation curves for PCR marker, GenBac3 in Trial 1, IRR and NIR cowpat supernatants (2 kg initial wet weight) and Trial 2 supernatant and rainfall runoff from 1kg ww cowpats. Detection limits are represented as the LOD at the end of the experiment, which was dependent on the volumes filtered

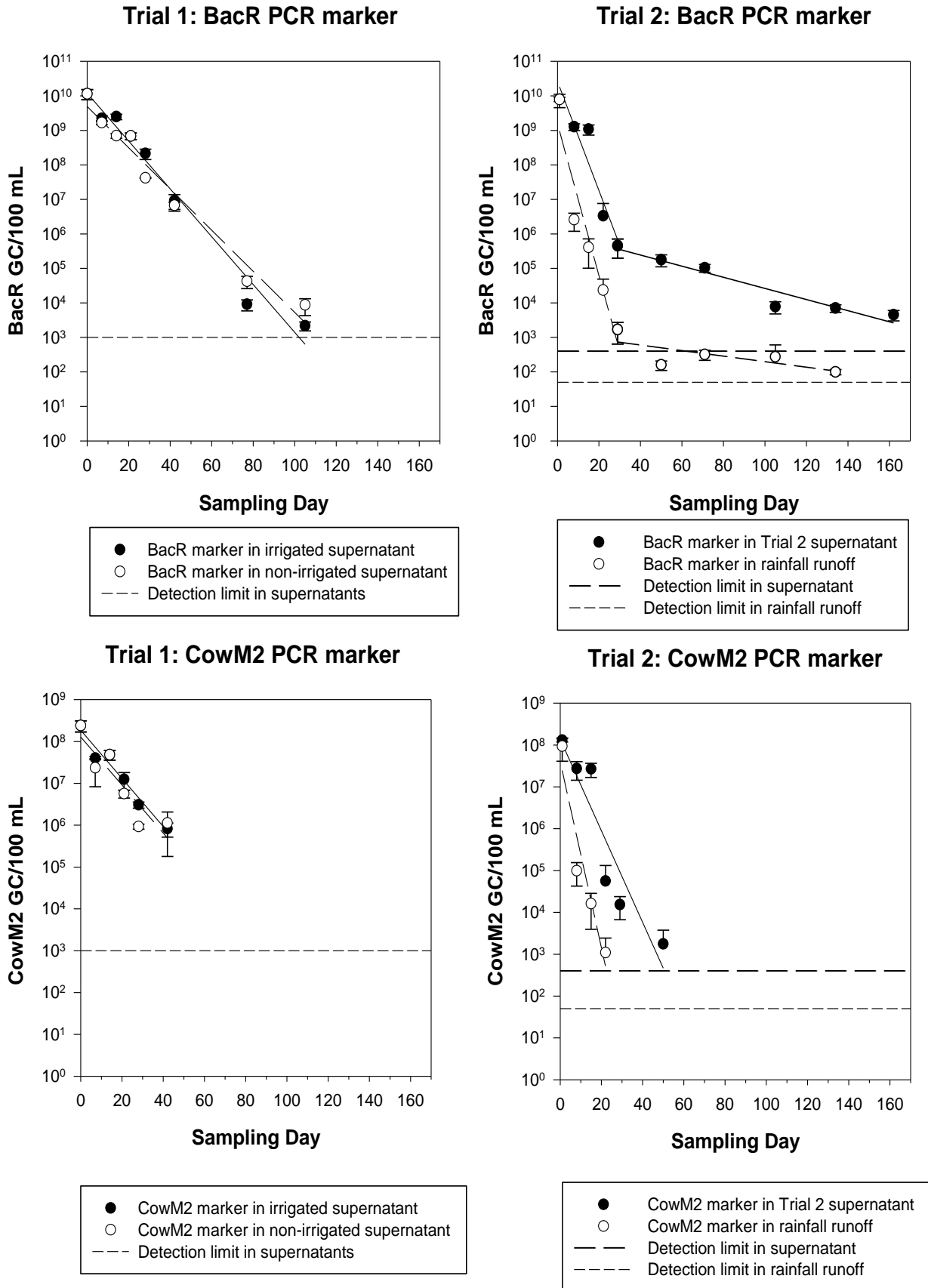


Figure 26: Mobilisation curves for BacR and CowM2 PCR markers in Trial 1, IRR and NIR cowpat supernatants (2 kg initial wet weight) and Trial 2 supernatant and rainfall runoff from 1kg ww cowpats. Detection limits are represented as the LOD at the end of the experiment, which was dependent on the volumes filtered.

4.3.6 Trial 2 only: Faecal Ageing Ratio AC/TC

The re-suspended cowpat supernatant and rainfall runoff were both diluted 1:10 into freshly collected river water to compare the number of total coliforms (TC) and river microflora (atypical colonies (AC)) to estimate the age of faecal inputs to river water. Prior to calculating the AC/TC ratio, the concentration of TC in the non-sterile river water was counted and subtracted from the final concentration of TC in the cowpat inputs diluted into river water. The TC concentrations in the river water used for dilution during Trial 2, had a mean of 2.7×10^3 CFU/100 mL (range 450- 1.4×10^4) prior to adding the supernatant or runoff. *E. coli* in the river water was also higher than expected (based on samples collected prior to Trial 2) with a mean of 915 CFU/100 mL (range 0 to 3.5×10^3).

The faecal ageing ratio, AC/TC, in the supernatant diluted into river water was 0.10 on Day 1 and remained <1.8 till Day 22 suggesting fresh faecal pollution (Figure 27 and Appendix Table 36). Between Days 29 and 105 the AC/TC ratio fluctuated between 3.1 and 6.8, increasing to 55 and 212, respectively on the final two days of sampling.

The AC/TC ratio in the cowpat rainfall runoff (0.11) (Figure 27 and Appendix, Table 37) was similar to the supernatant on Day 1 but by Day 8 it was above 2.0 and fluctuated between this value and 5.8 until Day 50 when it was 56, and *E. coli* was 1200 CFU/100 mL. On Day 71, only one replicate sample had a TC concentration greater than the TC concentration in the river water blank and therefore the AC/TC ratio (5.8) was based on a single sample. From the next sampling, Day 105 onwards, concentrations of *E. coli* were below 30 CFU/100 mL in the cowpat runoff and AC/TC ratios ranged from 4.8 to 126 over these final three sampling events. Using a Student's *t* test for unequal variances, the AC/TC ratios were not significantly different between the supernatant and the rainfall runoff ($p = 0.38$).

4.3.7 %BacR/TotalBac

Total Bacteroidetes was measured by the GenBac3 marker and the percentage of BacR/Total Bacteroidetes (TotalBac) was analysed to see if the ratio changed over time due to differences in survival of the bacteria targeted by the two markers. In fresh faeces the %BacR/TotalBac was 17-23% in the cowpat supernatant and 21% in rainfall runoff (Appendix, Table 35 to Table 37). %BacR/TotalBac incrementally decreased during Trial 1 until Day 28 (3.2%) and Day 77 (1.0%) in IRR and NIR supernatants, respectively, before increasing to 18% prior to BacR becoming undetectable on Day 133 onwards (Figure 28).

In Trial 2, all ratios of %BacR/TotalBac were above 16% (Figure 28) in the supernatant and 21% in the rainfall runoff, with the exclusion of Day 22 in the supernatant (1% BacR/TotalBac). By Day 29 in the supernatant, BacR had returned to 18% of Total Bacteroidetes composition and remained above 16% till the end of the experiment. In the rainfall runoff, the %BacR/TotalBac increased from 21% on Day 1 to range between 28 and 67% until the PCR markers became undetectable on Day 162.

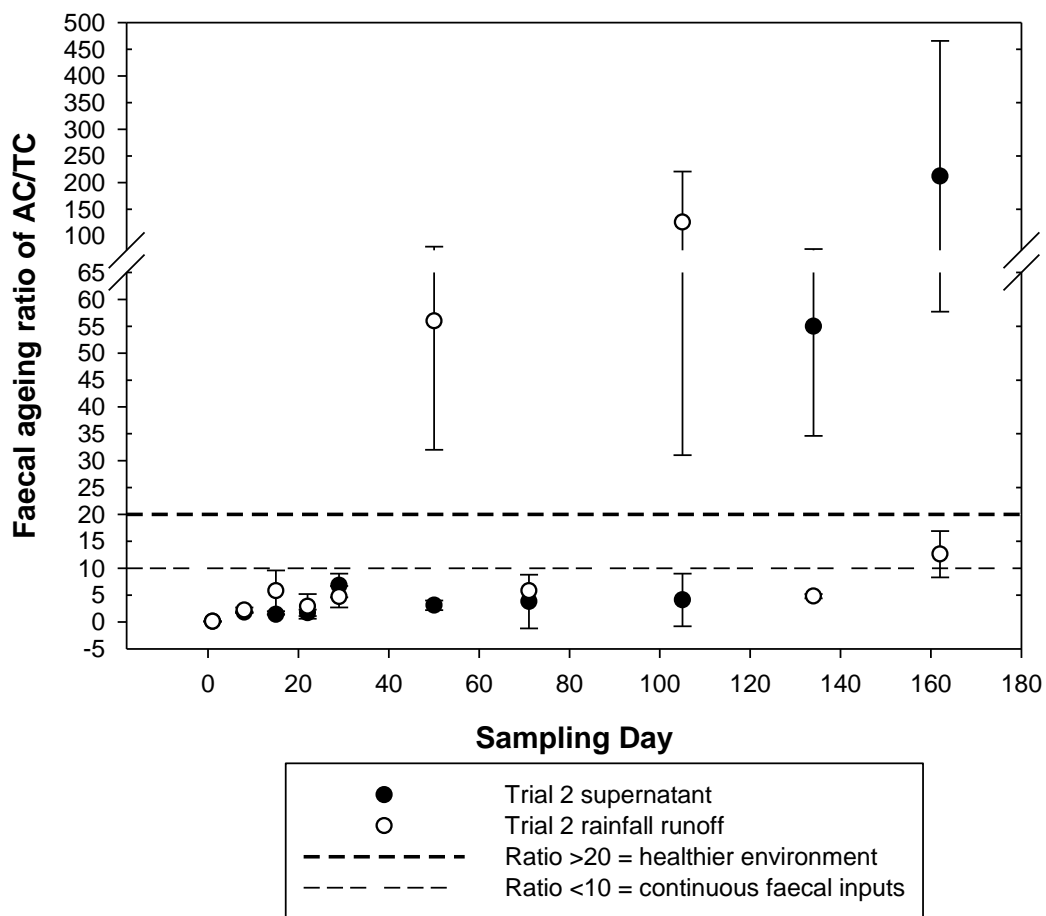


Figure 27: Trial 2 - AC/TC faecal ageing ratio of supernatant and rainfall runoff. The threshold values are taken from research on values of AC/TC ratio in river water (>20) that indicate a healthier environment with less likelihood of pathogen detection (Black et al., 2007), and a ratio of <10 indicative of ongoing faecal inputs (Brion, 2005).

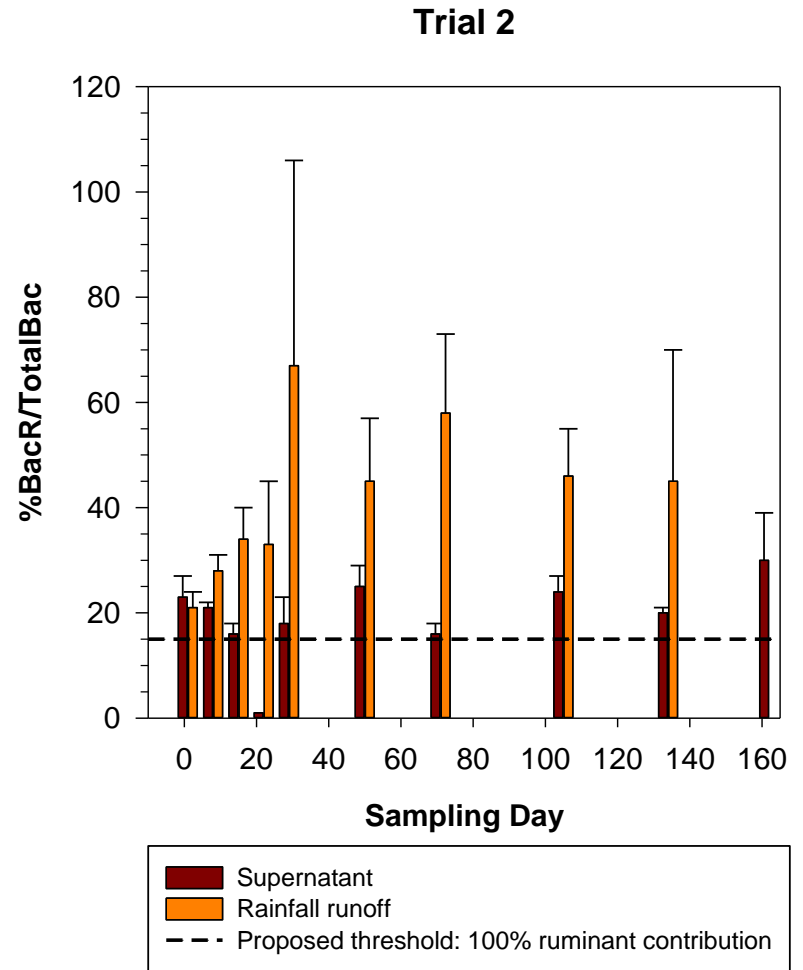
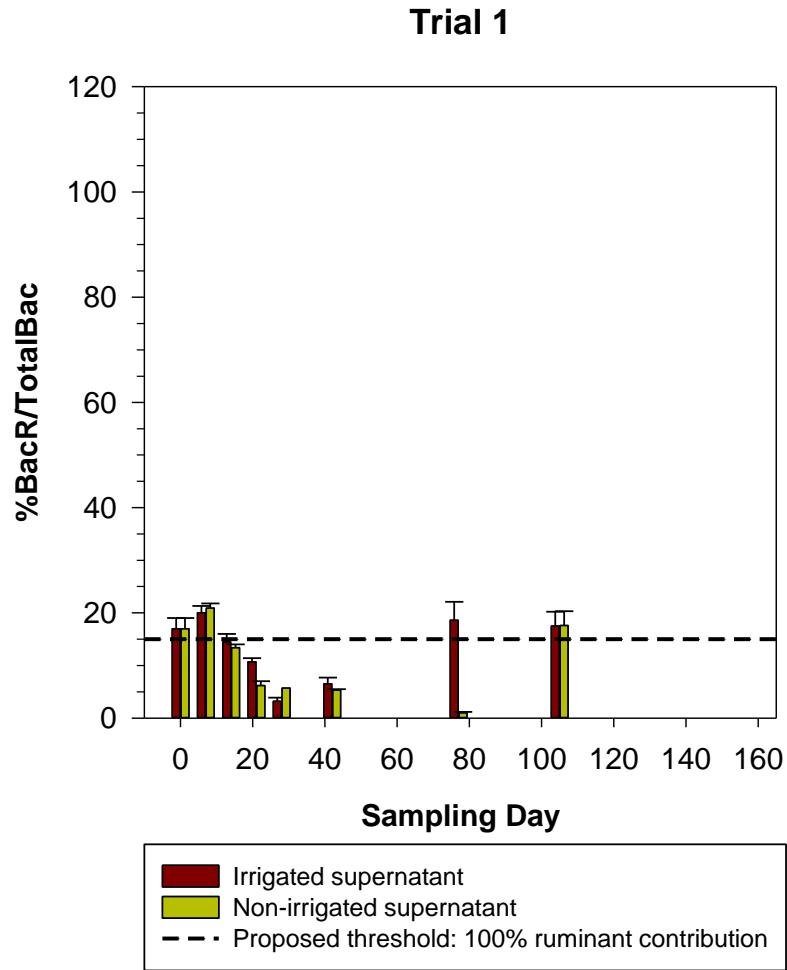


Figure 28: %BacR/TotalBac in Trials 1 and 2. It is proposed that at a threshold of >15% detection of BacR/TotalBac would be indicative of 100% contribution from fresh bovine sources subject to runoff after light rainfall and flood conditions.

4.3.8 Steroids mobilised from cowpats

The major steroids of importance to FST analysis are presented as mean percentages of steroid to total steroids in Figure 29 to Figure 31 with all steroid percentages and FST steroid ratios presented in the Appendix, Table 38 to Table 43. The recommended lower limit of concentration of total steroids in a sample is 2000 ng/mL prior to adjusting the concentration by the volume analysed (Devane et al., 2015). Total steroid levels below this need to be interpreted with caution. There were three sampling events in Trial 1 where the levels were <2000 ng/mL: Day 28 for NIR and Day 42 for IRR and NIR supernatants. It is difficult to determine prior to analysis the volumes of supernatant to analyse especially when dessication of the cowpat reduces mobilisation of steroids. Therefore, the results from these events require caution during interpretation, as it is impractical to repeat sample analysis. All triplicates for each sampling event, however, recorded similar percentages for each steroid and were consistent with trends of the averages of steroid percentages on sampling days either side. The exception was %24-ethylcholesterol, which is discussed in the following results. All samples in Trial 2 had total steroids above 2000 ng/mL prior to adjusting for volume.

The rainfall runoff blank which had been previously tested for PCR markers and *E. coli* was also tested for steroid concentration on six out of ten sampling occasions. The mean steroid concentration in the blank was 162 ng/L (SD 96) and was composed of cholesterol and 24-ethylcholesterol with mammalian-associated faecal steroids such as cop and 24-Ecop at or near the LOD in all blank rainfall runoff samples.

Throughout the two trials, 24-ethylcoprostanol (24-Ecop) was the dominant steroid in all cowpat matrices, with initial mean on Day 0 of 62.0% 24-Ecop/total steroids (SD 8.8) and overall range for all days of 41 to 65% in IRR supernatants and 30 to 62% in NIR (Figure 29). Maximum percentages of 24-Ecop occurred on either Day 0 or 7 and minimum percentages on Day 77 for both irrigation treatments. In Trial 2 on Day 1, percentages of 24-Ecop were 47.0% (SD 7.2) with overall range throughout the trial of 32 to 47% in the supernatant, and mean of 43.6% (SD 3.2) in the rainfall runoff with overall range 16 to 44%. In Trial 2, the minimum occurred on Day 8 in the supernatant, whereas in the rainfall runoff, the minimum occurred on the last day of sampling.

All of the other nine steroids in Trial 1 had overall means of $\leq 11\%$. The plant sterol, 24-ethylcholesterol in NIR did show a large increase to 23 and 16% on Days 42 and 77, respectively, but then dropped back to $\leq 10\%$ for the remainder. Day 42 recorded <2000 ng/mL

total sterols and therefore this high percentage for 24-ethylcholesterol should be interpreted with caution.

In the Trial 2 supernatant and rainfall runoff, excluding 24-Ecop, the overall means of the steroids in the cowpat supernatant generally remained below 11%, similar to Trial 1. Exceptions in Trial 2 supernatants were 24-ethylcholestanol (range 12-18%) (Figure 30) and 24-ethylepicoprostanol (range 12-16%) (Figure 31). In the rainfall runoff, percentages of 24-ethylepicoprostanol generally decreased from 13% and 24-ethylcholestanol concentrations fluctuated within the range 6-13%. In the rainfall runoff, the plant sterol %24-ethylcholesterol increased markedly from a minimum of 7% on Day 1 to a maximum of 28% on Day 105 decreasing to 11% by Day 162 (Figure 30). In addition, 24-methylcholesterol had an overall mean of 3.9% (SD 0.5) in supernatant but 13% (SD 13) in the rainfall runoff. The high variability in the rainfall runoff was affected by identification of 43-63% 24-methylcholesterol in the triplicates collected on the final day of sampling, which was an unexpected increase compared with the previous sampling of <8% (Figure 31). Whether this increase was an anomaly (perhaps due to plant contamination) or would have continued with additional monthly sampling past the six month mark remains unknown.

Using a Student's *t* test to compare between each of the Trial 1 steroids in the IRR and NIR cowpats, a significant difference between the two treatments was observed for cholesterol only ($p = 0.012$). Using linear regression analysis of %steroid/total steroids, the only steroid that showed a significant change in %composition over the course of the experiment was 24-ethylepicoprostanol ($p = 0.028$) in the IRR cowpat supernatant. In Trial 2, the plant sterols (24-ethylcholesterol, 24-methylcholesterol and stigmasterol) were present in higher concentrations in the runoff compared with the supernatant ($p \leq 0.0013$).

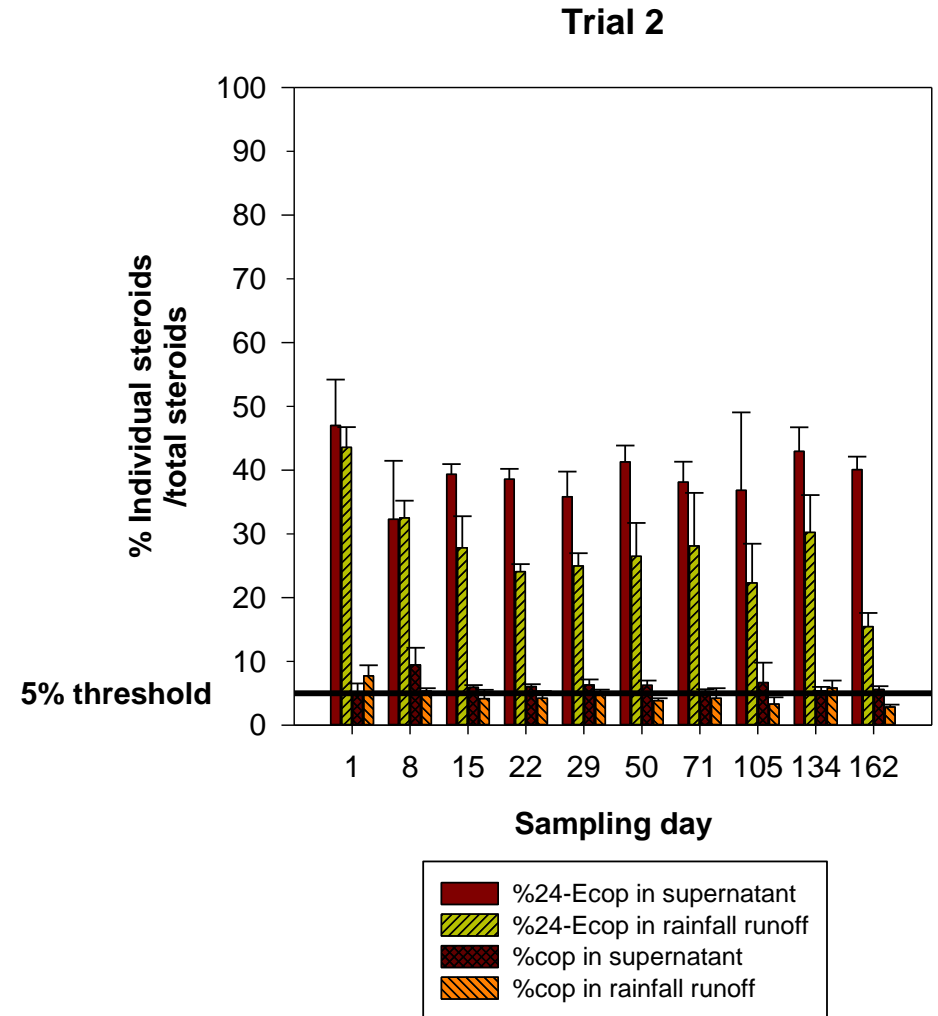
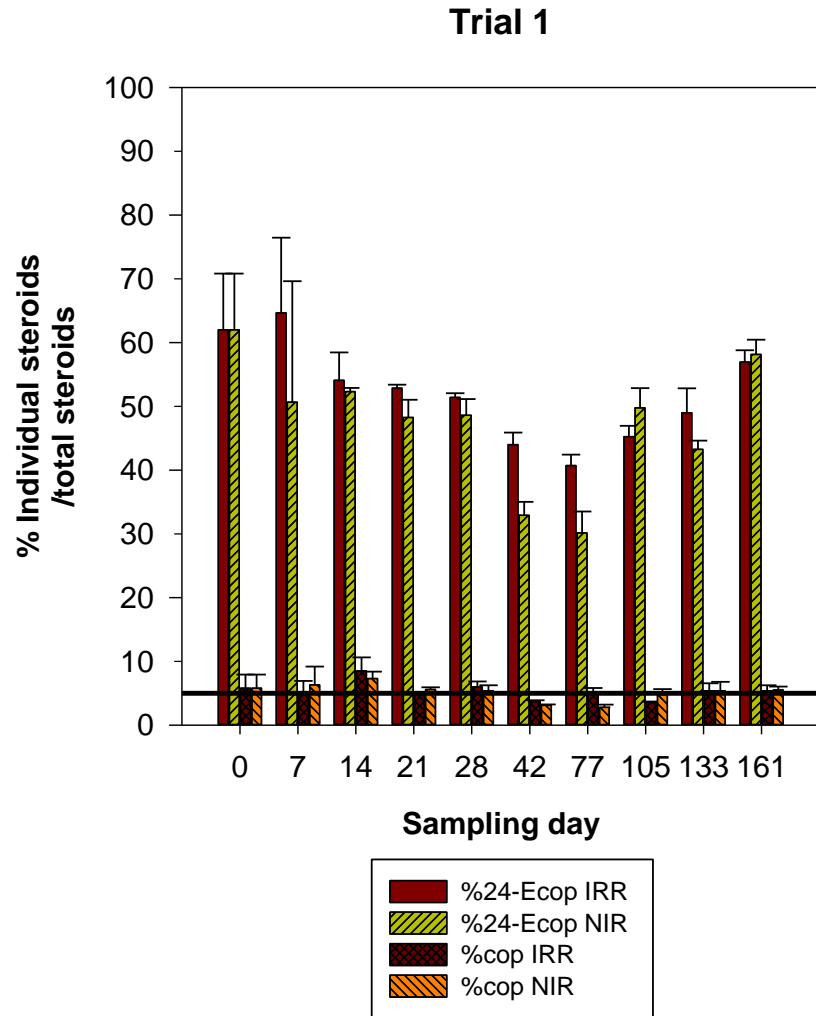


Figure 29: Percentages of mammalian stanols/total steroids important for FST analysis. The threshold of 5% characterises herbivore faecal pollution if 24-Ecop is above 5%; or human pollution if %cop is above 5% and 24-Ecop is less than 5%. In these trials, where both steroids are greater than 5%, then 24-Ecop dominates at a higher percentage compared with %cop, verifying the identification of herbivore pollution.

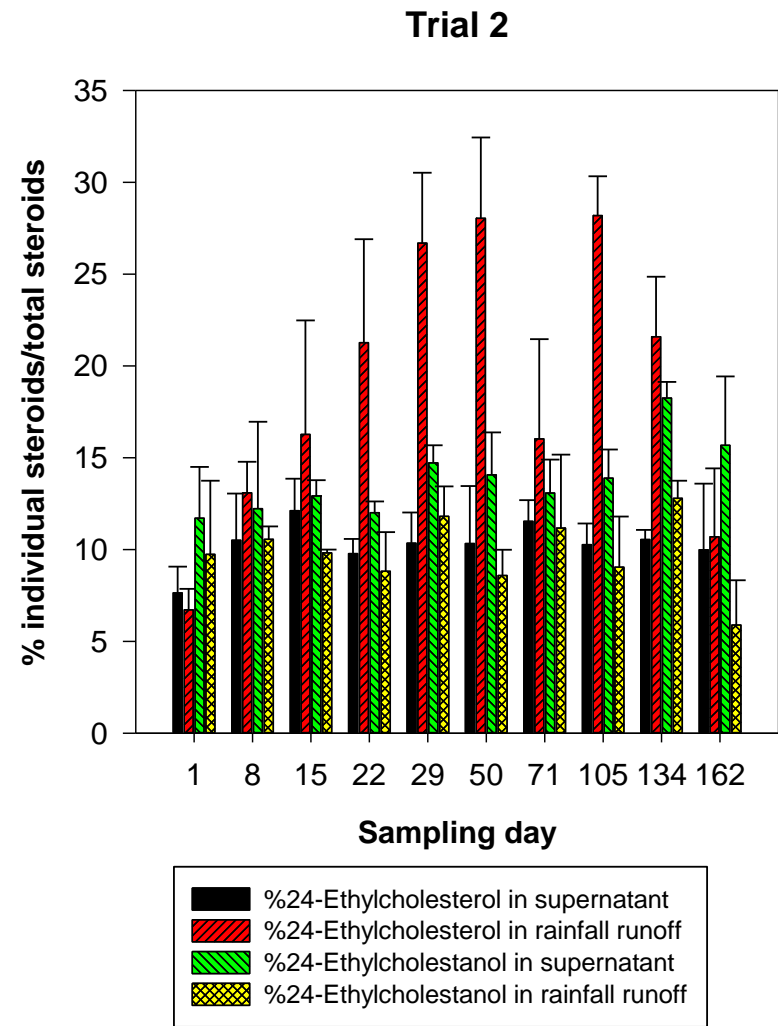
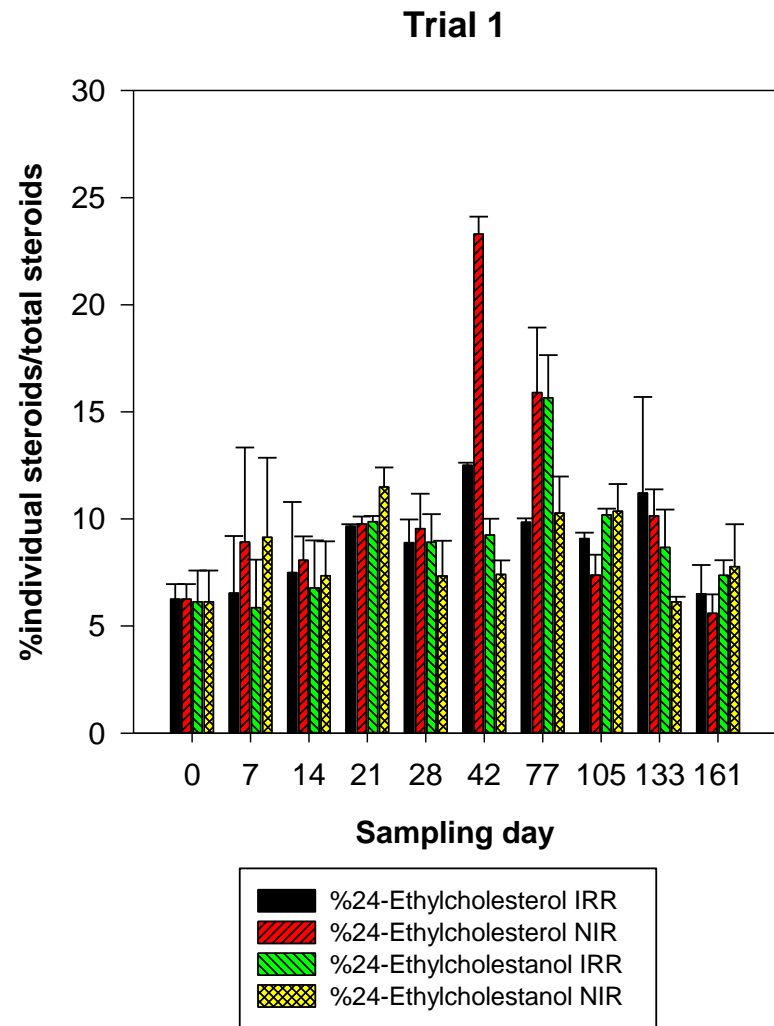


Figure 30: Percentages of plant sterols and stanols/total steroids in mobilised cowpat runoff from Trials 1 and 2. Take note, there is a difference in scaling of axes between the two trials.

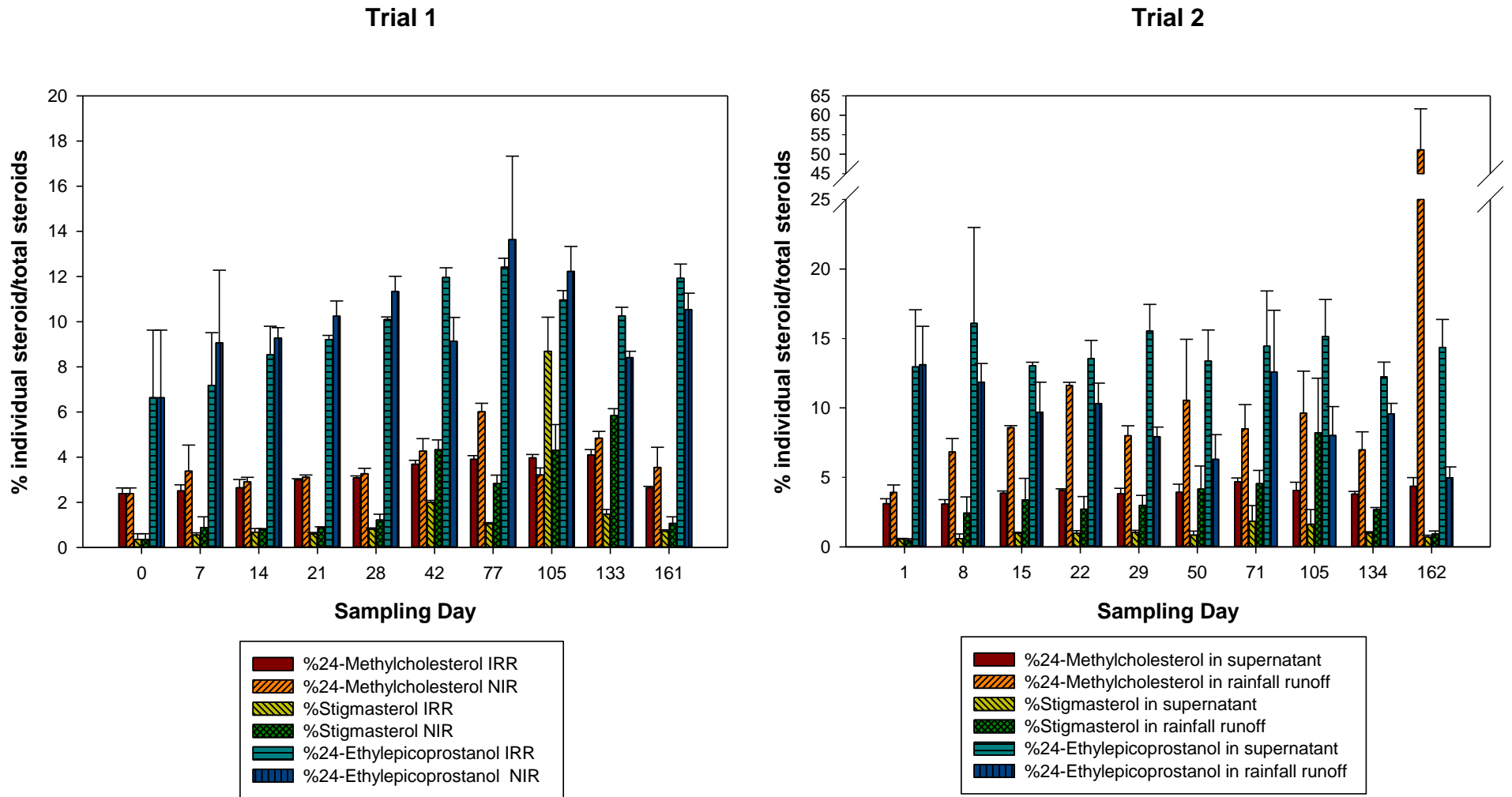


Figure 31: Percentages of plant and bovine steroids/total steroids in mobilised cowpat runoff for Trials 1 and 2. Take note, there is a difference in scaling of axes between the two trials.

4.3.9 Steroid ratios for discriminating faecal sources in cowpat runoff

Steroid ratios used to discriminate between human, herbivore mammal and avian faecal sources were analysed for both trials. Table 3 in Chapter One contains the steroid ratios referred to in this section with respective references. The two general steroid faecal ratios (F1 and F2) identify non-specific mammalian faecal contamination with the criteria for detection for either ratio, being ≥ 0.5 . F1 and F2 were not observed below 1.0 in the cowpat supernatants or rainfall runoff of both trials (Figure 32). In both Trials, F1 and F2 showed minima values during the period, Days 42 to 105, in the re-suspended cowpat supernatants. In addition, the data for FST ratios can be found in the Appendix, Table 39 and Table 40 for Trial 1; Table 42 and Table 43 for Trial 2.

The percentage of the major human steroid, coprostanol (cop, ratio H1) in both trials was on occasion above the 5-6% threshold for identifying human pollution but this was always in association with percentages of 24-Ecop (R1) in excess of 30% (Figure 29). In general, %cop in the rainfall runoff were highest in the first month of the experiment and decreased progressively through the experiment, whereas there was greater fluctuation and no obvious trends in the cowpat supernatants. The R1 ratio measures %24-Ecop/total steroids with percentages $> 5-6\%$ indicative of herbivore sources if the H3 ratio of cop/24-Ecop is < 1.0 . R1 was consistently above 30% in all supernatants and 15% in rainfall runoff (Figure 29). Therefore, at no stage, in either matrix, did R1 fall below the criteria for identifying faecal pollution as derived from herbivores.

The H2 stanol ratio, (cop/(cop + cholestanol)), discriminates between mammalian and environmental sources of coprostanol. In the supernatants of both trials and in the rainfall runoff, H2 identified mammalian faecal sources (H2 > 0.7) on most occasions (Figure 33), and values were always above the truly environmental source threshold of 0.3. However, in all supernatants, particularly mid-experiment, H2 did lower to 0.66, and showed greater fluctuations in the rainfall runoff.

Ratios H3-H5 specifically discriminate between human and herbivore mammals by comparing cop and 24-Ecop, with ratios H4 and H5 apportioning contribution from herbivore and human faecal sources based on these two stanols. For the H3 ratio (cop/24-Ecop), the mean for all supernatants in both trials and in rainfall runoff was ≤ 0.4 , for all replicates, which indicates herbivore faecal contamination (human contamination would be > 0.99) (Figure 33). There was a similar finding for H4 (cop/(cop+24-Ecop)) in all of the matrices where the only source for cop was attributed to herbivore faeces (Figure 34). Therefore, the ratios H5 and R2, which use a formula to estimate animal contributions, indicated 0% human (H5) and 100% herbivore (R2) attribution in all supernatants and rainfall runoff samples as expected for bovine

sources (Appendix, Table 39 to Table 43). The ratio H6, which measures either the identification of human pollution (criterion of >1.5) and/or ageing of human pollution based on cop and its isomer, epicoprostanol (cop/epicop), had mean ratios ranging from 3.3 to 9.4 in Trial 1 supernatants and 3.7 to 6.0 in Trial 2 supernatants and runoff. Although the values of cop are suggestive of human pollution, as explained previously this is in conjunction with cop/24-Ecop ratios indicative of herbivore (bovine) sources.

The ratio R3 (24-ethylcholestanol/cop) is used in association with the H4 ratio (cop/(cop/24-Ecop)) to discriminate between bovine, porcine and human faecal contamination. $R3 >1.0$ indicates bovine pollution and <1.0 suggests either human or porcine, requiring the discriminatory power of H4 ($>60\%$ human, $<60\%$ porcine/bovine). The mean values of R3 in all supernatants and rainfall runoff were similar ranging from 0.9 to 3.8 (Figure 34). All matrices, therefore, were within the limits of the criterion for bovine faecal identification by R3 (>1.0) with one exception on Day 14 in IRR supernatant but even then, H4 was indicating bovine, negating misidentification of the source.

The P1 ratio which discriminates between herbivore runoff (≤ 1.0) and plant runoff (≥ 4.0), was <0.7 in all supernatants from both trials (Figure 35). In the Trial 2 rainfall runoff, mean values of P1 were 0.8 (SD 0.4) with the period between Day 22 and Day 105 recording several occasions where the ratio was >1.0 but well below the threshold for identifying plant runoff. Threshold criteria for determining avian faecal pollution were monitored and mean maximum values of avian sterol ratios remained below the criteria for identifying avian faecal sources throughout the two trials (Figure 35).

The novel ratio of 24-ethylepicoprostanol/24-Ecop (R4) was investigated to see if there was conversion of steroids to 24-ethylepicoprostanol in the ageing environment of the cowpat over five and a half months as has been noted in human faeces with the conversion of cop to epicoprostanol (McCalley et al., 1981). In Trial 1, mean ratios of R4 were initially 11% on Day 1 and 18% by Day 161 with maximums observed on Day 77 (sampling period 7 on the figure) in both IRR and NIR (Figure 36). Although, similar high values for R4 were noted midway in the Trial 2 matrices there was also greater variability in the ratio throughout the trial. Linear regression did not show significant differences in R4 ratios in supernatants or rainfall runoff over the course of the experiment, therefore, the fluctuations in the ratio negated the identification of ageing trends in this ratio for any matrices.

In general, the highest variations in steroids were seen during Days 42-105, with most ratios recording either the maximum or minimum values during this period. These observations prevented the use of any sterol ratios as indicators of an ageing environment, for example,

stigmasterol/24-Ecop in Figure 36. The ratio cop/epicop was observed to be a potential faecal ageing ratio in sediment for human sewage in the urban river study. In this rural study, however, it followed a similar pattern as other steroid ratios in cowpats, preventing its application to signalling an ageing runoff event (Appendix, Table 39 and Table 42).

4.3.10 Correlations between all FST markers mobilised from cowpats

Spearman Ranks was used to generate correlations between non-normal FST marker data. In all matrices, *E. coli* had significant, moderate to strong positive correlations with the three PCR markers (range r_s 0.62 to 0.78, $p \leq 0.004$). *E. coli* had moderate to strong positive correlations with total steroids ($r_s > 0.83$, $p < 0.0001$) and %24-Ecop (r_s 0.65, $p = 0.001$), but only weak correlations in NIR supernatants for %24-Ecop. In rainfall runoff, *E. coli* had significant weak to moderate positive correlations with herbivore and human steroid ratios (%24-Ecop, %24-ethylepicoprostanol and %cop) (r_s 0.43 to 0.53, $p \leq 0.003$).

The three PCR markers were strongly correlated with each other ($p < 0.0001$) at r_s 0.88 to 0.97 in supernatants and rainfall runoff. In all matrices except for the NIR supernatant, PCR markers were moderately to strongly correlated with the total steroids and the herbivore steroid %24-Ecop (range r_s 0.59-0.82, $p < 0.002$). In the NIR supernatant, *E. coli* and the PCR markers were only significantly correlated with the total sterols ($r_s > 0.90$, $p < 0.0001$). There were no obvious patterns of significant correlations between other steroids and the other FST markers.

In Trial 2 supernatant, the AC/TC faecal ageing ratio had significant strong, negative correlations with *E. coli* (r_s -0.85, $p = 0.0001$), and weak to moderate, negative correlations with PCR markers ($r_s < -0.65$, $p < 0.017$). In contrast, there were no consistent significant correlations with steroids or steroid ratios for the AC/TC ratio. In the rainfall runoff, the AC/TC faecal ageing ratio had significant moderate but negative correlations with *E. coli* (r_s -0.57, $p = 0.002$), herbivore and human steroids (r_s -0.60 to -0.74, $p < 0.0003$), and strong but negative correlations with PCR markers ($r_s > -0.82$, $p < 0.0001$). In comparison, AC/TC had moderate, positive correlations (r_s 0.51 to 0.72, $p \leq 0.006$) with P1 and most of the plant sterols.

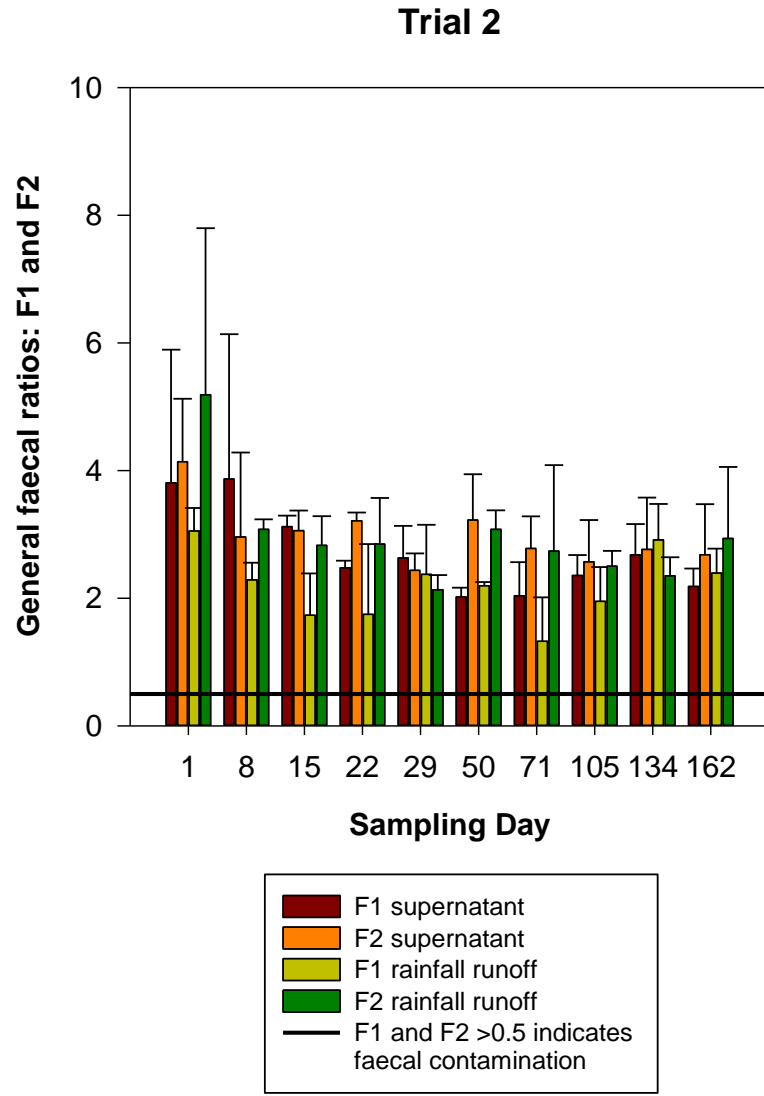
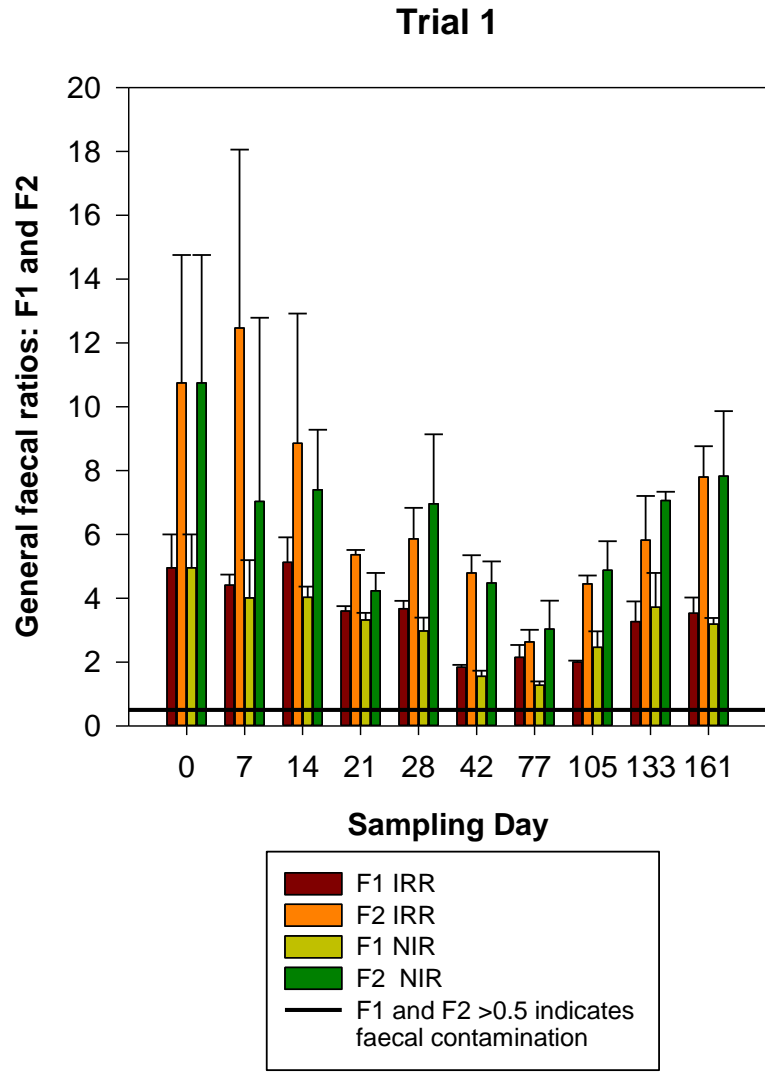


Figure 32: Steroid ratios that identify general (non-specified) faecal contamination; F1: cop/cholestanol; F2: 24-Ecop/24-ethylcholestanol. Take note, there is a difference in scaling of axes between the two trials.

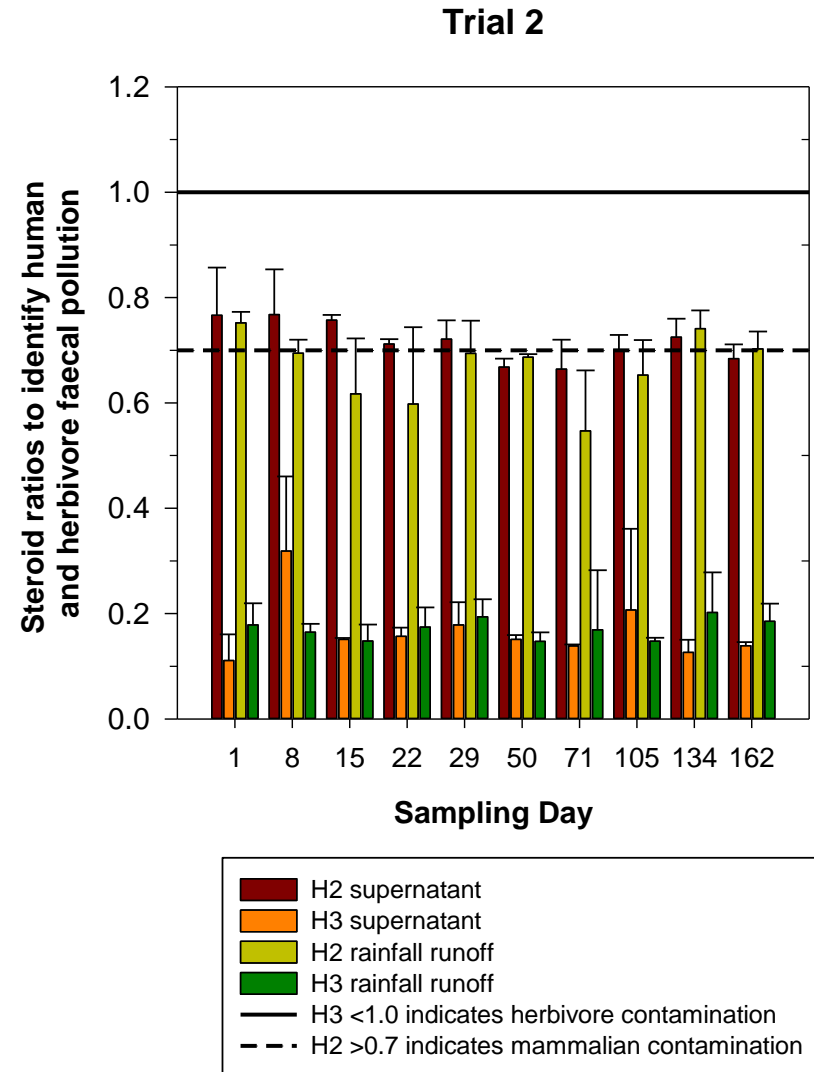
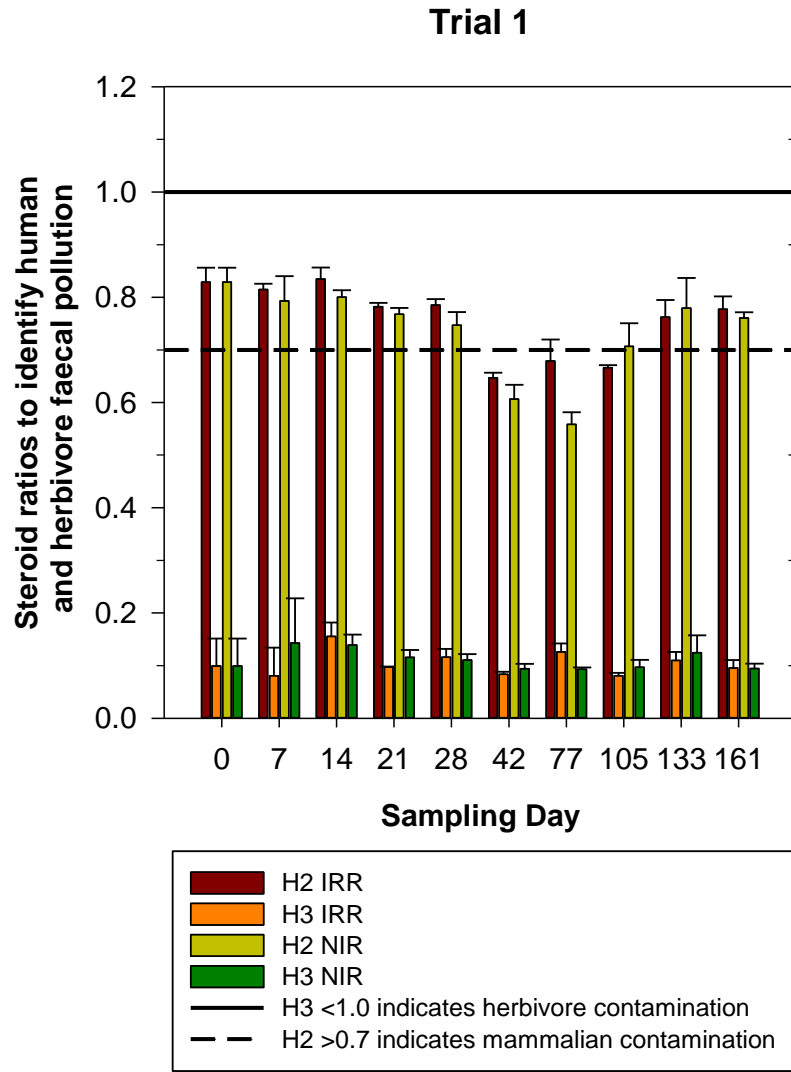


Figure 33: Steroid ratios that identify human and herbivore pollution. H2 ratio: $\text{cop}/(\text{cop} + \text{cholestanol})$ and H3 ratio: $\text{cop}/24\text{-Ecop}$

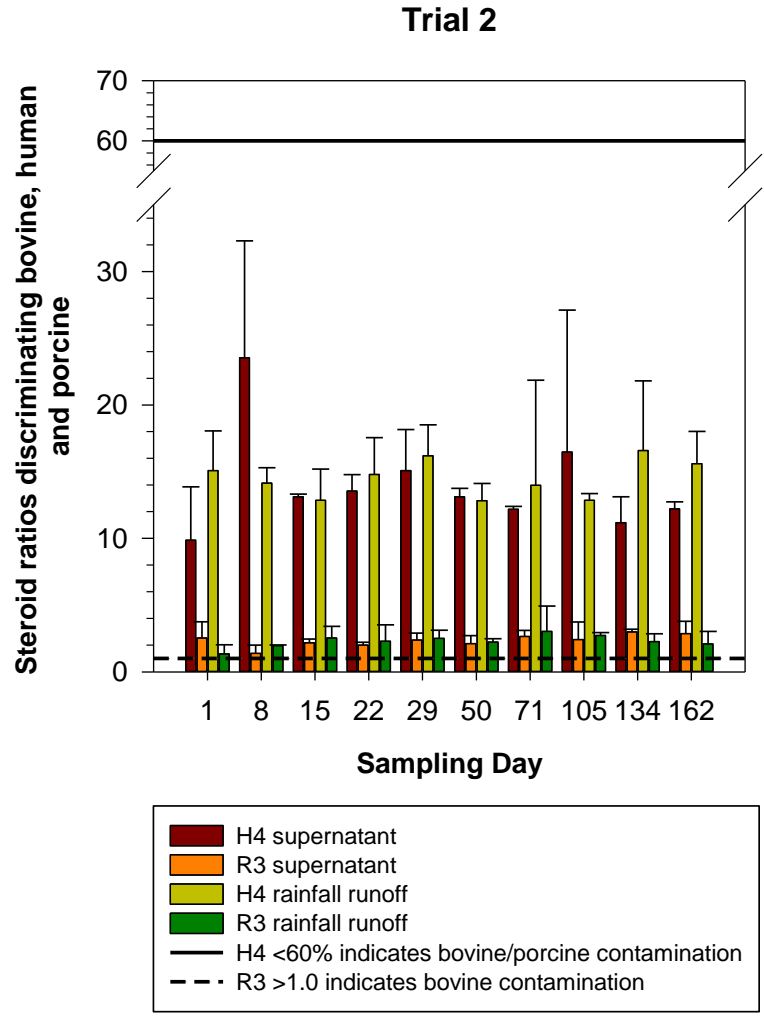
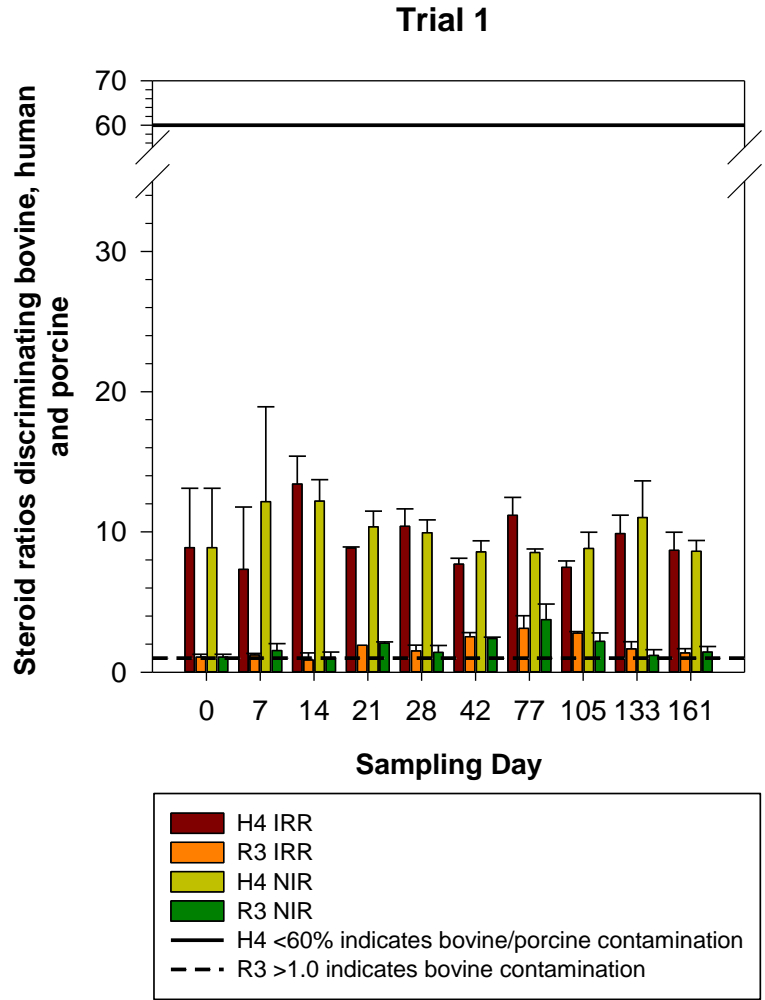


Figure 34: Steroid ratios for discriminating between bovine, human and porcine: R3 (24-ethylcholestanol/coprostanol) and %H4 (cop/(cop/24-Ecop)).

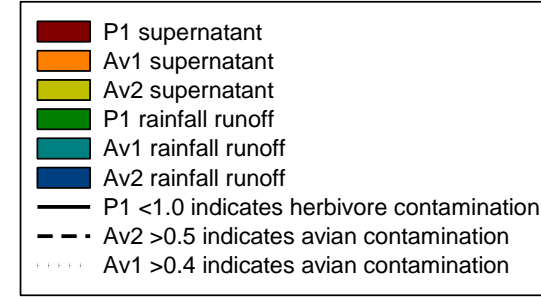
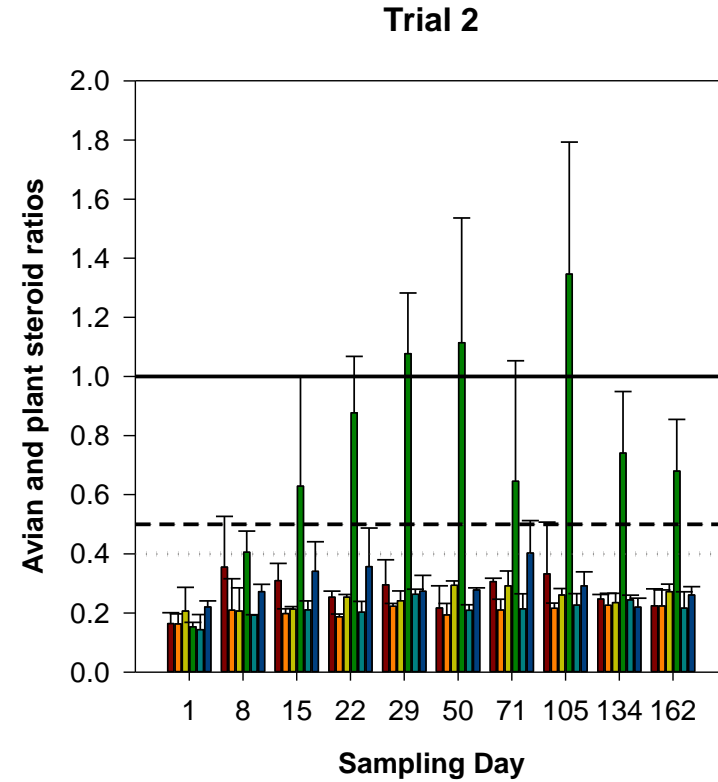
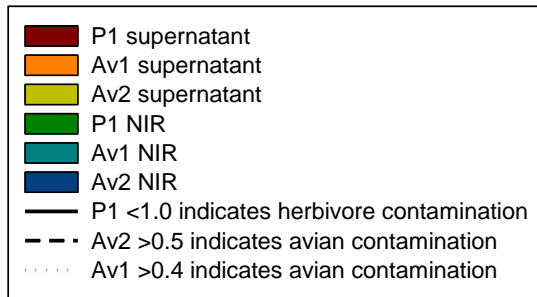
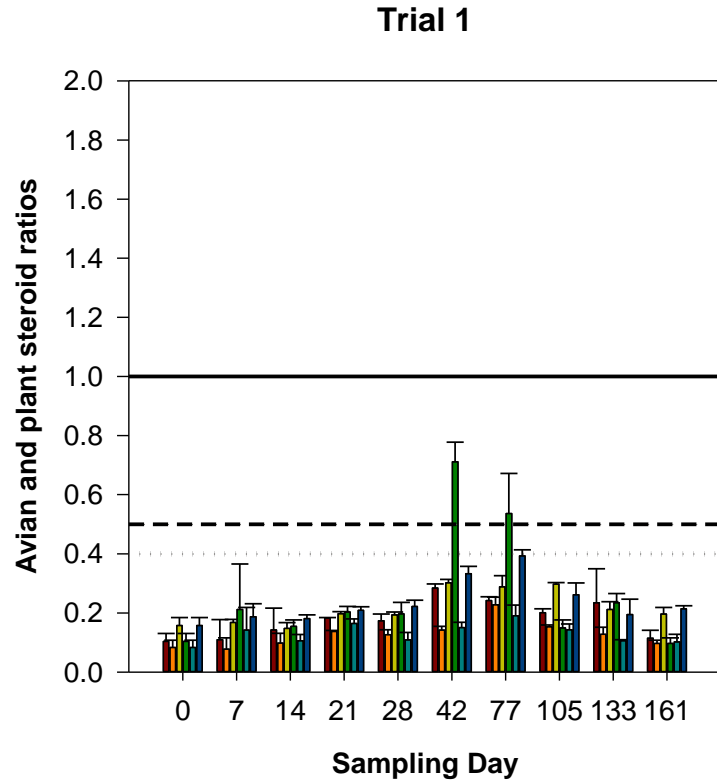


Figure 35: Plant ratio (P1, 24-ethylcholesterol/24-Ecop) and Avian ratios (Av1 and Av2). The P1 ratio must be above 4.0 for the identification of plant rather than herbivore runoff.

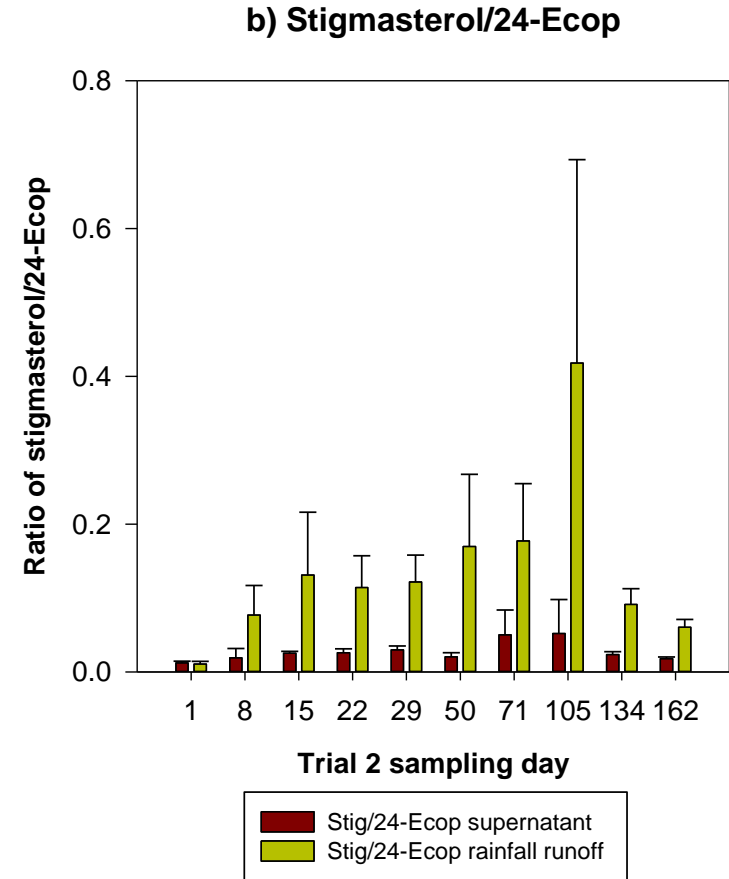
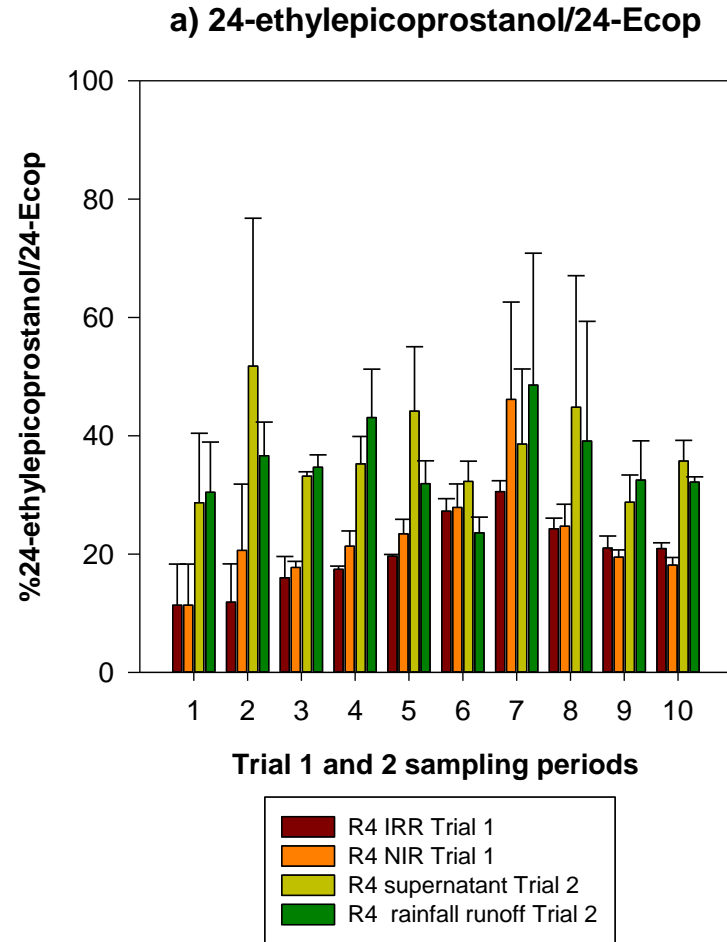


Figure 36: Sterol ratios investigated as potential faecal ageing indicators: R4 (24-ethylepicoprostanol/24-Ecop) and Stigmasterol/24-Ecop. Maximum ratios were observed mid-experiment and negated their use as indicators of ageing.

4.3.11 Inactivation coefficients for steroids

The null hypothesis was tested that steroid mobilisation rates in cowpat supernatants were statistically equivalent between all ten steroids and total steroids within the same treatment trial; and between treatments within each trial. Selected steroids of significance for FST analysis are presented in Figure 37 to Figure 39 as mobilisation decline curves. Tables of all the mobilisation rates (k) and coefficient of determination (r^2) values for decay of steroids in cowpats are presented in Table 26 for Trial 1 and Table 27 for Trial 2.

The mobilisation curves for steroids in Trials 1 and 2 show two-stage curves. In Trial 1, linear regression analysis of the second stage (k_2) of the mobilisation curves for the cowpat supernatant samples, revealed that in general, the slopes of the regression (range 0.003-0.011) were not significantly different from zero ($p > 0.05$) (11 of 11 steroids in IRR and 10 of 11 steroids in NIR). Therefore, in Trial 1, from Day 42 (IRR) and Day 77 (NIR) onwards, the steroid concentrations fluctuated above the detection limit. Initial concentrations of total steroids were 1.5×10^5 ng/mL in both Trial 1 matrices, and by Day 161, the total steroid concentration was 330 ng/mL and 300 ng/mL in the IRR and NIR supernatants, respectively (Appendix, Table 39).

Similarly, In Trial 2, regression analysis of the second stage of the mobilisation curves for the cowpat supernatant and rainfall runoff samples, also revealed that, in general (11 of 11 steroids in the supernatant and 10 of 11 steroids in the runoff), the slopes of the regression (≤ 0.003) were not significantly different from zero ($p > 0.05$). Therefore, in Trial 2, from Day 29 onwards the steroid concentrations fluctuated above the detection limit. Initial concentrations of total steroids were 2.7×10^5 ng/mL in the supernatant and 1.1×10^8 ng/L in the rainfall runoff (Appendix, Table 42). By Day 162, total steroids concentration had decreased to 1400 ng/mL and 8900 ng/L in the supernatant and rainfall runoff, respectively. Mobilisation rates were not calculated for the second stage of the process in either Trials 1 or 2, and the first stage was treated as a monophasic mobilisation decline curve.

In Trial 1, the slopes of the regression analyses were not significantly different ($p > 0.05$) when individual steroids were compared between the IRR and NIR supernatants. There was a similar finding in Trial 2, when individual steroids were compared between the two matrices ($p > 0.05$). This suggested similar mobilisation rates for individual steroids between the supernatant and rainfall runoff.

The slopes of all the steroids were compared within the same treatment regime using the procedure of Zar (2010) to perform a multiple comparison of more than two slopes by

ANCOVA and a significance level of $\alpha < 0.05$. In the IRR treatment, analysis of the individual steroids and the total steroid concentration over Days 1-42 (the first phase of the mobilisation decline curve) showed that they all had statistically similar mobilisation rates. In the NIR treatment, the mobilisation rates of the same steroids and total steroid concentration were also statistically similar (Days 1-77). In the Trial 2 supernatant, and in the rainfall runoff, all steroids (Days 1-29) within each matrix had statistically similar mobilisation rates.

T_{90} values were calculated for each of the steroids and are shown in Table 26 for Trial 1 and Table 27 for Trial 2. The time taken for a one log reduction in total steroid concentration in IRR and NIR supernatants were similar at 34.5 and 33.3 days, respectively. Overall T_{90} values ranged between 25.0 to 45.5 days for individual steroids except stigmaterol, which had much higher values. In Trial 2, the steroid T_{90} values were a lot lower compared with Trial 1, as was observed for the PCR markers (Table 25). All T_{90} values in the Trial 2 supernatant ranged between 13.5 to 16.7 days, with lower values in the rainfall runoff of 7.5 to 10.0 days.

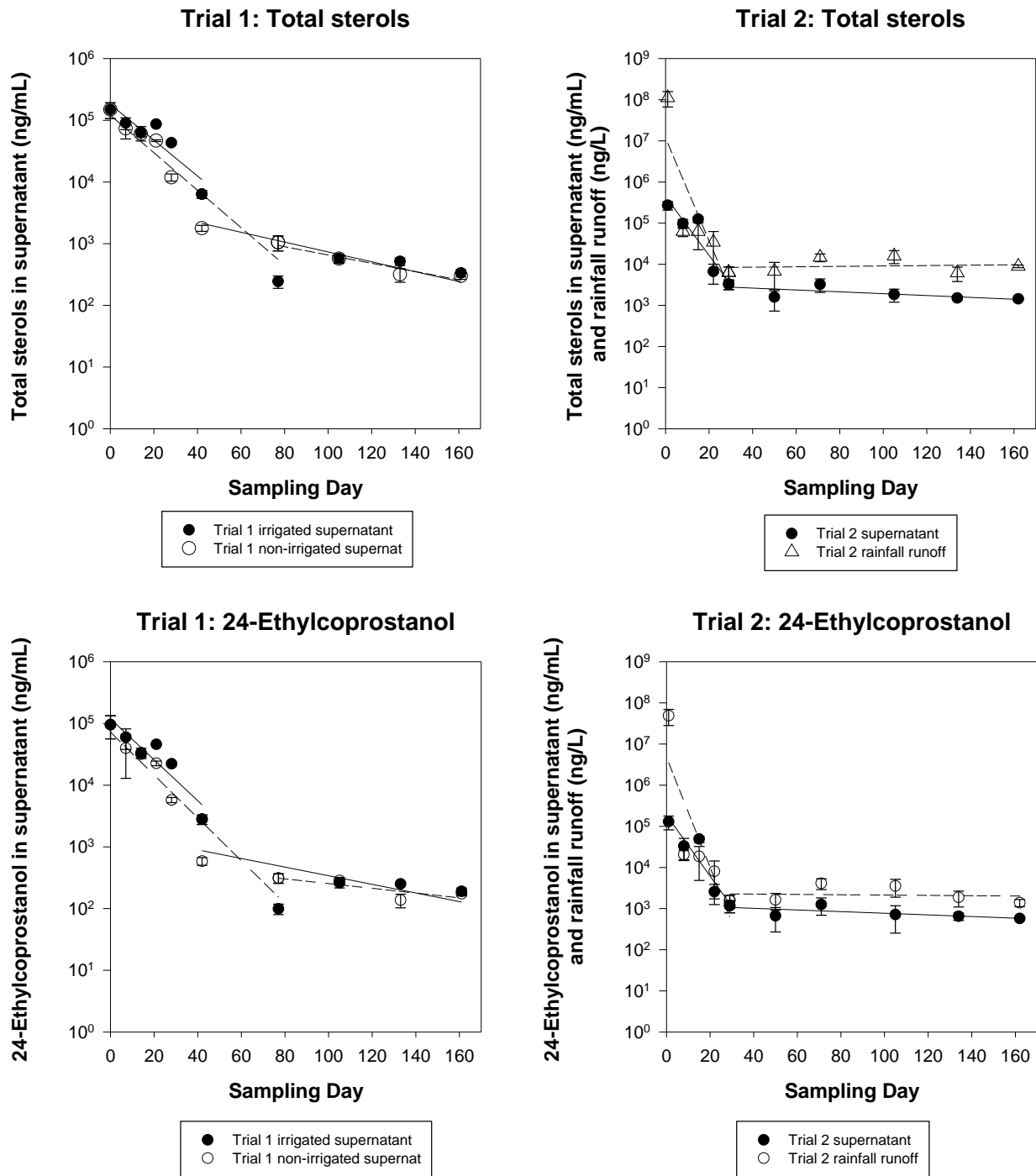


Figure 37: Mobilisation decline curves of total steroids and the major herbivore stanol, 24-ethylcoprostanol in Trials 1 and 2. Note that supernatants are measured as ng/mL and rainfall runoff as ng/L.

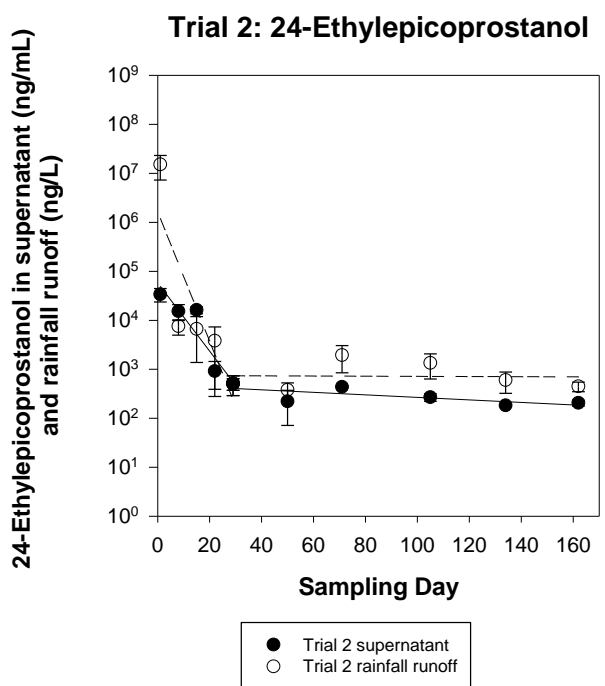
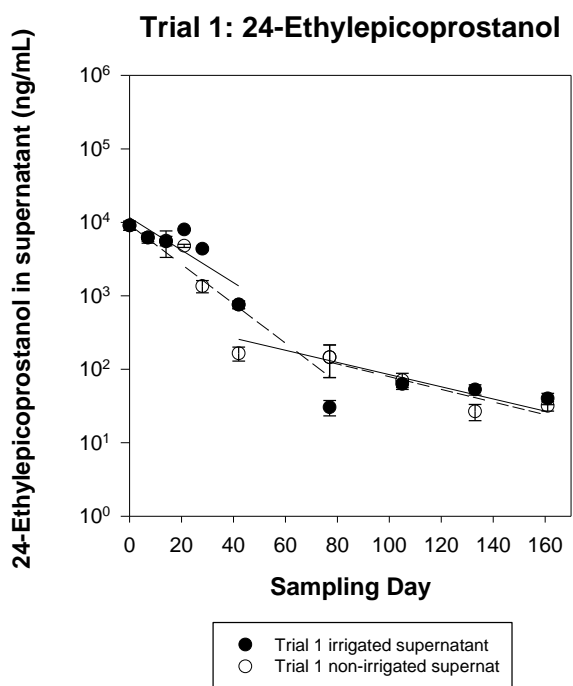
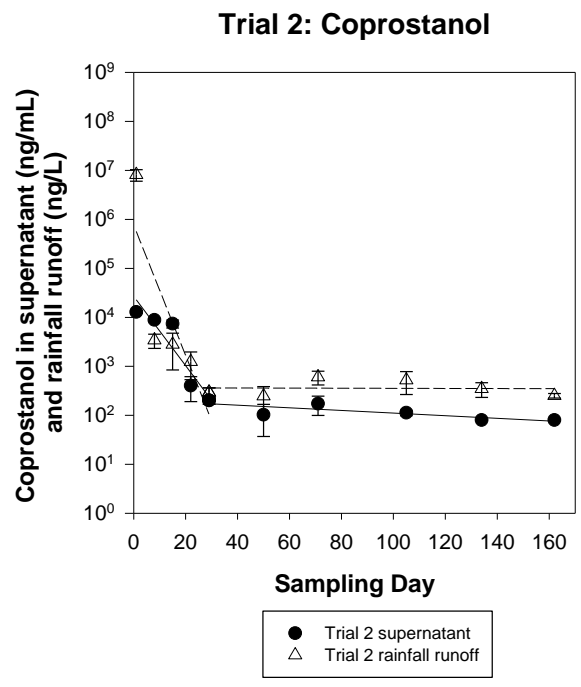
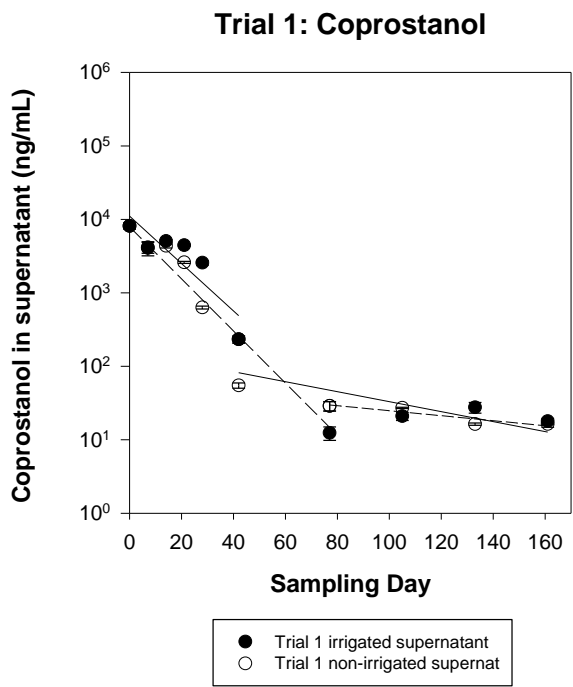


Figure 38: Mobilisation decline curves of coprostanol and 24-ethylepicoprostanol in Trials 1 and 2. Note that supernatants are measured as ng/mL and rainfall runoff as ng/L.

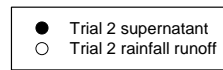
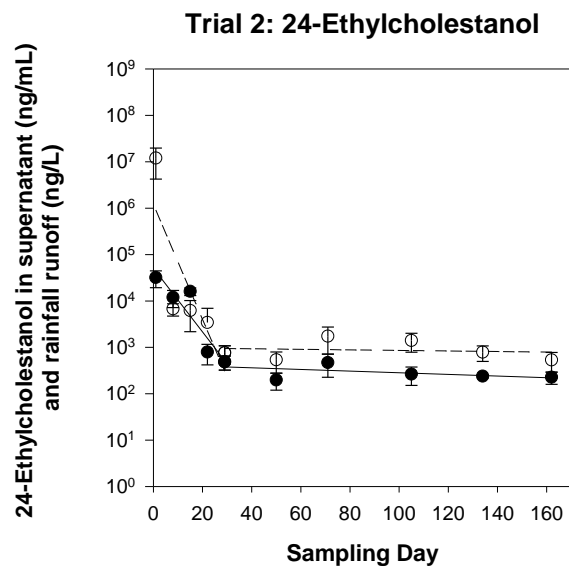
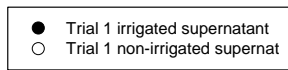
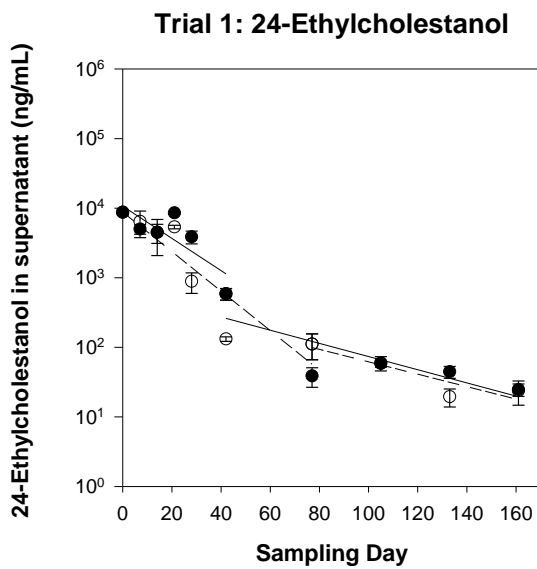
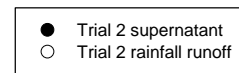
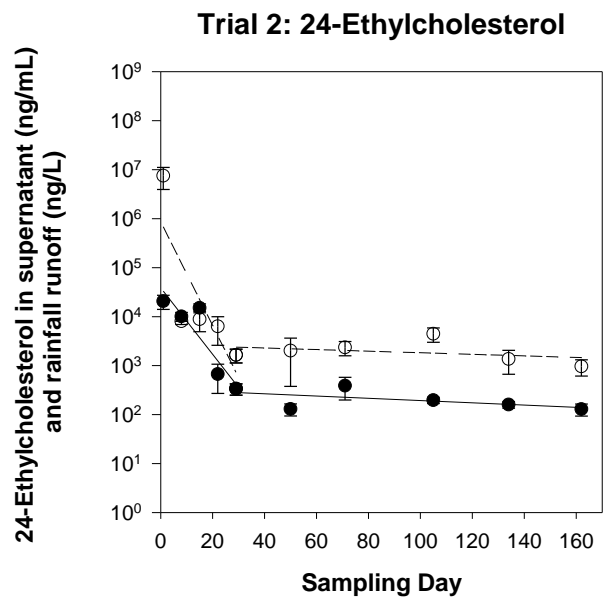
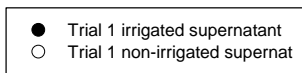
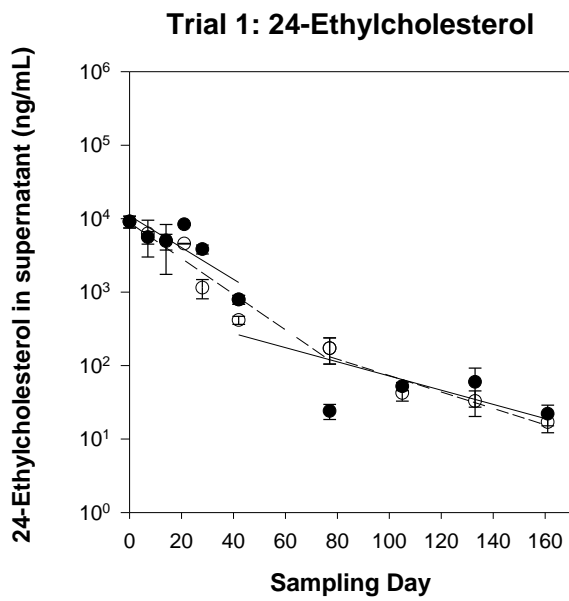


Figure 39: Mobilisation decline curves of plant derived steroids in Trials 1 and 2. Note that supernatants are measured as ng/mL and rainfall runoff as ng/L.

Table 26: Trial 1: mobilisation decline rates of steroids from irrigated and non-irrigated re-suspended cowpat supernatants

	Total sterols		Cop		24-Ecop		Epicop		Cholesterol		Cholestan	
	k* (r ²)	T ₉₀ †	k (r ²)	T ₉₀	k (r ²)	T ₉₀	k (r ²)	T ₉₀	k (r ²)	T ₉₀	k (r ²)	T ₉₀
IRR	-0.029 (0.83)	34.5	-0.032 (0.78)	31.3	-0.033 (0.86)	30.3	-0.025 (0.77)	40.0	-0.026 (0.81)	38.5	-0.023 (0.74)	43.5
NIR	-0.030 (0.89)	33.3	-0.035 (0.88)	28.6	-0.035 (0.89)	28.6	-0.04 (0.89)	25.0	-0.027 (0.87)	37.0	-0.027 (0.87)	37.0
	24-Mcholesterol		24-E-epicop		Stigmasterol		24-Echolesterol		24-Echolestan			
	k (r ²)	T ₉₀	k (r ²)	T ₉₀	k (r ²)	T ₉₀	k (r ²)	T ₉₀	k (r ²)	T ₉₀		
IRR	-0.024 (0.78)	41.7	-0.022 (0.72)	45.5	-0.013 (0.67)	76.9	-0.022 (0.71)	45.5	-0.023 (0.66)	43.5		
NIR	-0.026 (0.85)	38.5	-0.027 (0.84)	37.0	-0.018 (0.92)	55.6	-0.024 (0.92)	41.7	-0.028 (0.83)	35.7		

Table 27: Trial 2: mobilisation decline rates of steroids in re-suspended supernatant (super) and rainfall runoff from cowpats

	Total sterols		Cop		24-Ecop		Epicop		Cholesterol		Cholestan	
	k* (r ²)	T ₉₀ †	k (r ²)	T ₉₀	k (r ²)	T ₉₀	k (r ²)	T ₉₀	k (r ²)	T ₉₀	k (r ²)	T ₉₀
Super	-0.072 (0.87)	13.9	-0.071 (0.86)	14.1	-0.074 (0.88)	13.5	-0.067 (0.85)	14.9	-0.073 (0.89)	13.7	-0.067 (0.87)	14.9
Rainfall	-0.125 (0.71)	8.0	-0.133 (0.72)	7.5	-0.134 (0.73)	7.5	-0.131 (0.71)	7.6	-0.125 (0.74)	8.0	-0.125 (0.70)	8.0
	24-Mcholesterol		24-E-epicop		Stigmasterol		24-Echolesterol		24-Echolestan			
	k (r ²)	T ₉₀	k (r ²)	T ₉₀	k (r ²)	T ₉₀	k (r ²)	T ₉₀	k (r ²)	T ₉₀		
Super	-0.067 (0.85)	14.9	-0.070 (0.87)	14.3	-0.060 (0.78)	16.7	-0.068 (0.82)	14.7	-0.069 (0.85)	14.5		
Rainfall	-0.112 (0.69)	8.9	-0.132 (0.73)	7.6	-0.100 (0.72)	10.0	-0.106 (0.66)	9.4	-0.124 (0.70)	8.1		

*k, Mobilisation decline rate; †T₉₀ measured in days

4.3.12 Trial 1 only: Metagenomic assay of irrigated cowpat supernatants

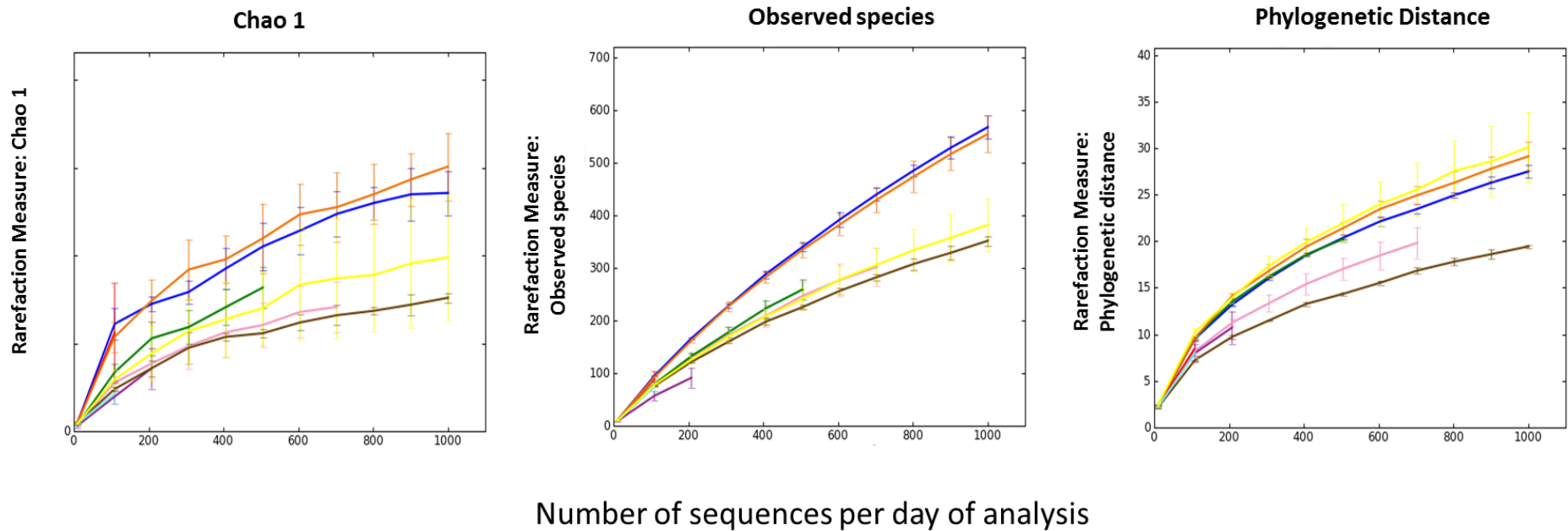
The similarities in marker decay between the two irrigation regimes from Trial 1, led to the decision to process only the irrigated cowpat supernatant samples for an amplicon-based metagenomic study. This pilot study investigated the microbial communities of the decomposing cowpats by targeting the V1-V3 hypervariable region of the 16S rRNA gene. A total of 287,541 sequences were generated by pyrosequencing the DNA extracts of 30 samples on a Roche 454 GS FLX sequencing platform. The 30 samples consisted of the triplicate samples collected from each irrigated cowpat supernatant analysed on ten sampling days during Trial 1. The combined runs from two sequence batches contained 225,878 sequences after hamming barcodes were removed and files concatenated, and quality files removed those with quality Phred scores below 25. On average 4.6% (SD 2.1%) of sequences were removed from each of the 30 irrigated samples due to identification of potential chimeras, leaving a total of 216,500 sequences for analysis. There was a wide variability in the number of sequences generated by each sample with a mean of 7217 and a range of 23 to 32,123 sequences/sample. The average read length for each sequence was 458 base pairs, and 116,203 OTUs were identified and analysed for microbial community diversity. The negative controls of faecal extraction blanks from the sampling events did not produce amplicons when amplified with eubacterial primers Bac8F and Univ529R, and consequently, were not sent off for sequencing.

Diversity analyses

Three methods were used to calculate the microbial diversity within a sample, which is defined as the alpha (α) diversity, and reflects the diversity based on the abundance of taxa within that sample. The first two methods for α -diversity were based on assessing microbial diversity using species-based qualitative indices (Chao1 and Observed species) (Chao, 1984). The third method used a scheme relevant to molecular analyses of sequence and is a qualitative divergence-based index (Phylogenetic distance) based on the sum of the branch lengths that separate two microorganisms in a phylogenetic tree (Lozupone and Knight, 2008).

The alpha diversity of the individual irrigated cowpat supernatant samples is displayed in Figure 40. A sampling depth of 1000 was used for alpha diversity measures to identify whether sufficient sequencing depth had been achieved and hence most of the microbial diversity within a sample captured. Day 133 had low mean numbers of sequence and is not represented in this diversity figure. Rarefaction plots for each sample analysed ($n = 30$) are presented in Figure 41 to show the numerical depth of sequence sampling for the majority of cowpat faecal samples.

Similar to α -diversity, a number of methods can be used to define beta (β) diversity which seeks to measure the number of sequences shared between different samples. These methods can use qualitative measures based on the presence/absence of data to compare the microbial community between samples; and/or quantitative measures, which take into account the relative abundance of each microorganism based on the number of sequences representing that organism. In this study, the qualitative method of unweighted unique fraction metric (UniFrac) is presented in Figure 42 as a plot of the principal co-ordinate analysis (PCoA) of the β -diversity between the irrigated cowpat supernatant samples over time. Some of the sampling events are only represented by two replicates in the β -diversity plot because the other sample contained a low number of sequences (<540). The first coordinate of the PCoA accounted for 38% of the diversity, with the next two components contributing 8.8% for PC2 and 5.3% for PC3. Ordination of samples by PCoA indicated that they clustered according to the day of sampling, and sequentially with the progression of time from fresh to aged cowpats.



Colour Key for Day of analysis									N/A	
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 42	Day 77	Day 105	Day 133	Day 161

Figure 40: Rarefaction plots to evaluate alpha-diversity of microbial communities in irrigated cowpat supernatants Days 0-161. Day 133 had low mean numbers of sequence and is not represented in this diversity figure.

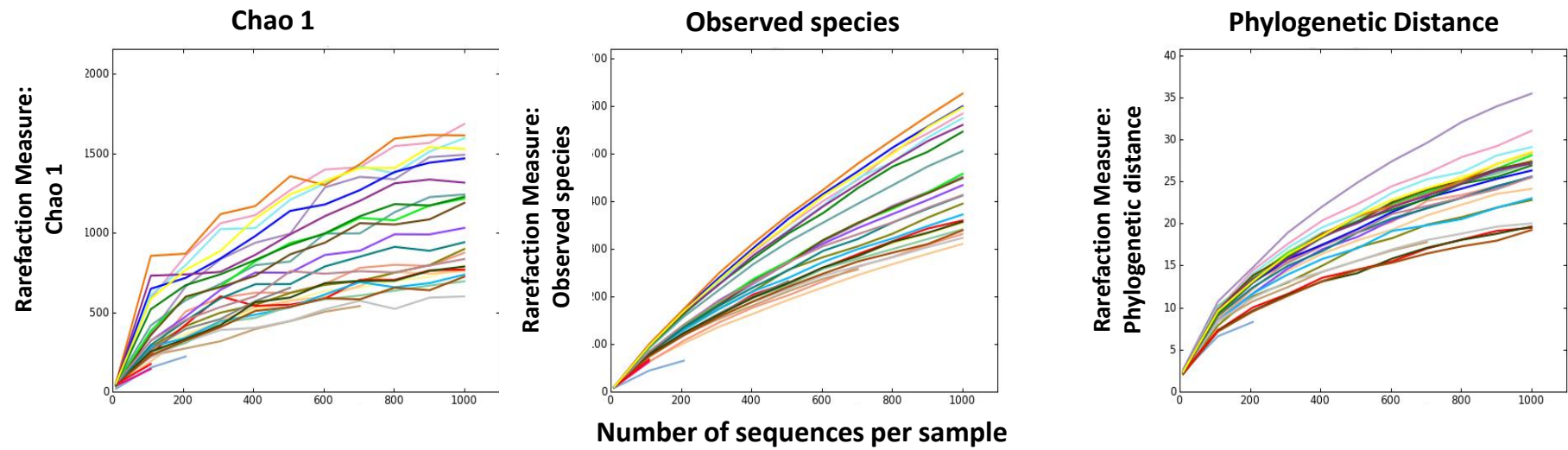


Figure 41: Rarefaction plots to evaluate alpha-diversity of microbial communities in irrigated cowpat supernatants for each sample analysed (n = 30). There is no key to identify samples as this complex figure is provided to show the depth of sampling for the majority of samples.

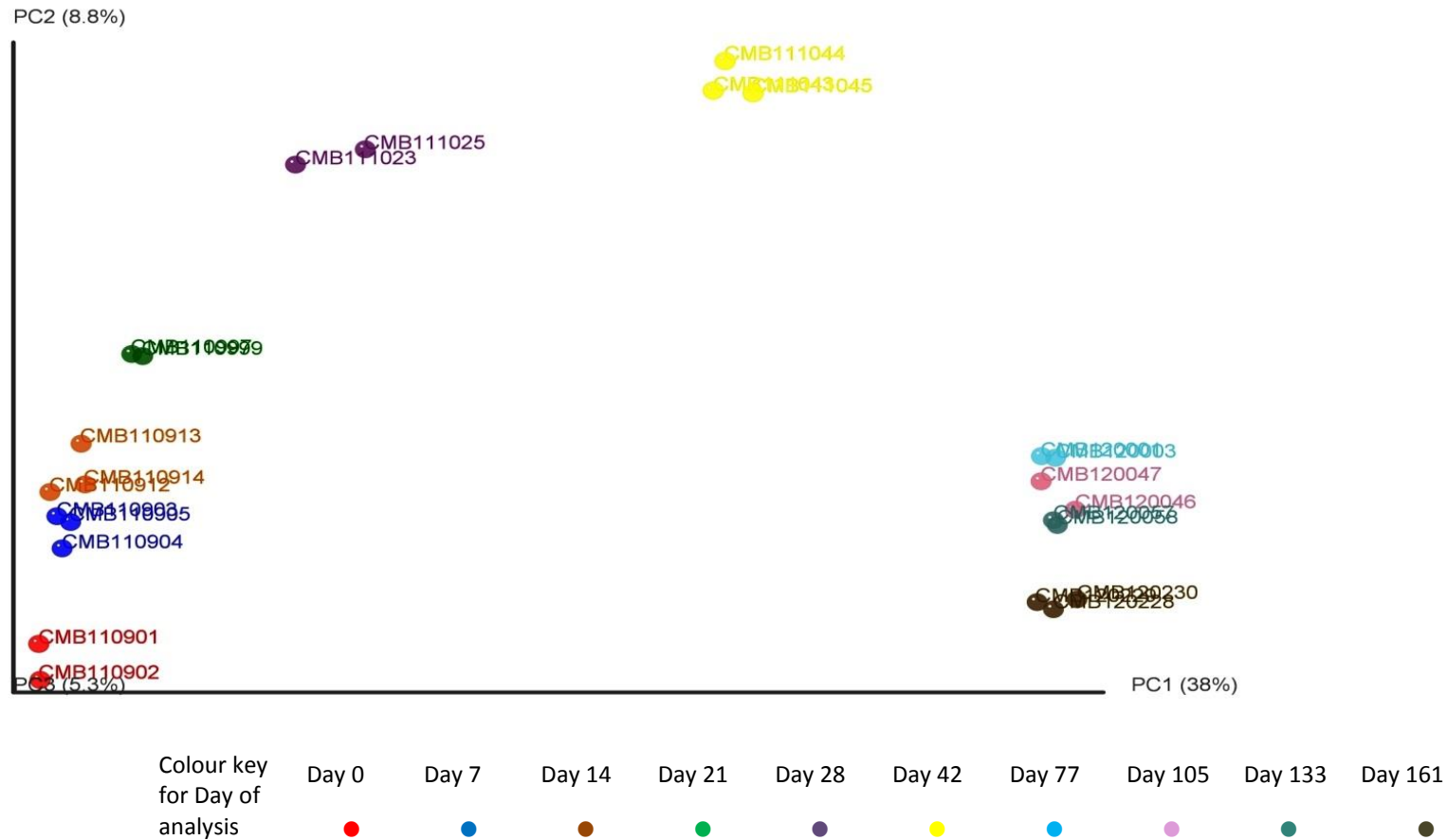


Figure 42: Unweighted UniFrac analysis of beta-diversity by Principal coordinate analysis of microbial communities identified in samples collected from irrigated cowpat supernatant over a five and a half month period.

4.3.13 *Microbial Taxa identified in decomposing cowpats*

Bacterial Phyla in decomposing cowpat

There were 29 Phyla identified in the irrigated cowpat supernatant over a five and a half month period and the major bacterial Phyla are presented in Figure 43. On Day 0, the Phylum Firmicutes was represented by the highest mean percentage of sequences at 50% (SD 10%), with Bacteroidetes the next most common phylum at 38% (SD 9%), followed by the Tenericutes, 6% (SD 2%) and Proteobacteria, 3% (SD 2%). All other phyla were present at $\leq 1\%$ of the sequences and included Lentisphaerae, Spirochaetes, Cyanobacteria, Fibrobacteres, Actinobacteria, Verrucomicrobia and Planctomycetes.

The Tenericutes increased to 17% (SD 2%) by Day 28, but then dramatically reduced to $<0.05\%$ from Day 77 onwards and were undetectable by the last day of sampling. The Phyla, Firmicutes, decreased over time with a concomitant increase in Proteobacteria and Actinobacteria as the cowpats aged. Actinobacteria were present on Day 0 with a mean number of sequences of 0.04% (SD 0.05%) and remained at less than 1% until Day 42 when there was a shift in its dominance to representing 18% (SD 4%) of the Phyla sequences and from then onwards stabilised on 25-26% for the remainder of the experiment. In comparison, the Firmicutes declined, after Day 21, to 18-22% on Days 28 and 42 before steadily decreasing over the remaining sampling events to 7% (SD 2%) on the last day. Members of the phylum Bacteroidetes were a dominant part of the microflora over the entire sampling period. The lowest percentage of sequences for Bacteroidetes was on Day 42 (27%, SD 5%) but increased again to reach 43% (SD 3%) by Day 161.

Bacterial Orders in decomposing cowpat

Figure 44 presents the Orders of bacteria with 167 taxa identified at the Order level, with only eight of these taxonomic units represented by $>1\%$ of the sequences on Day 0. The Order, Clostridiales, which belongs in the Firmicutes phylum, was represented by the highest mean percentage of sequences 46% (SD 10%) on Day 0 and decreased from Day 28 onwards till it reached 5% (SD 1%) of sequences on Day 161.

The Order Bacteroidales in the phylum Bacteroidetes was represented by 38% (SD 9%) of sequences on Day 0, and decreased sharply from Day 42 (17%, SD 2%) onwards to $<1\%$ by Day 161. However, other members of the same phylum of Bacteroidetes: the Flavobacteriales and Sphingobacteriales increased as the Bacteroidales order decreased. These two Orders were minor components of the cowpats on Day 0 at 0.03% (SD 0.02%) for Flavobacteriales and

0.03% (SD 0.04%) for Sphingobacteriales but steadily increased over the course of the experiment to 11% (SD 2%) and a notable 31% (SD 0.9%) respectively by the last day of sampling.

The Order Actinomycetales was represented by only 0.03% (SD 0.04%) sequences on Day 0, but this had increased to 18% (SD 4%) on Day 42 and 24% (SD 3%) by Day 161. The Actinomycetales was the dominant Order in the Phyla Actinobacteria throughout the course of the experiment. The Orders Sphingobacteriales and Actinomycetales dominated the microbial communities identified from Day 77 onwards. There were also small increases (<5% by Day 161) in members of the Proteobacteria phylum: Burkholderiales, Xanthomonadales and Rhizobiales after Day 28.

Bacterial Genera in decomposing cowpats

There were 582 OTUs generated by the metagenomic analysis at the genus level, of which only 61 OTUs had a mean level >1.5% on at least one sampling occasion. Species of the potentially pathogenic *Campylobacter* genus were identified in low prevalence on Day 0 at 0.04% (SD 0.05%) and maintained a similar prevalence until no longer detected after the Day 14 sampling event. Another member of the Campylobacteraceae family, a species of *Arcobacter* had a spike on Day 28 of 8% (SD 2%) despite having low prevalence on other days (<1%) and being below detection on Day 0. A few other bacterial groups showed a spike in prevalence midway through the experiments (Days 21-42) reducing to $\leq 1\%$ by the end of the experiment including those of the family Anaeroplasmataceae of the Phylum Tenericutes (7%, on Day 28), and fluctuations in prevalence for a member of the Peptostreptococcaceae family up to 13% on Day 21 and another increase on Day 42 (10%). A member of the *Bacteroides* genus increased slowly from 2% on Day 0 to 10% on Day 28 dropping to zero prevalence from the next sampling interval until the end of the experiment.

Taxa identified as dominant in fresh faeces

As expected from the analysis of the higher taxa, it was genera and families belonging to the Bacteroidetes, Firmicutes or Tenericutes that had mean percentages of sequences >2% on Day 0. None of these groups, however, were prevalent towards the five and a half month mark of the experiment with the exception of the Peptostreptococcaceae family (3%, SD 0.9% on Day 161), which is a member of the Clostridiales Order in the Firmicutes phylum. Figure 45 illustrates the shift in the bacterial community over time as the cowpats aged.

On Day 0, a *Ruminococcus* sp. had the highest mean percentage of sequences at the genus level (21%) decreasing to <1% by Day 42 (Figure 45). Other members of the

Ruminococcaceae family were also identified at approximately 5% in the early stages of the trial. Taxa in the Order Bacteroidales had the next highest number of sequences on Day 0; including the genus *Paludibacter* (8%) and the family of *Rikenellaceae* (5%). Sequences from the genus *Prevotella* were identified infrequently and at low abundance (<2%), even in the early stages of the experiment.

Taxa identified as dominant in aged faeces

An unknown family in the Order Actinomycetales had the highest mean percentage of sequences on the last day of sampling (Day 161) with 14%, but a very low prevalence in the cowpat on Day 0 (0.01%) (Figure 45). A similar trend was noted for genera of the Flavobacteriaceae family, which had 4% on Day 161. These two taxa exhibited small increases in prevalence at each sampling interval, but from Day 42 onwards they increased more rapidly with an unknown Actinomycetales recording 7% of the sequences and Flavobacteriaceae >2% on Day 42. Members of the Sphingobacteriaceae family including the genus *Pedobacter* (9% on Day 161) were also identified with increasing prevalence as the experiment progressed, although they had low prevalence (<0.07%) on Day 0. The genus *Pedobacter* was identified at <0.03% on Day 0.

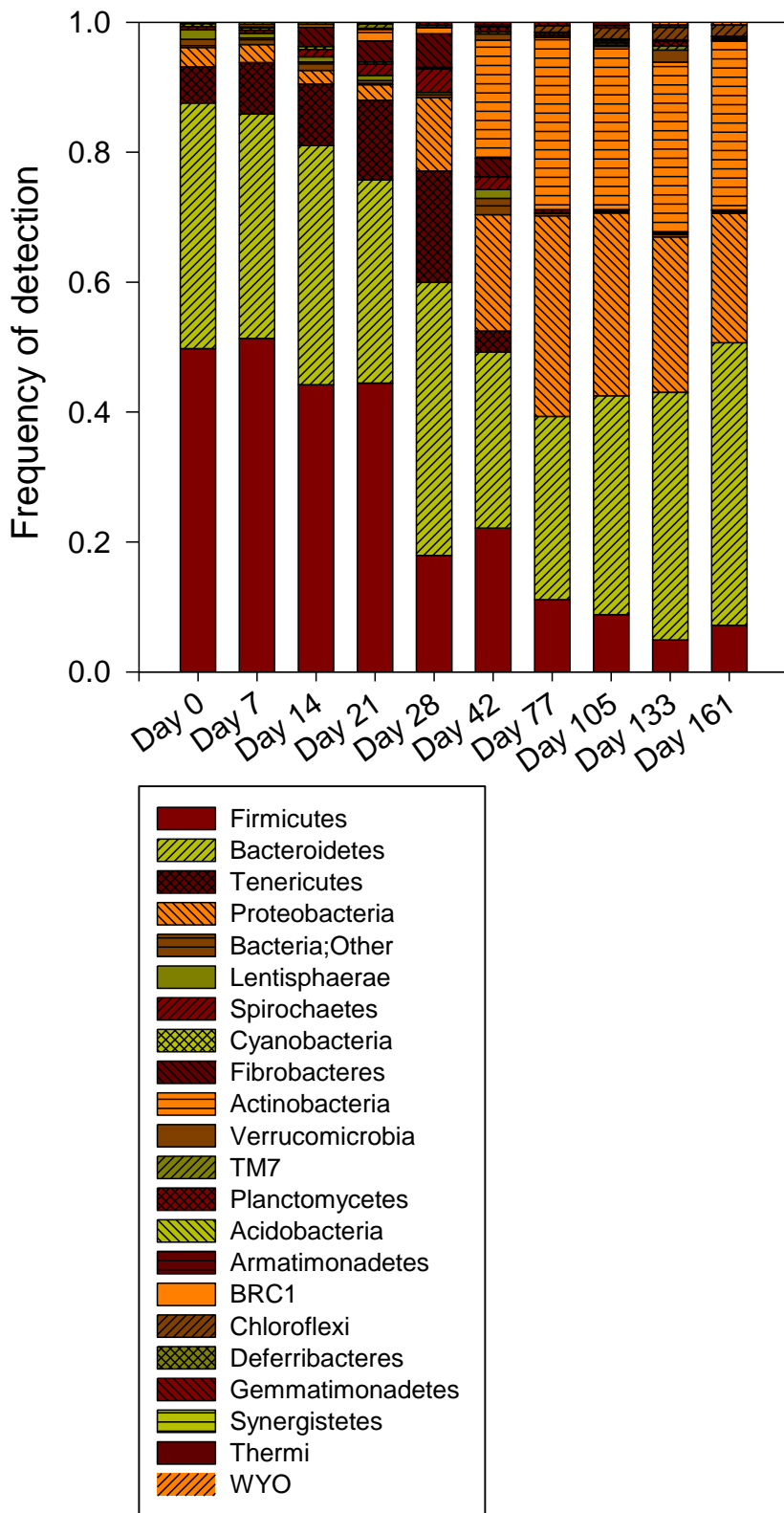


Figure 43: Microbial Phyla identified by metagenomic sequencing of decomposing cowpat faeces.

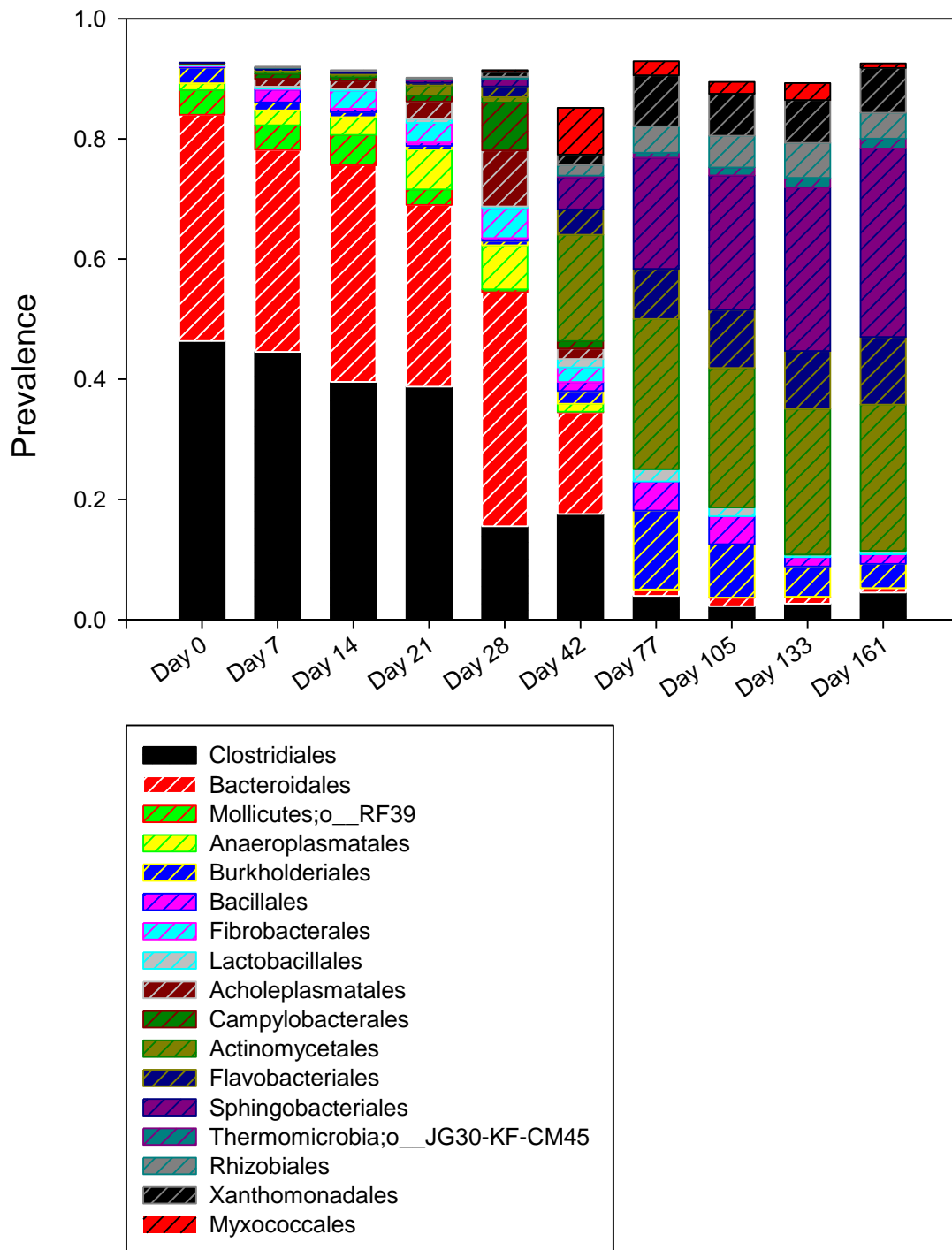


Figure 44: Microbial Orders identified by metagenomic sequencing of decomposing cowpat faeces. Orders represented are only those that recorded a prevalence of >2% on at least one sampling occasion.

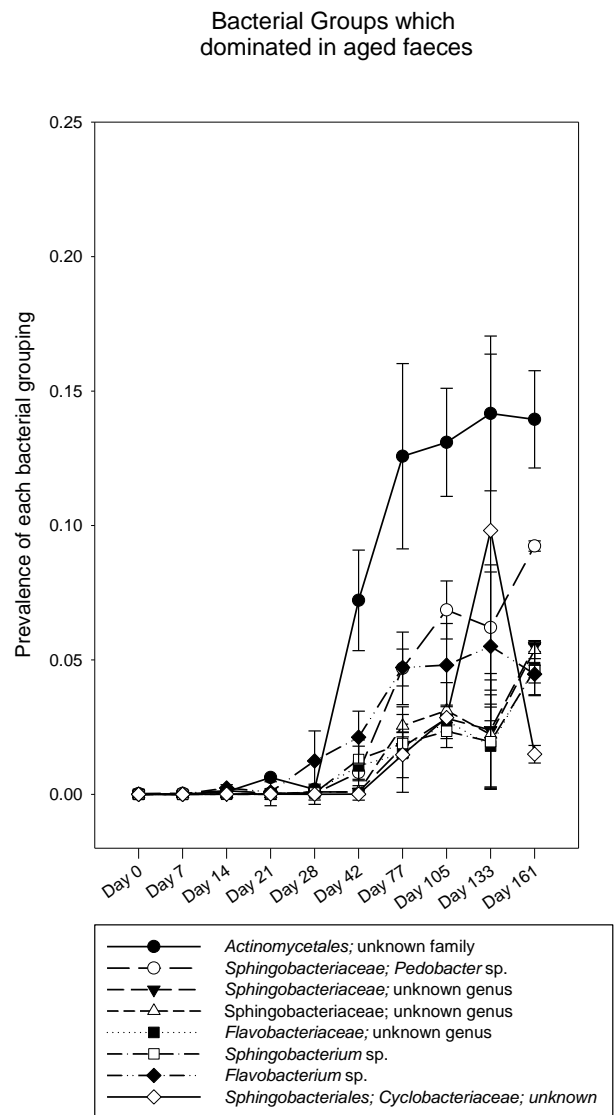
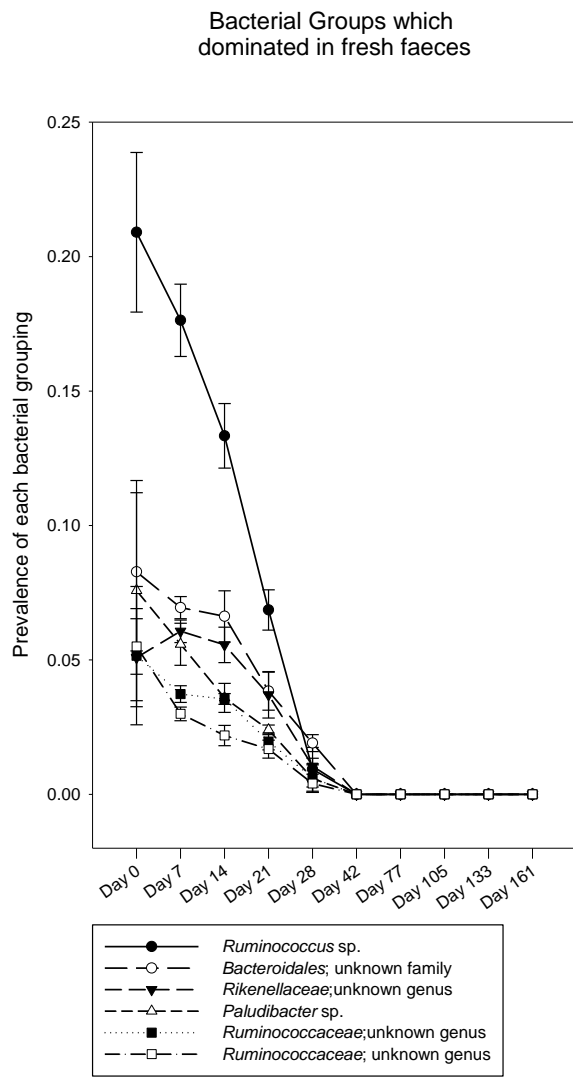


Figure 45: Bacterial OTU sequences in the genus category that were identified as dominant in fresh and aged cowpats after mobilisation from decomposing cowpats.

4.4 Discussion

This summertime study described the impacts of simulated flood and rainfall events on the mobilisation of faecal indicators from ageing cowpats. It also presented the first metagenomic analysis of the microbial community shifts that occurred as cowpats decomposed under field conditions. Taxonomic shifts in the microbial communities of ageing cowpats were hypothesised to alter the markers used for faecal source tracking: *E. coli*, AC/TC faecal ageing ratio, the PCR markers and the faecal steroids. Once deposited into the environment as cow faeces, the FIB and microbes in the bovine intestine, which are the targets of the AC/TC ratio and PCR markers, were expected to undergo processes of growth and decay. The detection rates of these microbes, therefore, were hypothesised to alter as the cowpat aged. Furthermore, it was hypothesised that the ageing of cowpats would provide an environment for steroid biotransformations, similar to the conversion of faecal sterols to other steroids in the cow intestinal environment. These conversions would, therefore, impact on the degradation rates of individual steroids, and hence the faecal signatures generated by ratio analysis of the ten steroids analysed in this rural study.

In a review of nutrient release from cowpats, Haynes and Williams (1993) noted that the greater encrustation of cowpats that occurs in summertime conditions results in longer degradation times compared with other seasons. Degradation of the cowpat occurs through the two mechanisms of physical and microbial breakdown, with rainfall being an important factor in the physical degradation (Haynes and Williams, 1993). The formation of a crust not only inhibits degradation processes but also prevents rewetting of the cowpat by rainfall and thus can limit microbial growth, and subsequently processes of decomposition. Initial moisture content in the cowpats was approximately 90% in all cowpat trials, and by the end of the trials at five and a half months had stabilised at approximately 40% for the 2 kg cowpats of Trial 1 and 25% for the 1 kg cowpats of Trial 2 (Figure 23 and Figure 46). A crust had formed on the cowpats by the second sampling (Days 7 and 8) and later when the cowpat was dessicated, there was not complete re-suspension of the cow faeces in the water after minimal breaking up and stirring for 10 minutes. This would have reduced the release of microbes from the cowpat into suspension compared with earlier sampling occasions where the cowpat completely integrated with the water suspension. It is likely that the results from the supernatant provided a model of the field situation when flooding re-suspends microbes from the cowpat.

For both trials, entire cowpats were analysed at each time interval rather than taking subsamples as in other studies (Oladeinde et al., 2014; Sinton et al., 2007b). There were two key reasons for this approach. First, spatial differences in habitat within the cowpat can contribute to

variations in the microbial composition. For example, samples removed from the outer circumference of the cowpat are likely to be more aerobic compared with the anaerobic centre of the cowpat. Second, if the same cowpat is repeatedly sampled, then sample removal may introduce differential environmental conditions. These variations include changes in aerobic/anaerobic conditions and temperature differences. Maintaining the integrity of the cowpat allowed this study to investigate the mobilisation rates of microbes and FST markers from entire cowpats, rather than the concentration of these variables within the cowpat.

4.4.1 Metagenomic assay of irrigated supernatant from aged cowpats

Thirty irrigated cowpat supernatant samples from Trial 1 were analysed by amplicon-based metagenomic analysis of the V1-V3 region of the 16S rRNA gene to identify the taxa present in the microbial community as the cowpat decomposed. Triplicate samples were analysed from each cowpat supernatant at each of the ten sampling events over 161 days. The mean number of sequences per faecal sample was 7217 with a maximum of 32,123 sequences/sample. This study was, therefore, able to detect microbial populations to at least 0.03% relative abundance to provide a comparatively thorough analysis of the microbial populations in the cowpat as they aged under field conditions. Alpha diversity provides an estimate of the abundance of microbial taxa identified within a sample. Plotted curves of the α -diversity metric versus the number of sequences tend to plateau (slope of zero) as the microbial diversity is captured by the sequencing effort. The shape of the rarefaction curves in Figure 40 suggested that in most cowpat samples the maximum sequencing depth was not achieved, particularly as illustrated by the Observed Species metric, where the plots for all samples were still trending upwards. The lack of sequence saturation probably reflected the high diversity of the microbial community within a single cowpat.

Beta diversity provides a measure of the number of sequences shared between different samples (Lozupone et al., 2007). Figure 42 illustrates the biological β -diversity between different ageing cowpat environments with the noted clustering of samples collected from three different cowpats on the same day of analysis. There was also a time-dependent sequential pattern from fresh to aged faeces illustrating the successive changes in the microbial cowpat community as the cowpat decomposed.

The dominant taxa mobilised from fresh cowpats

The dominant Phyla mobilised from the fresh dairy cow faecal samples were the Bacteroidetes and Firmicutes each comprising at least 30% of the Phyla identified over the first 21 days (Figure 43). This is consistent with a number of other studies of dairy and beef cattle faeces and

ruminal fluid (Durso et al., 2011; Jami et al., 2013; Oikonomou et al., 2013; Ozutsumi et al., 2005; Pitta et al., 2014). These Phyla would appear to be a significant part of the rumen microflora of dairy cows and therefore dominate the microflora in fresh cow faeces.

The Phyla of Proteobacteria (which includes members of the total coliforms) and Tenericutes were also represented by bacterial OTUs at >1% of the total sequences. There was a marked reduction in members of the Firmicutes from Day 28 onwards, concomitant with an increase in the Proteobacteria and the Actinomycetes. In comparison, the Bacteroidetes maintained their dominance as part of the microflora throughout the 161 days. This Phyla is the target of the PCR markers for the universal marker, GenBac3 and ruminant markers used in this study (Reischer et al., 2006; Shanks et al., 2008; Siefring et al., 2008).

The dominant bacterial Orders identified in this study for the fresh dairy cow faeces were the strict anaerobes Clostridiales and Bacteroidales, which are involved in plant fibre degradation (Deng et al., 2008) and belong to the Phyla Firmicutes and Bacteroidetes, respectively. Clostridiales was represented by the families of Ruminococcaceae (including the genus *Ruminococcus*), the Lachnospiraceae and Peptostreptococcaceae. The Order Bacteroidales was represented by Rikenellaceae, Porphyromonadaceae (including the genus *Paludibacter*) and Paraprevotellaceae at greater than 1% of sequences. These bacterial taxa are similar to those identified in another metagenomic study of fresh faeces from mature dairy cows (Dowd et al., 2008) where the strict anaerobes were the dominant Orders identified. However, in the current study, *Ruminococcus* was the most prevalent genus identified at a mean of 21% of the sequences in fresh faecal cowpats compared with *Clostridium* species (19% prevalence, n = 20 dairy cows) in the study of Dowd et al. (2008).

The dominant taxa mobilised from aged cowpats

By the end of Trial 1, the proportions of the initial groups of Bacteroidales and Clostridiales had decreased markedly. The major groups identified in the aged cowpat faecal material were the Actinomycetales, Sphingobacteriales and Flavobacteriales, with the last two being members of the Bacteroidetes phylum (Figure 45). This suggests that although the Bacteroidetes appeared to maintain its dominance in the ageing cowpat there was in fact a major community shift within this phylum from the Order Bacteroidales, which contains many FST PCR marker targets, to the Sphingobacteriales and Flavobacteriales. This decrease in the Bacteroidales was also observed in the mobilisation decline curves of the FST bovine PCR markers, (Figure 26).

The proportion of sequences attributed to the Actinobacteria phylum (<0.05%) in the fresh faeces on Day 0 was lower compared with other studies where percentages ranged between

1-10 % of bacteria identified from dairy/beef cattle of all ages (Chambers et al., 2015; Durso et al., 2011; Ozutsumi et al., 2005). An OTU in the Order of Actinomycetales (which made up the largest proportion of the Actinobacteria) was recognised as a major bacterial taxon in the aged faeces (7-14% of the total sequences between Days 42 and 161) (Figure 45). This Actinomycetales OTU, therefore, may have significance in the identification of a signature PCR marker for faecal contamination from ageing cowpats.

Members of the Sphingobacteriaceae family including the genus *Pedobacter*, were also identified with increasing prevalence as the experiment progressed (Figure 45). However, they were not identified in the metagenomic studies of cow faeces discussed above, although these studies did not investigate aged faeces. The *Pedobacter* sp. has been identified as a common contaminant in the DNA extraction kits used prior to sequencing (Salter et al., 2014). The faecal extraction blanks that were extracted as negative controls with each batch of samples in this study were amplified alongside supernatant samples with primers targeting the V1-V3 region of the 16S rDNA to monitor for potential contamination. No amplicons were produced by the faecal extraction blanks, and therefore they were not sent for sequencing.

Salter et al. (2014) suggested that low amounts of DNA template ($<10^3$ to 10^4 copies) can lead to erroneous amplification of contaminants present from DNA extraction kits. The microbial community shift on Day 42 was in conjunction with the detection of 10^8 GC/100 ml of GenBac3 and 10^7 CFU/100 mL of *E. coli* in the irrigated supernatant. By Days 133 and 161 there were still 10^3 GC/100 mL of GenBac3 and 10^4 CFU/100 mL of *E. coli* present in the supernatant. The presence of substantial DNA template originating from faeces, therefore, reduces the likelihood of low level contaminants dominating the matrix during sequencing, and validating the identification of the bacteria as part of an aged community associated with the cowpat.

Actinobacteria and Sphingobacteriaceae are known to be common inhabitants of the soil community and aquatic environments as are many of the bacterial families identified in dairy cow faeces (Domínguez-Mendoza et al., 2014; Kukharensko et al., 2010; Uroz et al., 2014). Therefore, it could be possible that these OTU have multiple sources including the cow faeces or the pasture soil where they took advantage of the high nutrient conditions associated with the cowpat as it disintegrated on the field. Another possibility is that they are contaminants of the DNA extraction procedure as observed by Salter et al. (2014). Specificity studies of these particular OTUs as PCR marker targets would, therefore, need to include soil and aquatic environments to ascertain their usefulness as markers of aged cowpat runoff in water.

Furthermore, to exclude the possibility of sequencing artefacts, future sequencing studies should include all DNA sample extraction blanks.

Conclusions from metagenomic study of ageing cowpats

This pilot metagenomic study of the ageing cowpat microflora has shown major alterations in the microbial community from the initial dominance of the anaerobes belonging to the Orders of Clostridiales and Bacteroidales. The increasing number of sequences identified as Actinomycetales, Sphingobacteriales and Flavobacteriales bacteria in the latter part of the ageing experiment suggest a shift in the cowpat from a strictly anaerobic environment to an environment supportive of microbes capable of switching between aerobic and anaerobic conditions dependent on the availability of oxygen. It also coincides with moisture changes in the cowpat as seen in Figure 23 where, after Day 28, in the irrigated cowpat there was a striking increase in total solids to >60% reflecting a decrease in moisture content and an increase in sunshine hours and global radiation for December (Day 42) and January (Day 77), in conjunction with lower rainfall. These weather conditions would probably have increased day time internal cowpat temperatures as noted during measurements in Trial 2 (Figure 22), where fluctuations of >15°C between day and night time temperatures occurred during the summer period (December to February). The combination of stresses associated with decreased moisture content and increased cowpat temperatures may have precipitated a microbial community shift as appears to have occurred from Days 28 to 42 onwards (Figure 45). In the following sections the impacts of these microbial shifts seen in the cowpat extracts is discussed in terms of their effects on the FIB, PCR and steroid markers used for faecal source tracking.

4.4.2 E. coli mobilised from cowpats

The cowpat supernatants represented the reservoir of *E. coli* likely to be re-suspended from a cowpat during a heavy rainfall and flood event. *E. coli* concentrations in the initial cowpat supernatants (Trials 1 and 2) on Day 1 were 10^7 CFU/100 mL decreasing to 10^4 , 10^5 and 10^3 by the last day in irrigated (IRR), non-irrigated (NIR) and Trial 2 supernatants (respectively) (Figure 24). In previous studies, concentrations of *E. coli* in fresh cow faeces have been identified at ranges of 10^5 - 10^7 CFU/g dry weight (dw) (Oladeinde et al., 2014; Sinton et al., 2007b; Soupir, 2008) and 10^3 - 10^4 CFU/g dw of faeces in cowpats after 150 days ageing on the field (Sinton et al., 2007b).

Sinton et al. (2007b) noted microbial growth occurred in dairy cowpats only when moisture content was >80%. Oladeinde et al. (2014) noted increases in cowpats of culturable and PCR markers of *E. coli* within the first five days of deposition of natural cowpats under both

shaded and unshaded conditions. In the current study, the only treatment which registered a sustained increase in the release of *E. coli* from the cowpat was the NIR supernatant between Days 7 and 21 when moisture content of the Trial 1 cowpats was 80% for IRR and 76% for NIR. Reasons for an increase in the NIR only, could include reduced losses of *E. coli* from non-irrigated cowpats in the early stages before hardening of the cowpat crust. Overall, there was a notable reservoir of *E. coli* released into the supernatants ($>10^3/100$ mL) from ageing cowpats, even after five and a half months with the additional impacts of irrigation. Cumulative totals of natural rainfall were 258 mm and 420 mm for Trials 1 and 2, respectively. It was noted, however, that the Trial 2 rainfall total was heavily impacted by a downpour of 158 mm recorded several days prior to the final sampling day. Subtracting this deluge from the Trial 2 rainfall total would suggest cumulative rainfall was similar between the two trials.

After the first three sampling events in Trial 2, the integrity of the dessicated cowpat was maintained during the simulated rainfall events when the moisture content of the cowpat had reduced to 25% by Day 22 (Figure 23). The rainfall simulated in this study could be described as light rainfall in comparison to the flood simulation of the supernatants. In Trial 2, initial levels of *E. coli* in the rainfall runoff were still substantial at 10^7 CFU/100 mL. The rainfall runoff had a notable decrease of three orders of magnitude in *E. coli* on Day 8 of the experiment and numbers of *E. coli* plateaued until Day 105, after which *E. coli* were less than 27 CFU/100 mL but still detectable by Day 162.

Muirhead et al. (2006) noted a geometric mean of 2.1×10^5 CFU/g dw in fresh dairy cow faeces with a strong correlation ($r^2 = 0.90$, $p < 0.001$) between the concentration of *E. coli* in the faeces and in simulated rainfall runoff from fresh faeces. In a previous study which included rainfall runoff from both fresh and aged (30 days) cowpats, Muirhead et al. (2005) noted a lower but still significant correlation ($p < 0.001$) between *E. coli* in the cowpats versus rainfall runoff. Although not directly comparable with the Muirhead et al. (2005) study, there were moderate, significant correlations of 0.64 between the supernatant and the rainfall runoff from aged cowpats in Trial 2. Muirhead et al. (2005) attributed the lower correlation for aging cowpats to the formation of a hard crust on the cowpat surface reducing *E. coli* mobility compared with freshly formed cowpats, which in this rural study were noted to completely disintegrate under rainfall. Both studies of (Muirhead et al., 2005; Muirhead et al., 2006) reported findings that confirmed *E. coli* are largely transported from cow faeces as single cells, and would therefore, be highly mobile during overland flow events. From the current study, the levels of *E. coli* ($>10^3/100$ mL) present in both supernatant and rainfall runoff after two and a half months deposition, combined with the high mobility of the single *E. coli* cells suggested that aged

cowpats would still be a significant source of FIB, months after deposition. In several studies, the highest levels of *E. coli* in cow faeces have been noted during warmer months compared with wintertime, increasing the burden of *E. coli* to overland runoff during seasons when waterways are more likely to be used for recreational purposes (Oliver et al., 2012; Sinton et al., 2007b).

E. coli mobilisation rates from cowpats

Mobilisation rates of *E. coli* from cowpats into supernatants (range Log₁₀ 0.019-0.023 CFU/100 mL/day) were lower compared with the cowpat runoff after rain at 0.184/day, which was reflected in the low T₉₀ of 5 days for the rainfall runoff. This was supported by a statistically significant difference in *E. coli* mobilisation rates between the Trial 2 supernatant and the rainfall runoff. Although not directly comparable with studies of decay rates measured directly in cowpats, the T₉₀ for mobilised *E. coli* (range 44 to 52 days for all supernatants) was similar (46-48 days) to a study of *E. coli* decay rates measured as g/dw of faeces from 2.1 kg cowpats conducted over a six month period in a NZ summer (Sinton et al., 2007b). In contrast, a US summer study of *E. coli* within natural cowpats (range 0.6-1.5 kg) had much shorter T₉₀ of 7.8 and 13.0 days for shaded and unshaded treatments, respectively (Oladeinde et al., 2014). They suggested that the mixing of the cow faeces prior to the formation of homogeneous simulated cowpats may lead to a greater oxygenated environment in the cowpat, which would support persistence/growth of *E. coli*, which is able to grow in both aerobic and anaerobic conditions. Kress and Gifford (1984), however, did not identify any significant differences in the release of faecal coliforms from either constructed cowpats or naturally deposited cowpats when subjected to rainfall. In addition, the metagenomic study illustrated that the strict anaerobes, Clostridiales were still a dominant Order in the supernatant up to Day 28, suggesting the maintenance of an anaerobic environment within the cowpat. However, the aerobic/anaerobic environment of the internal cowpat could not be confirmed because monitoring of oxygen levels was not undertaken in this rural study.

4.4.3 FST PCR markers mobilisable from cowpats

Overall, FST PCR markers were highly correlated with each other in all experimental treatments, and in general, with *E. coli*, the total sterols and the major herbivore steroid, 24-Ecop. The strong association between these microbial and FST markers supports their use as indicators of bovine faecal sources.

Throughout the trials, unlike *E. coli* in NIR cowpats, there was no increase in mobilisation of any of the FST PCR markers from the cowpats (Figure 25 and Figure 26).

Concentrations of PCR markers (GC/100 mL) on the initial day of sampling in all matrices were 10^{10} GenBac3 $>10^9$ and 10^{10} BacR $>10^7$ and 10^8 CowM2. These concentrations were approximately 10 to 1000 fold higher than the *E. coli* in the equivalent supernatant and rainfall runoff samples. The PCR marker concentrations were also similar to the orders of magnitude noted in fresh swine slurry and cattle manure (Jaffrezic et al., 2011) and fresh faeces (Reischer et al., 2006; Stea et al., 2015) but lower compared with the fresh faeces in the study of Oladeinde et al. (2014). The general faecal marker GenBac3 was detectable in two of three supernatants until the last day of sampling. This persistent signal for GenBac3 is related to the large pool of taxa in the phylum Bacteroidetes that this marker targets (Dick and Field, 2004; Siefring et al., 2008). In comparison, BacR and the bovine specific, CowM2, target smaller genetic pools of bacteria. In particular, CowM2 targets a gene that is involved in energy metabolism and transport and that is present in low copy number per bacterium (Shanks et al., 2008), whereas GenBac3 and BacR both target the gene 16S rRNA in the phylum Bacteroidetes (Reischer et al., 2006; Siefring et al., 2008). Bacterial species in this phylum are known to carry 3.5 ± 1.5 copies of the 16S rRNA gene per bacterial cell (Větrovský and Baldrian, 2013), increasing the sensitivity of the assays compared with the putative single copy of CowM2.

The CowM2 marker was identified as having high specificity but low abundance in target faecal sources in a multi-laboratory evaluation of FST PCR markers (Boehm et al., 2013). This low abundance of CowM2 was contrary to the high numbers detected in fresh faeces in this rural study. CowM2 could be useful as a marker of fresh bovine/ruminant pollution, but should only be assayed when the BacR marker is detected in water at $>10^4$ GC/100 mL. This concentration of BacR is suggested, because CowM2 was generally one to two orders of magnitude lower in cow faeces compared with BacR, and the detection limit of many PCR assays is approximately 500-1000 GC/100 mL, depending on the volume of water filtered.

FST PCR marker mobilisation rates from cowpats

Statistically similar mobilisation rates for all three PCR markers were noted in this study within all treatments, as has been observed in a study of PCR marker decay within naturally deposited cowpats conducted over 57 days (Oladeinde et al., 2014). Furthermore, in Trial 1, there were no statistically significant differences in the mobilisation decline rates between the two irrigation regimes for individual PCR markers. In these Trial 1 supernatants, T_{90} for mobilisable *E. coli* was two to three-fold longer than the time for the PCR markers. Although a similar T_{90} was noted for *E. coli* in the two trials, the disparity between T_{90} for *E. coli* and PCR markers was even greater in the Trial 2 supernatant at more than five-fold (Table 25). In this rural study,

therefore, *E. coli* was a more conservative indicator of mobilisable faecal contamination compared with the PCR markers.

The lower T_{90} values observed for PCR markers in Trial 2 compared with Trial 1, may be a factor of the reduced weight of the cowpat creating a greater surface area to volume (SA:V) ratio for the Trial 2 cowpats. In addition, microbes on the surface of the cowpat are inactivated by sunlight irradiation reducing overall concentrations, especially during rainfall mobilisation, where the crust reduces rainfall impact on the cowpat's interior. The higher SA:V ratio may also have decreased habitat protection within the 1 kg cowpats by impacting on temperature fluctuations and moisture regimes, leading to more rapid inactivation of microbes in the initial decline phase of the Trial 2 cowpats. In comparison, *E. coli* in the Trial 2 supernatant had a similar T_{90} to Trial 1 supernatants, which may be explained by its greater thermotolerance, again supporting it being a more conservative indicator of faecal microbes mobilised from ageing cowpats.

Decreasing mobilisation rates in the second phase of decline may be due to a persistent population of bacterial targets for GenBac3 and BacR markers as noted in studies of microbial die-off in soil and water (Easton et al., 2005; Rogers et al., 2011). Reasons for this observed effect have been proposed, and include that the low population in the second decline phase are better supported by nutrient availability or secondly, the presence of a sub-population which has better survival characteristics compared with the majority of the strains in that population (Easton et al., 2005). The viable but non-culturable (VBNC) theory has also been postulated, whereby rapid decline in viability is followed by cells entering a reduced metabolic state to conserve energy, the VBNC state. In this state, the DNA in cells is still detectable by PCR (Oliver, 2010) resulting in detection as they are mobilised from the cowpat.

The results of this current rural study suggest that the bovine marker CowM2 is abundant in fresh dairy cow faeces in the NZ environment and would be useful in FST monitoring with its detection indicative of relatively fresh faecal sources. After 42-50 days post-defecation, CowM2 would not be expected to be detected in water where agricultural runoff from flood events contributed to the signal detected from GenBac3 and the BacR markers and *E. coli*. In the rainfall runoff, there was a three log reduction in levels of CowM2 from Day 1 to Day 8, and non-detection after Day 22. Mobilised levels of this bovine specific marker generated during lighter rainfall may be difficult to detect in the aquatic environment after the initial defecation event due to soil transport and degradation processes (including predation and microbial competition for resources) that occur during overland transport (Pachepsky et al., 2006; Rogers et al., 2011; Tyrrel and Quinton, 2003; Unc and Goss, 2004).

Rapid decline of CowM2 and other host-associated qPCR markers has been noted in other longitudinal studies of environmental matrices including manure-amended soils (Piorkowski et al., 2014b; Rogers et al., 2011) and naturally formed cowpats (Oladeinde et al., 2014). This decline in detection has led Rogers et al. (2011) to suggest that non-detection of host-associated qPCR markers, (which generally target single copy genes) in waterways where there are elevated FIB levels may underestimate the contribution of agricultural sources of faecal contamination. It also suggests a greater reliance is required on the less host-specific marker of the ruminant BacR for detection of bovine faecal contamination.

4.4.4 %BacR/TotalBac

The GenBac3 marker targets a large proportion of the Bacteroidetes Phylum (designated Total Bacteroidetes (TotalBac) in this rural study), which includes members of the Order of Bacteroidales. GenBac3 has been used as a general FST marker of non-specific faecal contamination (Siefring et al., 2008). If the general PCR marker and the host-specific PCR markers were shown to decay at similar rates, as suggested by the similar mobilisation rates from the cowpats in this study, then a ratio between these markers could be used to apportion the contribution from each faecal source. However, in recent studies, there has been conflicting evidence about similar decay rates for the Bacteroidetes markers in aquatic systems, which would preclude using these ratios to apportion the contribution of faecal pollution (Dick et al., 2010; Green et al., 2011; Silkie and Nelson, 2009). Importantly, Dick et al. (2010) observed differences in the persistence of the general and host-specific PCR markers associated with sediments, which could underestimate the contribution of sources if a ratio approach was applied. Re-suspension of the sediments at the end of their experiment returned the general Bacteroidales PCR marker concentrations to 50% of the initial concentration, compared to 1% for the specific host-markers.

The percentage of the BacR/TotalBac was $\geq 17\%$ in this study of fresh faeces, suggestive that at this value, all of the faecal signature derived from the GenBac3 marker could be attributed to BacR. The value of $\geq 15\%$ BacR/TotalBac was adhered to throughout Trial 2 for both the supernatant (with one exception) and all of the rainfall runoff (21% on Day 1 to range between 28 and 67%) (Figure 28). In the Trial 1 supernatants, however, there was a much greater fluctuation in BacR/TotalBac above and below 15%, showing reduced mobilisation of the bacterial target of BacR compared with Total Bacteroidetes. These fluctuations of the ratio in re-suspended cowpat supernatants may have been a result of stress from reduced water availability midway during the trial impacting on persistence/growth of the bacterium targeted

by the BacR marker, resulting in its reduced concentration in the cowpats and/or mobilisation into supernatant. This was also noted during the metagenomic assay where mobilisation of the Bacteroidales Order of microbes declined midway through Trial 1 and other members of the Bacteroidetes Phylum increased. In both trials, however, the %BacR/TotalBac remained $\geq 1\%$. These results were, therefore, not conclusive that a BacR/TotalBac of 15% was indicative of 100% ruminant contribution at all ageing stages of the cowpat, but they did support its use for attribution in known fresh faecal sources.

In the urban river study, however, the GenBac3 Marker was identified as ubiquitous in water and perhaps more useful as a marker of the absence of PCR inhibition, as observed in other international studies (Kirschner et al., 2015; Vierheilig et al., 2012). The observation of a low level ubiquitous population of Bacteroidales in drinking water has been confirmed using at least three different PCR markers targeting the 16S rRNA of Bacteroidales (van der Wielen and Medema, 2010). In the absence of animal and human FST PCR markers, this led the researchers to suggest that identification of Bacteroidales was indicative of a naturalised population rather than a faecally-derived source. These factors do not negate use of the %BacR/TotalBac for attribution from recent ruminant sources. In the event of a fresh faecal input to a waterway, a ratio of $>15\%$ BacR/TotalBac would be representative of 100% ruminant faecal contamination.

4.4.5 AC/TC ratio as a potential faecal ageing indicator

In Trial 2, the ratio of AC/TC in river water was investigated as a potential faecal ageing ratio for bovine pollution by dilution of the supernatants and rainfall runoff into freshly collected river water. The initial AC/TC ratios in the re-suspended cowpat supernatants and rainfall runoff from the cowpats were similar on Day 1 at 0.10, which is indicative of very fresh faecal inputs (Brion, 2005; Nieman and Brion, 2003) where the TC dominates the background river microflora represented by the atypical colonies (Brion and Mao, 2000). It is also similar to the ratios seen during the major continuous discharges of raw sewage in the urban river study of this thesis. Brion (2005) noted ratios of AC/TC <1.0 for cow manure leachate on Day 1 and by Day 14, ratios were 2.9, which was similar to the current study with ratios ranging from 2.2 to 5.8 for rainfall runoff and 1.4 to 1.8 for cowpat supernatants over 14 days.

The AC/TC ratio had significant strong to moderate but negative correlations ($p < 0.002$) with *E. coli* and the three PCR markers in the supernatant and rainfall runoff samples; and with mammalian-derived steroids in the rainfall runoff only. Increasing AC/TC ratios suggested an ageing event as TC numbers, including *E. coli*, declined in the cowpat. However, it was only after Day 105 and Day 50 that the AC/TC ratio in the supernatant and rainfall runoff,

respectively, was above 20. Investigation of human faecal contamination in a river suggested a ratio of 20 is indicative of aged inputs with less likelihood of pathogen detection (Black et al., 2007; Brion, 2005). This increase above 20, however, was not consistent in either matrix suggesting persistence/growth of TC in the cowpat. These results provided evidence that overland flow of faecal material derived from cowpats would register AC/TC ratios in surface waters of less than 10.0 for up to four months after deposition due to the persistence and growth of *E. coli* and TC in the cowpats. There were, however, lower levels of *E. coli* being detected in all trial runoffs in the latter stages of the experiment, therefore the AC/TC ratio should always be evaluated in relation to the microbial indicator concentration to determine its relevance in assigning faecal age.

4.4.6 Steroids mobilisable from cowpats

Overall, total steroids and the major herbivore steroid, 24-Ecop were significantly correlated with each other in all experimental treatments and with the three PCR markers and *E. coli*. Other correlations between steroids within treatments had less consistent patterns perhaps reflecting the dynamic nature of the internal cowpat habitat. The strong association between PCR markers and *E. coli* with 24-Ecop, the major herbivore steroid in NZ cow faeces supports the use of the 24-Ecop steroid ratios as indicators of bovine faecal sources.

The general pattern of dominant steroids noted in the mobilised steroids from fresh cowpats was %24-Ecop >%24-ethylepicoprostanol >%24-ethylcholestanol >%24-ethylcholesterol (Figure 29 to Figure 31). This pattern was similar to Australasian studies of steroids in fresh dairy cow faeces, and of herd home faeces, which were a collection of fresh and aged faecal material (Devane et al., 2015; Nash et al., 2005). In European studies, %24-ethylepicoprostanol and %24-ethylcholestanol have been identified as the dominant steroids in fresh cowpats and manure (a mix of cow faeces and straw) (Derrien et al., 2011; Gourmelon et al., 2010; Jaffrezic et al., 2011). These differences may be a product of regional variations in diet and microbial composition of the ruminant, although in Derrien et al. (2011) cows appeared to be pasture fed as in this study, with corn silage as an occasional additional feed.

Differences in steroid degradation and production (due to conversion from sterols) within the cowpat was postulated to occur and ratios between individual steroids were monitored in this rural study to identify any changes that suggested an aged environment. Derrien et al. (2011) observed the increase of epicoprostanol and 24-ethylepicoprostanol and the concomitant decrease of cop and 24-Ecop in pig slurry that had been treated and stored. McCalley et al. (1981) found an increase in epicoprostanol during the digestion process of human waste, which

they attributed to the conversion of cop or cholesterol to epicoprostanol. Derrien et al. (2011) suggested a similar process was occurring in the treated pig slurry, with the addition that sterols were converted to 24-ethylepicoprostanol. Therefore in this rural study, the initial decrease of 24-Ecop in the supernatants and the concomitant increase in 24-ethylepicoprostanol over the first three months was thought to herald this alternative conversion. However as Trials 1 and 2 progressed, it was noted that many steroids (including 24-ethylepicoprostanol) reached either their maxima or minima around Days 77-105, and then generally returned to similar percentages as observed within the first week. This finding discounted the ability to use ratio analysis between steroids to age the faecal material.

4.4.7 Steroid mobilisation rates from cowpats

Absolute concentrations of steroids mobilised from cowpats decreased progressively over the course of the two trials as can be seen in the mobilisation decline curves of Figure 37 to Figure 39. In Trial 1, the steroids within each irrigation regime declined at a similar mobilisation rate including the overall total steroid concentration. Furthermore, the irrigation treatment regime did not appear to affect the mobilisation rate of the steroids in the cowpats when the two treatments were compared. Similarly in Trial 2, the supernatant and rainfall runoff mobilisation rates were similar between all ten steroids and total steroids within and between the two treatments. This was an interesting finding, as steroid mobilisation from the rainfall impacted cowpat occurred at much lower concentrations compared with the re-suspended cowpat. Significantly different rates of mobilisation into these two matrices could have been expected due to the moisture loss and encrustation of the cowpat reducing the mobilisation from rainfall over time.

The steady decline of all ten steroid concentrations mobilised from the cowpat, suggests that there was a degradation of steroids occurring in the cowpat. This is in contrast to the oscillation of sterol production and degradation observed for sterols in sediment inoculated with sewage in marine mesocosm studies by Pratt et al. (2008). In that study, only coprostanol showed a steady trend of degradation, which was biphasic with a rapid decline in concentration in the first week, followed by a slower degradation rate, with minor fluctuations up to the end of the two month experiment. Pratt et al. (2008) ascribed the cycle of production and degradation of steroids to the activity of macrofauna, such as crabs, and microbial populations within the sediments resulting in the lysis of decaying organisms releasing steroids as a nutrient source for another growth phase. There may have been similar synthesis/degradation activity occurring within the cowpat habitat, but the time intervals between samplings did not enable detection of

these changes but rather a steady decline was observed over the longer time period of this rural study.

4.4.8 Stability of the FST signal from steroid ratio analysis

An important aim of this study was to confirm the stability of FST marker signatures when the faecal material was aged under environmental conditions. The effects of dilution can impact on the absolute concentrations of steroids when they enter a waterway making it difficult to establish concentrations of the human/herbivore steroids, which are indicative of specific faecal contamination (Furtula et al., 2012a). Furthermore, the different sizes of sediment grains and organic matter content can affect the distribution of associated steroids, confounding the use of their absolute concentrations (Bull et al., 2002). FST analysis, therefore, generally relies on ratio analysis between steroids to normalise data and determine sources (Furtula et al., 2012b).

The similarity of mobilisation rates for individual steroids from ageing cowpats supported the observation that the steroid ratios used for FST analysis remained stable throughout the cowpats' degradation over the five and a half months of the two trials. This included a stable FST signal independent of irrigation conditions. Prominent examples of the stability of the FST steroid signature included the avian steroid ratios remaining below the criteria for identifying avian pollution, showing that aged faecal material derived from bovine sources would not be misidentified as avian faecal pollution (Figure 35). The dominant herbivore steroid, %24-Ecop (R1) was lower in the initial fresh cowpat supernatant in Trial 2 at 47% compared with 62% in Trial 1, but consistently identified herbivore pollution on all sampling days (Figure 29). The same was true of ratio P1 (24-ethylcholesterol/24-Ecop), which discriminates between herbivore and plant runoff. P1 was unaffected by the substantial increases of plant sterol, 24-ethylcholesterol (6.7% to 28.2%) in the rainfall runoff (Figure 30) because these increases were offset by the high levels of 24-Ecop in the bovine faeces.

Due to the dominant levels of the steroid, 24-Ecop, the ratios H3-H5, which specifically discriminate between human and herbivore mammals by comparing cop and 24-Ecop, were always identifying herbivore mammal sources throughout both Trials (Figure 33 and Figure 34).

The ratio R3 (24-ethylcholestanol/coprostanol) is used in association with the %H4 ratio (<60% for bovine) to discriminate between bovine, porcine and human faecal contamination (Gourmelon et al., 2010). As expected for bovine sources, the ratio R3 was >1.0 in all supernatants and cowpat runoff experiments except for one sample when the mean of the triplicate samples was 0.9, but in this case, the %H4 ratio was <16% negating its misclassification as either porcine or human. The stability of the ratios discriminating bovine

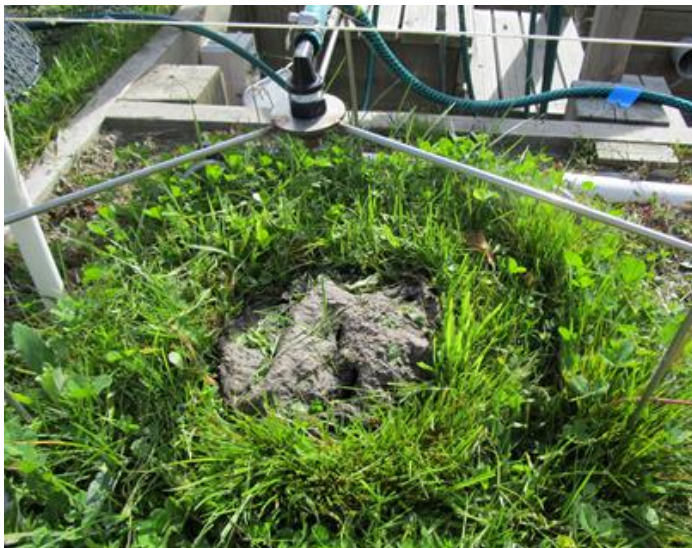
from human and porcine was also observed by Jaffrezic et al. (2011) in runoff from soils which had been amended with swine faecal slurry and cattle manure two hours prior to a simulated rainfall event. However, 100 L microcosms of seawater and freshwater inoculated with pig manure and monitored over 55 days were only stable for the specific porcine versus bovine stanol signatures for 6 days (Solecki et al., 2011). They found monophasic decay compared with the biphasic decline in mobilisation of steroids noted in this study but they also identified insignificant differences between decay rates for individual steroids when compared between freshwater and seawater matrices. In a study of the decay of FST markers in 100 L seawater and freshwater microcosms inoculated with human wastewater, Jeanneau et al. (2012) noted differential decay between cop, 24-Ecop and 24-ethylcholestanol. The ratios for human faecal contamination were only characteristic of human pollution up to Day 13 in seawater and Day 6 in freshwater. Differential decay between steroids may occur, therefore, dependent on the type of faecal contamination and once steroids are transported overland into waterways. In this rural study, however, steroid FST signatures were maintained when mobilised from decomposing cowpats up to five and a half months after deposition.

4.4.9 Conclusions

- The microbial community in the cowpat revealed shifts in community composition as the cowpat aged under field conditions over summertime.
- A member of the *Ruminococcus* genus was noted to be dominant in fresh faeces, but was replaced by members of the bacterial Orders, Actinomycetales, Sphingobacteriales and Flavobacteriales, which dominated aged cowpat runoff. These bacterial groups could be targeted as potential indicators of fresh and aged pollution runoff from bovine sources.
- Decomposing cowpats aged up to five and a half months contained appreciable amounts of *E. coli* that was available for mobilisation under flood conditions (10^3 to 10^5 CFU/100 mL). *E. coli* was mobilised from cowpats by lighter rainfall for at least two and a half months post-defecation.
- The effect of irrigation on the mobilisation rates of *E. coli*, steroids and PCR markers into cowpat supernatants was statistically insignificant when compared with non-irrigated cowpats.
- The steroid ratios used as FST markers of bovine faecal pollution did not change over time in either the re-suspended cowpat supernatant or rainfall runoff, validating tracking of fresh and aged bovine faecal runoff by steroid analysis.
- Individual PCR markers were mobilised at similar rates in all matrices, although CowM2 was noted to decrease below the detection limit in both supernatants and rainfall runoff much sooner than the GenBac3 and BacR PCR markers.
- CowM2 is useful for the confirmation of fresh bovine/ruminant pollution but it is suggested that CowM2 should only be assayed when the BacR marker is detected in water at $>10^4$ GC/100 mL.
- The ratio of BacR to GenBac3 PCR marker was analysed as a surrogate of BacR/Total Bacteroidetes to ascertain the ruminant contribution of faecal pollution. %BacR/TotalBac of $>15\%$ was a stable indicator of 100% contribution from fresh bovine sources subject to runoff after light rainfall and flood conditions.
- During ageing of the cowpat, *E. coli*, the three PCR markers, total steroids, and the herbivore steroid 24-Ecop, all had moderate to strong, positive correlations with one another ($p < 0.05$) supporting this toolbox of microbial and FST markers as indicative of fresh and aged bovine faecal sources.
- AC/TC had significant negative correlations with *E. coli*, PCR markers and mammalian-derived steroids supporting low AC/TC ratios as indicative of recent faecal contamination. Overland flow of faecal material derived from cowpats would register AC/TC ratios of less than 10.0 in

surface waters for up to four months after deposition due to the persistence and growth of *E. coli* and TC in the cowpats.

- After the first week post-defecation, the rainfall runoff samples from cowpats contained significantly lower concentrations of each of the FST markers compared with the re-suspended cowpat supernatants.
- It is recommended that where runoff from non-flood conditions may confound water quality monitoring, application of the Bacteroidales host-associated PCR markers for monitoring purposes is preferable to assessments relying on the environmentally persistent *E. coli*.



Trial 1: Aged irrigated cowpat on Day 161



Trial 2: Aged non-irrigated cowpat on nybolt mesh, Day 134

**Figure 46: Photos of dessicated cowpats in the last months of Trials 1 and 2.
Photo credit: Megan Devane.**

5 Chapter Five:

Health implications in relation to water quality monitoring

5.1 Limitations in current faecal contamination assessment methods

This chapter discusses the findings from the urban river and rural studies and the practical application of those results within the context of water management in NZ. The introduction discusses current water quality monitoring procedures in NZ and some of the limitations associated with contemporary methods.

5.1.1 Current water quality monitoring approaches

The National Policy Statement for Freshwater Management (NPS-FM) was released by the NZ Ministry for the Environment in 2014 to address the challenges facing our rural and urban waterways where anthropogenic activities have led to degradation of freshwater (www.mfe.govt.nz/). The NPS-FM provides a national objectives framework (NOF) to assist local government bodies and communities to plan freshwater objectives for the improvement/maintenance of a range of attributes essential to ecological health and human health in recreational freshwaters. An attribute is defined as a measurable chemical, biological or physical characteristic of water such as nitrate, phosphate and *E. coli* concentrations. National Bottom lines for all of the attributes have been established and provide an upper limit of permissible contamination. For assessment of microbial water quality for human health, the national bottom line for *E. coli* is 1000 CFU/100 mL. Where the water is used for activities with occasional immersion, freshwater objectives must be set at, or below the national bottom line based on an annual median concentration of 1000 *E. coli*/100 ml. In water bodies where full immersion activities such as swimming take place, then the minimum acceptable state is the 95th percentile concentration of 540 *E. coli*/100 ml. If water bodies exceed these limits, regional authorities and communities are tasked with providing action plans to lower exceedances over appropriate timeframes.

5.1.2 Need for a new toolbox?

To achieve the goals of reducing faecal contamination and meeting acceptable standards for recreational activity requires an understanding of the faecal sources impacting a waterbody and the persistence of the indicators used to identify that contamination event. Tracking down the

sources of faecal contamination requires additional tools such as faecal source tracking (FST) methods to aid water managers in prioritising which catchments pose the greatest health risk. Human faecal contamination presents the greater risk for infectious disease, followed closely by livestock faecal contamination from cattle and dairy cows (Schoen et al., 2011; Soller et al., 2010). The need for site-specific criteria using such tools as QMRA and epidemiological studies to link sources of illness with microbiological water quality was acknowledged in USEPA (2012) and further guidelines have been recognised as essential by Fujioka et al. (2015) in a paper outlining concerns about the current water quality criteria in the US. There is value in monitoring the policy changes in water quality monitoring by international groups such as the USEPA. Application of international research is often applicable to the NZ situation because the human vulnerability to health risk from faecally contaminated waters is universal and the knowledge transferable between geographies, with many similar pathogens impacting human health.

The current methods for evaluating FIB concentrations do not allow the identification or enumeration of the actual pathogens that cause disease. The presence of elevated levels of microbial indicators like *E. coli*, and even FST markers, does not necessarily signify the presence of pathogenic organisms. The absence of such indicators, however, does provide greater confidence that pathogens are not present in a waterway (Pachepsky et al., 2006). This aspect and their low cost, low technology testing regime are the reason why most models have been built around FIB to remove the prohibitive costs of testing for every pathogen likely to be present in a specific faecal sample.

Concern has been raised that recognised environmental reservoirs of FIB and FST markers such as sediments may be contributing to elevated levels of water quality indicators and may, therefore, overestimate the health risk from pathogens (Dick et al., 2010; Korajkic et al., 2014; Nevers et al., 2014). Equally, however, pathogens may also persist in an infectious form in environmental reservoirs. Pathogens such as *Cryptosporidium* are known to persist in environments such as beach sand (Abdelzaher et al., 2010; Sabino et al., 2014; Solo-Gabriele et al., 2016). Protozoa and viruses in the environment, however, have been shown to lose infectivity over time once released into the environment and there is little evidence to support their replication in non-host locations (King and Monis, 2007; Ogorzaly et al., 2010). These are factors that confound establishing a link between indicator and pathogen levels in a waterbody.

Investigations of the relationships between pathogens and FST markers and microbial indicators in water have noted varying results in regards to indicator-pathogen correlations (Harwood et al., 2014; Savichtcheva and Okabe, 2006; Till et al., 2008; Wu et al., 2011). There

is a consensus that no one indicator is sufficient to predict all pathogens because of the varying environmental characteristics of waterbodies and the differences in survival/persistence of all microbial and chemical indicators once they exit the warm-blooded host environment (Harwood et al., 2005). Differences between a recent and historical faecal input, including sewage treatment processes may, therefore, have an impact on whether the indicator and pathogen(s) are identified in water in combination.

In general, during treatment of sewage there is a greater reduction of FIB concentrations compared with some pathogens such as norovirus (Ottoson et al., 2006). The FIB concentrations may, therefore, be within water quality guidelines but there is still the potential for infection by pathogens. In contrast, some chemical FST markers, for example steroids, are known to increase in concentration in treated sludge wastes due to a reduction in organic matter (MacDonald et al., 1983). This increase raises the likelihood of detection as a biomarker when discharged into the environment without necessarily reflecting pathogen presence. Differences between types of faecal source (Korajkic et al., 2013a) may also impact on this indicator-health paradigm, which, in the case of FIB, was originally established based on human point sources (Dorevitch et al., 2010; Soller et al., 2010). Pathogen concentrations also vary due to the source of faecal contamination, hence the practicality of combining faecal source tracking with identification of contamination inputs. Furthermore, carriage of pathogens in an animal/human community varies over time and between geographies, impacting on the potential for pathogens to be detected in a particular source population (Wu et al., 2011). These factors all contribute to the discrepancies noted between indicator–pathogen correlations.

In summary, to attain a better assessment of health risk, there is a need for:

- improved identification of pathogen-related health risk by applying a suite of indicators
- improved identification of source(s) of faecal contamination
- identification of the environmental reservoirs for microbial indicators and pathogens
- an understanding of the persistence of microbial and FST indicators in the post-defecation environment
- discrimination between recent and historical faecal inputs, and treatment status of faecal waste

5.2 Urban river study: Improved identification of health risk

5.2.1 *E. coli* as an indicator of faecal contamination

In this current urban river study, large volumes of untreated human sewage were discharged for approximately six months into an urban river after earthquakes severely damaged the sewerage system. The Microbiological water quality guidelines for recreational areas (Ministry for the Environment, 2003) were based on the study of McBride et al. (2002), which ascertained that an *E. coli* concentration of ≤ 130 CFU/100 mL suggested a low risk of illness (0.1%) (Till et al., 2008). There was, however, an increasing risk of campylobacteriosis when *E. coli* was between 200 to 500 CFU/100 mL, becoming an unacceptable risk at >550 *E. coli*. The significant correlation between *E. coli* and *Campylobacter* (r_s 0.40) in the current urban river study was similar to that identified in the national survey of recreational freshwater sites in NZ (r_s 0.42) (Till et al., 2008). In the urban river study, the source of *E. coli* was attributed by FST markers to the untreated human sewage being directly discharged into the river. *E. coli* also had weak to moderate correlations with the protozoa, *Giardia* and *Cryptosporidium*. These findings suggested that *E. coli* was a useful frontline indicator of potential pathogen presence and hence public health risk in this scenario.

The good concordance between *E. coli* in river water and the identification of untreated human pollution allowed the generation of equations for the prediction of pathogen concentrations in river systems attributed to raw human sewage (Table 15). However, there is not always a simple linear relationship, with the effects of dilution, sedimentation and differing die-off rates of microbes being some of the factors that differentially impact pathogens and FIB, leading to an overestimation/underestimation of health risk from untreated sewage. Evidence from this urban river study supported the call for a cohort of indicators to better inform water managers of potential health risks. Indicators in this urban river study recognised as possible candidates for this cohort were the F-RNA phage.

5.2.2 F-RNA phage as indicators of faecal contamination in urban river study

In the urban river study, F-RNA phage monitoring by plate counts was identified as a useful, low cost, adjunct to the indicator *E. coli* for identifying untreated human-associated faecal pollution. There were significant, moderate to strong correlations between the two indicators and between F-RNA phage and the human FST markers, which was similar to *E. coli*. There were also significant weak to moderate correlations between F-RNA phage and the potentially pathogenic protozoa. In addition, there was a significant but weak correlation between *Campylobacter* and F-RNA phage during the continuous discharges. This correlation, however, was negated once

discharges ceased and the detection of *Campylobacter* was more likely attributed to wildfowl faecal inputs as verified by identification of wildfowl FST markers. This lack of a relationship between F-RNA phage and *Campylobacter* after major discharges ceased verifies the F-RNA phage as indicators of human pollution attributed to untreated sewage.

A subsequent study during 2015 of the same sites in this urban river, speciated the *Campylobacter* isolates by the PCR method of Wong et al. (2004) with all isolates collected during baseflow conditions being identified as the human pathogen *C. jejuni* (Moriarty and Gilpin, 2015). Further characterisation of *C. jejuni* isolates by the genetic subtyping scheme of Cornelius et al. (2014) was used to attribute the majority of *Campylobacter* to avian sources during baseflow conditions, in agreement with FST analysis (PCR and faecal steroids). These results suggest that wildfowl carriage of pathogenic *Campylobacter* may present a human health risk and should be accounted for in health risk assessments. The increasing recognition of wildfowl as vectors of *Campylobacter* and other pathogens (Gorham and Lee, 2015; Moriarty et al., 2011b; Zhou et al., 2004) may require a reassessment of the Soller et al. (2014) QMRA model for wildfowl and mixtures of wildfowl and human sources. However, studies in NZ of the subtypes of *C. jejuni* isolated from ducks and starlings has shown a low carriage of those subtypes also identified in human clinical cases, suggesting wild birds may not be a major contributor to health risk from campylobacteriosis (French et al., 2009; Mohan et al., 2013).

5.2.3 Recent practical applications of F-RNA phage in USEPA water quality monitoring

The use of plaque assays for F-RNA phage in conjunction with *E. coli* plate counts, as indicators of recent untreated sewage inputs to freshwater would be cost-effective for low technology laboratories, and could lead to better predictability of pathogens as part of the suite of indicator organisms suggested by Harwood et al. (2005). This finding has been further supported by the USEPA who released a report investigating the efficacy of coliphages, which include F-RNA phage, for the detection of faecal contamination (USEPA, 2015). The USEPA report concluded that coliphages were more suited to the detection of faecal contamination and potential pathogenic viruses than the enterococci and *E. coli*. Factors that increased their efficacy over traditional FIB included: less likelihood of replication in aquatic environments (Ogorzaly et al., 2010), better resistance to wastewater disinfection treatment and therefore, superior sentinels of virus presence in human wastewater, and in addition, they are a non-pathogenic organism. Further support for coliphages as indicators came from a review of eight epidemiological studies investigating the relationship between the identification of coliphages and cases of GI after

exposure to recreational water (USEPA, 2015). Five of the eight studies reported a statistically significant relationship between detection of F-RNA phages as indicators of illness.

5.2.4 F-RNA phage as indicators of recent human faecal inputs

In this urban study, F-RNA phage did not accumulate in the sediments during either discharge phase. This factor plus its lower levels in water post-discharge contributed to F-RNA phage being determined as an indicator of fresh human faecal inputs. *Campylobacter* also did not persist when discharged into the environment. The short term survival of *Campylobacter* in the environment has been observed in previous studies (Moriarty et al., 2011a; Moriarty et al., 2012). There are, however, conflicting reports on the persistence of F-RNA phage in water and sediment with the different phage subgroups displaying differential survival (Brion et al., 2002a; Muniesa et al., 2009; Ogorzaly et al., 2010). In contrast, *E. coli* was identified as more persistent in the environment, being identified in water and sediment, months after continuous sewage discharges ceased. Although, in general, the *E. coli* concentrations were greatly reduced in water and sediment post-discharge compared with active discharges.

In support of F-RNA phage as indicators of fresh human sewage, they had significant negative correlations with the bacterial faecal ageing ratio of AC/TC. For example, a low AC/TC ratio during active discharge corresponded with higher F-RNA phage levels. F-RNA phage were also not found at a swimming beach in Hawaii when >500 CFU/100 mL of *C. perfringens* was detected, prompting those researchers to suggest F-RNA phage as indicators of more recent sewage contamination (Fung et al., 2007).

5.2.5 Improved identification of sources of faecal inputs

Comparison of the different FST methods is essential for building a robust FST toolbox (Derrien et al., 2012). In this study, significant correlations were identified between faecal steroid analysis and human PCR markers in water samples during both discharge and post-discharge. There were also high correlations between all three human PCR markers.

Statistical analyses were performed on the combined data set from all three sites to determine if applying a reduced number of FST markers in water would be able to discriminate the sources of faecal contamination when applied to sewage discharges into a river system (Table 16). On the strength of evidence provided by PCA and logistic regression analysis it would appear that the three human PCR markers, and the steroid ratio analyses, are individually able to provide consistent discrimination of human pollution in waterbodies, to the exclusion of herbivore and avian inputs. In general, correlations between FST markers and protozoan

pathogens were stronger than those between *Giardia* and the microbial indicators and similar to those between *Cryptosporidium* and microbial indicators. Furthermore, logistic regression between *E. coli* and FST markers in water showed that *E. coli* provided predictive value of human pollution only at high levels $> 10^3$ CFU/100 mL and therefore, was a less useful parameter compared to the FST methods. It was shown that water managers could be confident in the results using either FST method to not only identify human faecal sources but also indicate risk to human health. In addition, the urban study provided verification of the 5-6% coprostanol level in water as indicative of human faecal inputs. These predictions, however, emphasised the need to base steroid analysis on a complement of steroids rather than a single steroid as a biomarker, such as coprostanol, which supports recommendations by Shah et al. (2007).

As noted in other studies (Ridley et al., 2014; Stea et al., 2015), the ubiquitous detection of GenBac3, the general faecal PCR marker, at high levels during discharge and post-discharge phases in the urban river study suggested it may be more useful as an indicator of the absence of PCR inhibition in this river matrix. These findings may support the research of Walters and Field (2006) who identified growth of Bacteroidales in raw sewage held for 24 hours, raising the question of whether the anaerobic Bacteroidales are able to grow/persist in the environment.

In this study, FWA in raw human sewage were identified in similar concentrations to international studies (Hayashi et al., 2002; Poiger et al., 1999), albeit at the lower end of the range. FWA in river water, however, were not identified in sufficient concentration to assign it to a human source. Due to the low detection of FWA in water, few correlation analyses with other indicators were able to be performed, calling into question their use in environments where there is a high rate of dilution.

In the urban river study, the steroid ratio of coprostanol/epicoprostanol in the water was indicative of inputs of untreated human sewage. Ratios of cop/epicop >20 were highly suggestive of untreated sewage; ratios <20 , however, require further validation to understand the impacts of dilution in a river system where untreated sewage is the contamination source. In this study it was shown that the elevated levels of *E. coli* attributed to untreated sewage were a good indicator of public health risk. In contrast, in sewage that has undergone treatment, the different decay rates and responses of microbes to the treatment process could undermine that relationship between *E. coli* and expected health risks. Recognition of human inputs that can be differentiated based on their treatment status is, therefore, useful when attempting to characterise health risks.

5.2.6 Sediment as an environmental reservoir of indicators and pathogens

C. perfringens was consistently identified at much higher concentrations than *E. coli* in the sediment, particularly, during the post-discharge phase. In comparison to *E. coli*, *C. perfringens* was identified in 10 to 100 fold lower levels in water during the active discharge phase. In addition, it was the only microorganism identified as not having a significant difference in concentration between the two discharge phases. These factors negated its use as an indicator in this temperate environment, whereas it has been identified as a better indicator than *E. coli* in tropical climates (Fujioka, 2001; Fung et al., 2007).

Potential pathogens accumulating in sediments

High concentrations of potentially pathogenic protozoa and *E. coli* were observed in the urban river sediments during the active discharges of sewage. Although *Cryptosporidium* was only detected in river water during the discharges, both protozoa were recognised in higher concentrations in sediment after active sewage discharges ceased when levels of *E. coli* in water were lower. Identification of protozoa in sediments, therefore, highlighted that despite non-detection in the water column there may be a health risk associated with re-suspension of sediments. Recovery of protozoa from sediments by current methods is also low, (typically <10%), indicating the true concentration of protozoa in sediment may actually be much higher. In addition, the low infectious doses of *Cryptosporidium* and *Giardia* (McBride et al., 2012) suggest that detection requires additional effort to determine infectivity of the protozoa detected. Not all species belonging to these protozoan genera will cause illness in humans (Kitajima et al., 2014) and identification of (oo)cyst infectivity in future studies would aid prediction of health risks.

The recognised transport of microbes between the sediment and water column may be a dynamic process occurring during base flow as well as high flow events (Litton et al., 2010; Piorkowski et al., 2014a; Yakirevich et al., 2013). Further research is needed to clarify the role of sediments on water quality and to quantify rates of continuous exchange of microbes between the underlying sediment and water column during base flow conditions. It would be important to characterise the protozoa and their redistribution via sediment transport processes as they may have different profiles of particulate attachment and sedimentation compared with bacteria partially due to the protozoan's larger (oo)cyst size and density (Medema et al., 1998).

A disconnect between chemical markers and pathogens in sediment

In the urban river study, the adsorption of FWA and steroids to particulate matter in sediments led to an accumulation of these chemical markers after discharges had ceased. There was a

strong positive relationship between FWA and the human-associated steroids in sediments but a lack of correlation between these chemical markers and either microbial indicators or pathogens in the sediments. Furthermore, there was a disconnect between high FWA levels observed in sediment and low levels of FWA in the overlying water. The evidence supported the hypothesis that in sediments, FWA and steroids were indicative of historical faecal sources. The lack of correlation between these chemical FST markers and pathogens in this sediment matrix also restricted their predictive value for health risks.

In general, in the absence of re-suspension events and with the proper sampling techniques, reservoirs of steroid and FWA markers in the sediments did not appear to be impacting on water quality testing in this urban river study. Sampling technique, however, must avoid re-suspending sediments.

5.3 Rural study: understanding the persistence of faecal indicators

5.3.1 Microbial and FST markers in the rural study

In this rural study of decomposing cowpats, significant, high correlations were identified between the general faecal marker and the two bovine-associated PCR markers; and significant correlations between PCR markers, the total steroid content and the major herbivore steroid 24-ethylcoprostanol. These findings supported those of the urban river study illustrating the efficacy of PCR markers and steroid analysis for determination of faecal sources.

Studies of the persistence of *E. coli* and FST markers mobilised from cowpats

Addition of FST markers to models of faecal microbial burden could overcome some of the known limitations of the indicator *E. coli*. These limitations include the persistence and growth of *E. coli* in the cowpats facilitated by high internal cowpat temperatures. The rural metagenomic study of cowpat faecal runoff showed that the Bacteroidetes Phylum decreased markedly in abundance as the cowpats decomposed. The FST PCR markers used in the rural study, target members of this Bacteroidetes Phylum, and along with the sterol markers exhibited steady decreases in mobilisation as cowpats aged on the field. For example, the host-associated PCR markers, BacR and CowM2 were no longer mobilisable from Trial 1 re-suspended cowpat supernatants after Days 105 and 42, respectively, while *E. coli* was still detectable at the end of the experiment at concentrations of 10^4 to 10^5 CFU/100 mL. However, in Trial 2 supernatants, the BacR and *E. coli* were both still detected in the mobilised phase on the last day of sampling, whereas CowM2 in Trial 2 was not detected after Day 50. Furthermore, the CowM2 marker was shown to be non-detectable after Day 22 in the rainfall runoff. These findings were supported by the work of Piorkowski et al. (2014b) who applied untreated liquid dairy manure to soil and

monitored *E. coli*, BacR and CowM2 concentrations over 72 days. They noted that CowM2 was no longer detected in soil six days after manure application. Evidence from this rural study and others, therefore, suggests that detection of the CowM2 PCR marker could be attenuated by decay and soil attenuation processes earlier than the other PCR markers. Therefore, when CowM2 is detected in a waterway, it may indicate recent faecal contributions from direct deposition or a runoff event. However, a timeline for the definition of recent faecal input cannot be provided by this rural study. Furthermore, bovine faecal studies of pathogen correlations with FST markers including CowM2 would need to be performed to confirm if there was a correlation between detection of CowM2 and pathogens associated with bovine faeces.

Caveats to the implementation of CowM2 as an FST marker of recent faecal inputs include the finding from Shanks et al. (2013a) that the more host-specific PCR markers, including CowM2, were absent from calf faeces up to Day 115 post-birth and were, therefore only identified in adult cows. In addition, there has been the recent finding of the CowM2 PCR marker in NZ farmed deer faeces (approximately 50% positive) (personal communication, Brent Gilpin), although CowM2 was not detected in deer faeces in US studies (Raith et al., 2013; Shanks et al., 2008). This finding of CowM2 in deer faeces may, therefore, suggest that CowM2 may be better represented as targeting cattle and deer when used for FST in the NZ environment.

The less host-specific PCR markers, GenBac3 and BacR, and the faecal steroids would be appropriate FST markers for monitoring the decline of faecal runoff following effluent/faecal mitigations. In addition, the quantitative assessments afforded by qPCR markers could enable monitoring to record the reduction of faecal loading from ruminant pollution once mitigations have been put in place.

%BacR/TotalBac as an indicator of 100% ruminant faecal contamination

Quantitative FST approaches are still evolving whereby the contribution of faecal contamination in a mixed source catchment can be apportioned to different sources (Soller et al., 2014). In the rural study, the ratio of %BacR/TotalBac as determined by the PCR markers was shown to have potential in estimating the quantitative contribution from fresh ruminant sources. In fresh bovine faeces, the %BacR/TotalBac was >15% in all cowpat runoff matrices, indicating that fresh inputs of cow faeces to a waterway may produce a similar ratio. Observation of this ratio could, therefore, indicate that all of the Total Bacteroidetes detected by the GenBac3 PCR marker could be attributed to fresh ruminant pollution. However, in regards to aged pollution, there was inconsistent evidence from the two trials that the %BacR/TotalBac ratio in the supernatants remained above 15% during on-field ageing processes, as this ratio was maintained only in the

supernatant and the rainfall runoff from Trial 2. Furthermore, in the urban river study, the GenBac3 Marker was identified as ubiquitous in water and perhaps more useful as a marker of the absence of PCR inhibition, as observed in other international studies of Total Bacteroidetes/Bacteroidales general markers (Kirschner et al., 2015; Vierheilig et al., 2012). These factors do not negate use of the %BacR/TotalBac for attribution from ruminant sources. When %BacR/TotalBac is observed at ratios >15% in agricultural watersheds, this rural study shows that it definitively identifies 100% ruminant faecal source(s).

5.3.2 Stability of steroid FST markers mobilised from cowpats

The stability of the steroid ratios used for FST analysis was an important finding from this study when assessing changes in steroid concentrations in cowpats over a five and a half month period. The stability of steroids was supported by the study of Derrien et al. (2011) who noted statistically insignificant differences between steroid percentages in fresh and aged cow manure (where faeces had been mixed with straw).

Mobilisation rates from cowpats for both supernatants and rainfall runoff were noted to be similar for all steroids thereby maintaining the ratios that discriminated between herbivore (in this case bovine) and human pollution. However, studies of sterol decay in simulated aquatic environments noted instability of sterol ratios between six and 13 days. These findings suggest that once FST markers reach aquatic environments, they may have differential decay rates in water compared with faeces (Jeanneau et al., 2012; Solecki et al., 2011) but also dilution and sedimentation effects will have an impact as noted in the urban river study.

5.3.3 Modelling of contaminants from agricultural sources

With the intensification of land use, researchers have noted that larger herd sizes and intensively managed grazing can lead to the degradation of both the soil and vegetation of grassland environments (Bilotta et al., 2008; Houlbrooke et al., 2011). Surface runoff from land is increased by the soil treading damage generated by grazing animals, with the larger size of cattle and dairy cows noted to generate greater land damage compared with sheep (Houlbrooke et al., 2011; McDowell and Houlbrooke, 2009). Soil compaction and a reduction in soil pore size occur with intensive grazing and damage increases in winter conditions. Monaghan et al. (2007) noted that extensive drainage systems under farm paddocks have the consequence of creating a pathway for transport of nutrients and microbial pollutants directly into watercourses without the attenuation afforded by vegetation planted alongside streams. In addition, effluent from dairy shed washing is increasingly a large contributor of potential waterway pollution. The high

numbers of microbial and chemical FST and FIB indicators observed in this study (10^7 - 10^{10} CFU/GC 100 mL⁻¹) in the supernatant re-suspensions of fresh faeces are reflective of the high concentrations of indicators that could be expected in this type of dairy shed runoff.

The mobilisation rates determined in this study for *E. coli* and FST markers will help the evaluation of mitigation strategies to reduce the effects of increased agricultural runoff. McBride (2007) has shown that limiting the runoff of *E. coli* into farm streams leads to a significant reduction in *Campylobacter* loads to waterways.

Modelling tools have been designed to assess nutrient and microbial contributions from livestock farming (Muirhead et al., 2011; Muirhead and Monaghan, 2012). Another toolkit has been developed that encompasses both the physical environment and the social aspects that contribute to faecal contamination from agricultural stock (Oliver et al., 2009). This dual toolkit evaluated four different aspects, firstly the burden of *E. coli* contamination as predicted by the daily excretion rate in faecal material, numbers of livestock per farm and the decay of *E. coli* in defecated faeces. The next two factors ascertained the likelihood of faecal transfer to watercourses via natural features, for example, slope and soil structure; and through farm infrastructure such as drains. The fourth criterion focussed on the social aspect to understand the obstacles such as debt financing and labour shortages that contribute to preventing appropriate mitigations being implemented.

The first two factors of the Oliver et al. (2009) toolkit were investigated in this current rural study being 1) the burden of *E. coli* and its decay in ageing faecal deposits and 2) the transfer of faecal pollution to waterbodies by overland runoff mechanisms. The mobilisation rates and T₉₀ concentration reductions defined for *E. coli* derived from cowpat runoff could be applied to such models and furthermore, the model accuracy could be enhanced by inclusion of the initial concentrations and mobilisation rates for the bovine-associated FST markers as determined by this study.

5.3.4 The effect of ageing faecal sources on water quality interpretation

In the rural study, the persistence of the total coliforms in the ageing cowpat affected the expected increases in the faecal ageing ratio AC/TC as the cowpat aged. There was still substantial mobilisation of TC including *E. coli* from the aged cowpat so although AC/TC ratios of fresh faeces were very low at <0.5, the ratio fluctuated between this value and 7.0 until Day 105 in the cowpat supernatant and Day 50 in the rainfall runoff. According to Brion (2005), AC/TC values <5.0 are indicative of fresh faecal inputs and ratios >20.0 of historical inputs.

The findings of this rural study suggested that during a flood event, the AC/TC ratio could be expected to indicate fresh faecal pollution derived from re-suspended decomposing cowpats. This suggests that during heavy rainfall events, the generation of substantial overland flow would invalidate the use of the AC/TC ratio as a faecal ageing tool. In contrast, lighter rainfall conditions would yield lower rates of mobilisation of TC from cowpats deposited on the field after a month, and consequently produce higher AC/TC ratios indicative of aged pollution. It is likely that microbes in overland runoff from non-flood conditions would be further attenuated by interaction with soil and particulate matter. Attenuation processes could include adsorption of microbes/microbial DNA to organic matter and degradation/decay reducing the microbial and FST PCR marker load entering waterbodies (Forge et al., 2005; Pietramellara et al., 2009; Piorkowski et al., 2014b). It is recommended that where runoff from non-flood conditions may confound water quality monitoring, application of the Bacteroidales host-associated PCR markers for monitoring purposes is preferable to assessments relying on the environmentally persistent *E. coli*. In the aftermath of flooding, however, *E. coli* and bovine-associated FST marker detection in waterways must be interpreted with caution as high levels of these indicators are likely to be indicative of aged faecal sources. Currently we do not have enough information to understand if these indicators would be associated with concomitant pathogen runoff from cowpats.

5.3.5 Microbial indicators and FST markers as indicators of health risk

Identification of aged sources needs to be placed in the context of health risk from pathogens, as aged pollution can still be concomitant with identification of pathogens as shown by the urban river study of sediments. Most of the agricultural runoff mitigations have been investigated for their effect on reducing nutrient sources (McDowell and Houlbrooke, 2009; Monaghan et al., 2007) rather than microbial runoff and, in particular, pathogen transport to waterbodies. There is a need for the further assessment of faecal pathogens in the farm cycle (Sobsey et al., 2006) including the ageing of dairy effluent in animal wastewater ponds and its effect on the survival of microbes, incorporating this knowledge into mitigation strategies in the NZ context.

The relationship between the FIB and FST markers and their correlation with pathogens could be further explored with FST PCR markers such as CowM2 as potential indicators of pathogens (such as *Campylobacter*) less tolerant of environmental conditions (Moriarty et al., 2011a; Moriarty et al., 2012). In contrast, the rural study showed that *E. coli* was a more conservative indicator, useful for those pathogens that persist post-defecation, particularly

pathogenic *E. coli* such as *E. coli* O157:H7, and possibly protozoa and viruses (Sobsey et al., 2006).

Temperature experiments have noted the inactivation of *Cryptosporidium* at temperatures >40°C (King and Monis, 2007; Li et al., 2005). Similar to the rural study, Li et al. (2005) detected very high internal cowpat temperatures of 40 to 70°C when the ambient air temperature reached 25°C and higher. They noted the inactivation of *Cryptosporidium* oocysts in cowpats at temperatures >40°C with >3.0 log removal of oocysts per day. Therefore, Li et al. (2005) suggested that based on the average oocyst concentration in both calf and adult cattle faeces, after two days the oocysts would have been inactivated by the high temperatures. This again suggests that *E. coli* monitoring may overestimate the health risk from *Cryptosporidium* associated with cowpats. Future studies targeting aged pollution sources and/or low FIB concentrations, therefore, need to be assessed in tandem with pathogen detection and infectivity for all microbial groups as suggested by other researchers (Corsi et al., 2015). Such assessments would enhance understanding of the potential for overestimation/underestimation of risk associated with FST marker detection.

5.4 Practical considerations for implementation

This section addresses the third objective of the thesis, which was to integrate the findings of the urban river and rural studies to provide a cohesive framework of recommendations for improving interpretations of current water quality tools. These recommendations include a discussion of indicators that can be employed to discriminate between recent and historical faecal sources. Tables are provided outlining the appropriate application of the various tools in the FST toolbox to particular contamination scenarios (Table 28 and Table 29). In addition, barriers to implementation of FST monitoring are discussed including the cost associated with FST tools and a comparison of the environmental stability of the individual tools, which impacts their suitability for a specific contamination event.

5.4.1 The application of indicators for ageing faecal contamination

AC/TC as a faecal ageing ratio

Table 28 presents AC/TC ratio values determined in this study and by other researchers as useful for evaluating the age of faecal inputs in water bodies. In the urban study, faecal ageing ratios of AC/TC <1.5 in river water were indicative of continuous inputs of faecal pollution from human sources. The significant negative correlations between the AC/TC ratio and all human FST markers and pathogens supported low AC/TC values <1.5 as useful indicators of potential pathogens.

The rural study showed that evaluation of faecal ageing in a water sample using the AC/TC ratio should only be performed during baseflow conditions when overland flow from rainfall is minimised. Application of the AC/TC ratio to only evaluate baseflow conditions, however, is likely to also apply to urban waterways where a high loading of dog faeces has been observed in rivers after rainfall (Moriarty and Gilpin, 2009). Investigations of ageing effects on dog faecal scats have not been performed, however, it is probable that, similar to cowpats, they will generate substantial FIB concentrations when mobilised by heavy rainfall, resulting in fresh faecal signals from the AC/TC ratio. Furthermore, where heavy rainfall generates sewer overflow into urban waterways, then the AC/TC ratio could also be indicative of fresh faecal inputs.

In sediments, the steroid ratio of coprostanol/epicoprostanol showed potential as a faecal ageing ratio when a waterbody was impacted by untreated human faecal inputs. There were significant differences in the cop/epicop ratio between the two discharge phases at the two active discharge sites but this ratio requires further validation in sediment. There was a lack of correlation in sediment between pathogens and the chemical FST markers, including the

cop/epicop ratio. There was also no association between indicators in the water and the underlying sediment. The conclusion drawn from this disconnect was that the water was detecting recent faecal inputs (albeit diluted by transportation processes), in comparison to the sediments, which were providing a historical or cumulative signature of past faecal events.

Table 28: AC/TC ratio values for assessing the age of faecal inputs

Source	Event	AC/TC ratio	Reference
Human sewage	Untreated sewage discharged into waterbody	0.3 - 1.7	As seen in urban river study during sewage discharges in association with HumM3 PCR marker
Human sewage	Sewage discharge into river	1.5 - 3.9	Brion (2005)
River water	Wildfowl and dog inputs to river	1.0 – 6.0 and 15.7	Urban river study in association with FST markers of avian and/or dog pollution
River water	River returning to a healthier environment, less likelihood of pathogen detection including viruses	>15.0	Black et al. (2007)
River water	Aged faecal events, river returning to a healthier environment	>20.0	Black et al. (2007)
River water	Heavy rainfall	3.0	Brion et al. (2002);
	Day 3 after storm	10.0	Nieman and Brion (2003)
	Day 7 after storm	79.0	
Fresh cow manure	Day 1	<1.0	Brion (2005)
	Day 14	2.9	
Cowpats flood runoff	Day 1	0.1	Rural cowpat study
	Days 7-22	<1.9	
	Days 29-105	3.1-6.8	
	Days 134-162	>55.0	
Cowpats rainfall runoff	Day 1	0.1	
	Days 8-29	2.2-5.8	
	Days 50 -162	4.8 - 126	

The host-associated PCR marker, HumM3 was identified in river water as an indicator of recent faecal inputs from human sources because it was detected on all occasions during active discharge but had a low rate of detection post-discharge when compared with the other two human PCR markers. Due to the ongoing intermittent discharge of raw sewage into the urban river, it was not possible to identify a timeframe, in terms of days/weeks for signalling a change from a recent to an aged faecal event.

In the rural study, the host-associated CowM2 PCR marker was also identified as signalling a recent ruminant faecal input. The CowM2 marker was identified in high abundance

(10^8 and 10^7 GC/100 mL) in supernatants and rainfall runoff (respectively) from fresh faecal cowpats. It was, however, no longer detected in supernatants after 50 days, or rainfall runoff after Day 22. Therefore, it was suggested that CowM2 would be useful for the confirmation of fresh bovine/ruminant pollution but should only be assayed when the BacR marker is detected in water at $>10^4$ GC/100 mL because CowM2 was generally one to two orders of magnitude lower in cow faeces compared with BacR and the detection limit of many PCR assays is approximately 500-1000 GC/100 mL.

The metagenomic study of ageing cowpats observed the identification of microbial community shifts in the cowpat. Dominance by bacteria that inhabited the cow rumen was shifted to those bacterial groups that out-competed the initial community by adaptation to the unique environment of the decomposing cowpat. Operational taxonomic unit (OTU) sequences assigned to the genus *Ruminococcus*, and to the families of Actinomycetales, Flavobacteriales and Sphingobacteriales were identified as potential indicators of fresh and ageing faecal environments (respectively) when water pollution was derived from runoff from bovine sources. Further investigation is required to determine if the OTUs dominant in decomposing cowpats are unique to this aged environment or are ubiquitous in soil and waterways. Future studies based on metagenomic assays of water will be able to draw on the sequence information generated in this rural study when river water samples are impacted by bovine pollution. The sequence data generated was derived from flood-like conditions and could be compared with river populations drawn from baseflow, to investigate river microbial populations under these two flow regimes.

5.4.2 Recommendations for a better approach

Overall, the findings from the urban and rural studies conclude that interpretation of faecal contamination in aquatic environments requires implementation of a cohort of microbial and faecal source tracking indicators to increase confidence in the assessment of both faecal source identification and the potential for health risk. Table 29 provides an assessment of some of the scenarios encountered by water managers with recommendations for which FST tools are appropriate for a particular contamination scenario. The recommendations for FST tools are based on practical experience and the findings from this current study. Also included is a suggested truncated version of FST analysis when cost is a limiting factor.

Table 29: Recommended approaches for FST tools under specified conditions

Specified condition	Recommendation for FST tool if FIB elevated	When cost is a factor
Fresh faecal input to water <i>E. coli</i> >500 CFU/100 mL	AC/TC, PCR markers, FWA and faecal steroids	AC/TC, Host-associated PCR markers (store water for steroids for one week or freeze one month, so can test if required)
<i>E. coli</i> ≤500 CFU/100 mL in water	AC/TC, Faecal steroids and PCR markers (unless aged sources suspected)	AC/TC, Faecal steroids
Untreated waste discharging into river water (diffuse pollution)	AC/TC, F-RNA phage, PCR markers (including HumM3) and faecal steroids (cop/epicop ratio confirms treatment status)	AC/TC, F-RNA phage and PCR markers (including HumM3 to indicate recent event)
Treated waste discharging into water (diffuse pollution)	F-RNA phage, Faecal steroids (cop/epicop ratio confirms treatment status) and PCR markers	F-RNA phage, Faecal steroids (PCR marker concentrations are reduced by treatment)
Water sample stored >24 hours	Faecal steroids and filter higher volumes for PCR markers (≥300 mL)	Faecal steroids
Stormwater	AC/TC, PCR markers, faecal steroids and FWA	AC/TC, FWA
Suspected environmental sources of <i>E. coli</i>	AC/TC, PCR markers and faecal steroids (which will provide an assessment of plant steroids)	AC/TC, PCR markers
Agricultural sources	AC/TC, faecal steroids and PCR markers (including host-associated PCR markers like BacR and CowM2 - if BacR >10 ⁴ GC/100 mL)	AC/TC, PCR markers
Suspected meatwork effluent	Faecal steroids, PCR markers	Faecal steroids
Indigenous avian faecal sources	Faecal steroids, avian PCR markers	Faecal steroids (when only avian pollution is suspected)
Heavy rainfall	PCR markers as faecal steroid signal will be dominated by plant steroids due to overland runoff	PCR markers

In the urban river system under study, the concentration of *E. coli* derived from untreated human sewage was shown to be a reliable indicator of public health risk from contaminated water. In addition, F-RNA phage were identified as suitable, cost-effective indicators to be measured in conjunction with *E. coli* as a signal of a recent faecal input. This finding is supported by recommendations by USEPA for the addition of coliphages, which includes the F-RNA phage, into the conventional monitoring water quality toolbox (USEPA, 2015).

In the urban and rural studies, the PCR markers and steroids were powerful indicators of faecal source inputs. Strong to moderate correlations between the two FST methodologies in the urban river study suggested they could be used individually or combined when greater confidence in the result was required. In addition to being source specific, in the urban river study, these FST markers showed weak to strong correlations with protozoan pathogens, which were similar and in some cases superior to correlations between *E. coli* and protozoa.

Campylobacter had the weakest associations with all microbial and FST indicators. It was observed, however, that where elevated *E. coli* levels were detected, identification of the HumM3 PCR marker in conjunction with F-RNA phage and a low AC/TC ratio <1.5 was indicative of fresh pollution and an associated health risk from *Campylobacter*. This requirement for a suite of indicators to predict *Campylobacter* presence was probably symptomatic of the observed short term persistence of *Campylobacter* in the aquatic environment. The FST PCR markers that target single copy genes, HumM3 and CowM2, were less persistent in the environment than *E. coli* and were shown to be useful indicators of recent faecal inputs to a waterbody.

5.4.3 Obstacles to implementation

Cost of analysis and stability of FST markers after sample collection

When cost parameters determine that only one FST method can be employed then the tools need to be assessed based on their merits, the catchment under investigation, and the likely contamination sources (Tran et al., 2015) (Table 29). PCR markers provide a more rapid evaluation of water samples compared with steroids; however, the advantages of faecal steroid analysis include their superior stability. Steroids are not degraded by chemical treatments and therefore maintain a signature for human waste in treated effluents (Sinton et al., 1998), and stable bovine steroid ratios in decomposing cowpats as shown by the rural study. In contrast, bacteria are susceptible to chlorination, and therefore, FIB and microbial PCR markers may not indicate the presence of pathogens in treated waste (King et al., 1988; Koivunen and Heinonen-Tanski, 2005; Tyrrell et al., 1995). Furthermore, steroid analysis (coprostanol/epicoprostanol ratio) in the urban river study was able to confirm the identification of untreated wastewater, allowing health risk assessments based on known pathogen-indicator correlations in raw sewage.

Steroids in water are stable for at least a week when refrigerated in the dark and unlike PCR markers, the water can be frozen for a month prior to processing (Gregor et al., 2002) or samples filtered and frozen. This strategy enables storage of water samples/filters for later

steroid analysis of selected samples if additional confidence is required when PCR is employed as the initial tool.

It has been observed that water samples containing levels of *E. coli* below the Action level of 550 CFU/100 mL may not contain detectable levels of PCR markers, however, levels of steroids in the sample can still provide useful quantitative data for interpretation of sources (Derrien et al., 2012; Gourmelon et al., 2010). This assertion was supported by observations in the urban river study at Owles Terrace post-discharge (Appendix, Table 32). This low detection of PCR markers could be an ageing or dilution effect, as in contrast, reasonable concentrations of PCR markers have been detected in river water at *E. coli* concentrations <500 CFU/100 mL (personal communication, Brent Gilpin).

PCR markers allow a finer detail of the species attribution of animal pollution compared with the broader discrimination of human versus ruminant versus avian afforded by steroid analysis (Devane et al., 2015). However, this specificity of the PCR markers may also mean that certain types of avian pollution such as indigenous birds may not be identified by PCR markers, because the majority of avian PCR markers have been designed for seabirds and ubiquitous waterfowl such as ducks (Ahmed et al., 2016; Devane et al., 2007; Green et al., 2012). Additional benefits of steroid analysis include that multiple steroids are analysed in one assay providing supplementary information, such as information on the impact of plant decay/runoff based on the proportions of plant sterols identified (Nash et al., 2005). Another example is the ability to identify meatwork effluents, when cholesterol on its own dominates the sterol profile of the water sample (Devane et al., 2015).

Fluorescent Whitening Agents (FWA) are susceptible to photodegradation (Kramer et al., 1996) and chlorine degradation (Burg et al., 1977) and in the urban river study were generally not detected in water even during continuous discharge of sewage. Degradation by sunlight, dilution and the reduced use of laundry detergents containing FWA due to the earthquakes, however, may have been factors contributing to the low levels in the urban river study. Previous studies have identified FWA in stormwater drains from leaking sewer pipes suggesting they are useful in the urban context of low volume stormwater drains (Gilpin et al., 2003).

Geographic differences between steroid concentrations in faeces

In Australasian studies, 24-Ecop was identified as the major steroid in cow faeces (Devane et al., 2015; Nash et al., 2005). In comparison, several European studies have recognised 24-ethylepicoprostanol and 24-ethylcholestanol as dominant (Derrien et al., 2011; Gourmelon et al., 2010; Jaffrezic et al., 2011). In this rural study and that of Derrien et al. (2011) similar

concentrations of steroids were identified in fresh and aged cowpats, and in cow manure, both fresh and aged (respectively). Differences in steroid composition between studies, therefore, may reflect regional differences in diet and microbial gut composition rather than ageing processes. These geographic differences, however, need to be taken into account when applying relevant ratios to discriminate faecal sources. The much higher proportion of 24-Ecop in the bovine faeces of this study would reduce the H4 ratio of %cop/(cop+24-Ecop) to a greater extent compared with the European faecal sources, which noted an overlap between porcine and bovine steroid ratio discrimination. Their analysis required the application of PCA to discriminate the different sources as they could not rely directly on steroid ratios. For the second ratio, R3 (24-ethylcholestanol/cop), cop and 24-ethylcholestanol were identified in similar concentrations in both studies, therefore, they should not prevent correct source assignment. We have little information on sterol profiles in pigs in the NZ situation, both feral and farmed, and this is a knowledge gap that requires research to ascertain if these two ratios, %H4 and R3, provide bovine/porcine/human discrimination in the NZ context.

5.5 The future of water quality monitoring

A waterbody is a complex biological system, which requires an understanding of the surrounding catchment and landuses within. Regional weather patterns, tides and river flows impact on water quality, with the water column being only one of the environmental matrices harbouring microorganisms within a waterway. Resuspension of FIB and pathogens from macrophytes and sediment/soil/beach sand reservoirs has been recognised as contributing to the microbial population detected during routine monitoring of water quality. These factors influence the multi-dimensional approach that is required to predict the health risk attributed to a waterbody used for recreational purposes, and influence the health advisories the water managers are required to make on a daily basis.

Automated systems for continuous monitoring of water quality

Recently there has been a move towards automated systems of predicting health risk with instrumentation collecting continuous data on site and the latest innovation is relaying that real-time data to water managers via the internet so they can update health advisories daily. These techniques overcome the draw backs of relying on FIB or quantitative PCR marker indicator tests that require at least 18 to 4-6 hours, respectively, for a result. Automated systems include those that measure FIB such as ColiMinder® OMS (Vienna Water Monitoring solutions). ColiMinder® measures enzymatic activity, for example, beta-glucuronidase for *E. coli* and can

operate in both fresh and marine water. Other systems rely on the collection of on-site hydrological data (wave height and period, turbidity and water temperature) and weather conditions (including wind direction and speed, rainfall and temperature) to predict the likelihood of high FIB concentrations (Shively et al., 2016). These systems rely on the well characterized impacts of tidal and wave effects on resuspension of FIB from sediments and beach sands. They also draw on the knowledge of land runoff created by rainfall events, which usually carry high FIB loads into nearby waters. Additionally, mobile droplet digital qPCR marker systems are being trialled to allow detection of FIB and faecal sources from water samples at beaches with potential turn around times of 2 hours, which would provide real-time data for the assessment of same day analysis of water quality. These advancements would allow closure of popular swimming locations on the actual day when health risk is deemed too high (Marx, 2015).

The systems above rely on indicators, which assess parameters considered likely to indicate the presence of pathogens. Future innovations based on the advent of new technologies such as next generation sequencing (NGS) may allow us concurrent detection of the faecal source indicators and actual pathogens, or rather representatives of each pathogen group (Aw and Rose, 2012; Fujioka et al., 2015; Newton et al., 2013; Shanks et al., 2013b; Vierheilig et al., 2015). The differential survival characteristics between pathogens groups (protozoa and *Campylobacter*) noted in the urban river study and other studies suggests that there is a need to include a broader range of pathogens as “indicators” of their respective group. It would be difficult to aim to detect all possible pathogens present in a water sample, due to 1) they are often present in very low concentrations, which still represent a health risk; 2) known pathogens may be absent/not detected but a faecal event has still occurred and must be identified to acknowledge its inherent health risks. This second point emphasises the continued requirement for proxy parameters indicative of faecal contamination.

Aiding the identification of indicators and pathogens with real-time streaming of data are the new portable technologies based on NGS techniques. One of these innovations is the MinION (Oxford, Nanopore Technologies), which is a portable device for molecular analyses that is driven by nanopore 'strand sequencing' technology, where the DNA/RNA molecule passes through a protein nanopore, sequencing in real time as the nucleic acid translocates the pore. The inventors of this technology are developing disposable sample preparation devices designed to convert complex samples such as blood or environmental samples directly onto a nanopore sensing device. It is proposed that the field deployed MinION would be vertically integrated with a cloud based service for real-time molecular analyses.

Isolation methods for a diverse group of pathogens in a water sample

The detection of diverse groups of pathogens such as bacteria, virus and protozoan groups is challenging and expensive but worthwhile to improve assessments of health risks (Corsi et al., 2015). There are technical problems that arise when trying to combine methodologies for the extraction and detection of all microbial groups from water for either FST/pathogen detection or NGS metagenomic assays. For example, these different method requirements would prove problematic if wanting to include all pathogen groups in the detection repertoire for the automated on-site qPCR machines discussed above. When dealing with faecal water contamination, the filtering of 100 to 500 ml of water is sufficient to identify many of the waterborne FIB and potential bacterial pathogens. Protozoa and viruses, however, require higher volumes (10 to 100 L) of water to be filtered to improve sensitivity (Ahmed et al., 2015a; Wong et al., 2012), which requires specialised field equipment. Filtering of such high volumes increases the likelihood of concentrating inhibitors, which will disrupt PCR analysis. Currently, researchers are investigating novel extraction procedures for simultaneous recovery of all pathogenic microbial groups from a range of water matrices including tap and river water. The aim is to improve efficiency and recovery, whilst reducing the inhibition of molecular assays such as PCR (Ahmed et al., 2015a; Gibson and Schwab, 2011; Polaczyk et al., 2008).

Increasing the sensitivity of qPCR methods may be enhanced by targeting actively growing cells, which carry multiple copies of ribosomal RNA. FST studies employing rRNA-targeted reverse transcription-qPCR assays have shown improved sensitivity compared with DNA-based qPCR methods (Kapoor et al., 2015; Pitkänen et al., 2013). Targeting the RNA rather than DNA overcomes the problems associated with detection of extracellular DNA or non-viable, and therefore, non-infectious cells. In a mixed source sample, however, the unknown number of multiple copies of RNA/cell may restrict the ability to quantitatively assign contribution from each faecal source. Incorporation of techniques for identifying RNA would also enable the detection of RNA virus groups (Aw and Rose, 2012).

These new technologies should not spell the demise of microbial indicator testing (FIB and coliphage), which is still a powerful sentinel of potential faecal contamination at least for the near future and for localities where access to sophisticated laboratory assays is limited. Current FST identification technologies such as PCR markers and faecal steroids and the AC/TC faecal ageing ratio will provide frontline weapons against waterborne disease in many territories. These FST tools provide not only information on the presence of faecal contamination at recreational sites but also identification and evidence for ageing of faecal sources, and association with pathogens. This faecal source information can then be translated into better predictability of

health risk based on QMRA-type analyses, which recognise the differences in pathogen potential between faecal types. In the future, it is hoped that these FST tools will be supplemented by next generation sequencing technologies that allow the concurrent screening of the indicators and pathogen groups in water.

6 Chapter Six:

Conclusions

The faecal source tracking (FST) studies conducted for this thesis have added to the knowledge about the environmental persistence of microbial indicators and FST markers used for water quality monitoring of freshwaters in NZ.

There has been concern that because *E. coli* is capable of long term persistence in the environment in temperate climates it is no longer a valid frontline tool for water quality monitoring. However, in the study of an urban river impacted by continuous discharges of untreated human sewage, it was demonstrated that *E. coli* was a suitable microbial indicator for establishing a public health risk. Furthermore, as an indicator, *E. coli* outperformed F-RNA phage and the ubiquitous *C. perfringens*. In the rural study, decomposing cowpats were shown to harbour high concentrations of *E. coli*, which were available for mobilisation after flood and lighter rainfall events. These results suggest that for at least five and a half months post-deposition *E. coli* could be mobilised from cowpats at levels that if washed into water by flood conditions could exceed water quality guidelines. It has yet to be established, however, whether the actual health risk from ageing cowpats is equivalent to that from fresh faecal deposits.

In the studies of urban and rural faecal inputs presented within this thesis, PCR markers and faecal steroids were shown to be reliable indicators of the source(s) of faecal contamination. In addition, in the urban river study, these two FST tools were good predictors of protozoan pathogen presence, and hence indicative of human health risk.

This research determined the temporal effect of post-faecal deposition on FST marker signatures, and therefore the impact of ageing on faecal source attribution. Faecal ageing tools were evaluated in the rural and urban environments, including assessing novel markers for discriminating between fresh and historical faecal inputs. The sequence information generated by the amplicon-based metagenomic assay of microbial community changes in decomposing cowpats will allow identification of microbial differences between river microbial populations under base flow and flood conditions. This will aid the identification of potential markers of aged/fresh sources of bovine/ruminant faecal pollution.

This research contributes to the interpretation of *E. coli* levels used for alerting water managers to a faecal contamination event. The results emphasise the differences between the types of faecal sources and that interpretation of elevated *E. coli* concentrations is dependent on

knowledge of the source of contamination. The differential fate and transport of microbial and FST markers noted in this study and others, supports the use of diverse FST tools to provide multiple lines of evidence for tracking the source(s) of faecal contamination and indicating the associated public health risk.

6.1 Key research findings

6.1.1 FST use in urban environments

The findings for the urban river study can only be applied to the discharge of untreated sewage into a river. Relationships between indicators, pathogens and FST markers would be altered if the source of discharge was treated human wastewater, and the persistence of all parameters would vary dependent on the type of sewage treatment applied.

Key observations from this urban river study included;

- There were moderate to strong, significant correlations observed between the microbial indicators of *E. coli*, F-RNA phage, pathogenic protozoa and FST tools (PCR and steroid markers) in water samples, suggesting that FST tools can be useful indicators of faecal source and potential pathogen presence when untreated human sewage impacts a river system.
- In general, PCR and steroid FST markers were better predictors of human pollution and health risk than the microbial indicators.
- There were few significant correlations between *Campylobacter* and other variables in water. In association with elevated *E. coli* levels, detection of the following suite of markers: the human PCR marker, HumM3; a low AC/TC ratio <1.5, and F-RNA phage, suggested recent human faecal inputs and increased health risk from *Campylobacter*.
- F-RNA phage were identified as indicators of recent faecal contamination with lower persistence in freshwater environments compared with *E. coli* and *C. perfringens*. This aspect makes them a useful, low cost, frontline tool to be used in association with *E. coli* levels for determination of health risk associated with recent, untreated human sewage discharges.
- There was a significant, substantial agreement between the two FST tools: Human-associated PCR and steroid markers when applied to water quality monitoring. Water managers, therefore, could be confident in the results using either FST method. In addition, the urban river study provided verification of the 5-6% coprostanol level in water as indicative of human faecal inputs.

- The faecal ageing ratio of AC/TC had value as a cost-effective tool in water quality monitoring to determine if elevated FIB were associated with fresh faecal inputs (AC/TC <1.5) during discharge of raw sewage.
- A coprostanol/epicoprostanol ratio of ≥ 20 in association with low levels of epicoprostanol (<1% of total steroids), identified untreated human sewage as the predominant faecal source in river water.
- The coprostanol/epicoprostanol ratio in sediment showed potential in discriminating between fresh and aged faecal inputs in sediment when the source was untreated human sewage.
- F-RNA phage and *Campylobacter* did not accumulate in sediments. *E. coli* persisted in sediments but levels were much lower post-discharge than during active discharge. *C. perfringens* was identified in high concentration in sediments during and after discharge.
- Potentially pathogenic protozoa persisted in river sediments after cessation of active sewage discharges for at least six months. Therefore, sediment re-suspension increases health risk from the re-mobilisation of pathogens.
- The FST markers: fluorescent whitening agents and faecal steroids, appeared to be stored in sediments after the major discharges had ceased but had few correlations with either microbial indicators or pathogens in sediment. Concurrent evaluation of water samples and underlying sediment showed a disconnect between FST markers in the two matrices and suggested that sediment represented a historical picture of pollution inputs.

6.1.2 FST use in rural environments

The impact of ageing on the microbial community in cowpats five and a half months post-defecation has not previously been investigated under field conditions. In particular, it had not been demonstrated whether faecal steroids maintain a stable bovine faecal signature during the long-term ageing process. This study described the first amplicon-based metagenomic assay of the microbial community in an ageing cowpat and revealed shifts in the community composition over five and a half months. The anaerobic Orders, Clostridiales and Bacteroidales are present in the cow rumen and were the dominant groups in the first month after cowpat deposition. Summertime conditions, with low rainfall and high sunshine hours, were recorded in conjunction with a community microbial shift to Actinomycetales, Sphingobacteriales and Flavobacteriales bacterial groups. The hypothesis that these microbial community shifts would impact on the steroid ratios used as FST signatures was disproved. There were, however, changes in the bacterial groups targeted by the PCR markers, and in *E. coli* concentrations. Decreasing

mobilisation from cowpats was noted for these microbe-based markers when cowpats were subjected to both flood conditions and rainfall.

Key observations from this rural study included;

- The metagenomic study of ageing cowpats observed microbial community shifts in the mobilised fraction from ageing cowpats and identified bacterial groups that out-competed the initial community by adapting to the unique decomposing cowpat environment. A member of the *Ruminococcus* genus was noted to be dominant in fresh faeces, but was replaced by members of the bacterial Orders, Actinomycetales and Flavobacteriales, which were predominant in aged faecal runoff. These bacterial groups could be targeted as potential indicators of fresh and aged pollution from bovine sources.
- After five and a half months of ageing under temperate field conditions, 1 kg and 2 kg original wet weight cowpats still contained appreciable amounts of *E. coli* that was available for mobilisation if subjected to flood conditions. The *E. coli* mobilised from a one kg cowpat by a lighter rainfall event was considerably reduced after the initial defecation, from Day 8 until two and a half months, from which time *E. coli* concentrations were very low but still detectable till the end of the experiment at five and a half months.
- The ten steroids assayed and the total steroid concentration, all had statistically similar decline rates when mobilised from the cowpats into re-suspended cowpat supernatants and rainfall runoff. In addition, irrigation treatment of decomposing cowpats did not affect mobilisation rates of individual steroids from re-suspended cowpats.
- The equivalent mobilisation rates observed for individual steroids validated the use of steroid ratios as FST markers of fresh and aged bovine faecal runoff.
- Individual FST PCR markers were mobilised at similar rates in both re-suspended cowpat supernatant and rainfall runoff, although CowM2 was noted to decrease below the detection limit much sooner than the GenBac3 and BacR PCR markers.
- Although, the CowM2 marker was identified in high abundance in the supernatant from fresh faecal cowpats, it was not detected in supernatants after 42 to 50 days, or rainfall runoff after Day 22. Therefore, CowM2 is useful for the confirmation of fresh bovine/ruminant pollution but should only be assayed when the BacR marker is detected in water at $>10^4$ GC/100 mL.
- It is recommended that where runoff from non-flood conditions may confound water quality monitoring, application of the Bacteroidales host-associated PCR markers for

monitoring purposes is preferable to assessments relying on the environmentally persistent *E. coli*.

- The ratio of BacR to GenBac3 PCR marker was analysed as a surrogate of BacR/Total Bacteroidetes to ascertain the ruminant contribution of faecal pollution. A BacR/TotalBac of >15% was a stable indicator of 100% contribution by ruminant sources when analysing rainfall runoff from cowpats, from one to five and a half months post-defecation. Results from the re-suspended cowpat, however, were inconclusive for all ageing stages of the cowpat supernatant. In conclusion, BacR/TotalBac of >15% was a stable indicator of 100% contribution from fresh bovine sources subject to runoff from either light rainfall or flood conditions.
- During ageing of the cowpat, moderate to strong correlations ($p < 0.05$) were noted between *E. coli*, the three PCR markers, total steroids, and the herbivore steroid 24-Ecop. The strong associations between these microbial and FST markers supports their use as a toolbox, indicative of fresh and aged bovine faecal sources.
- There were moderate correlations noted between the faecal ageing ratio, AC/TC and the toolbox of FST markers. Persistence/growth of total coliforms (TC) including *E. coli* as observed in the ageing cowpats, however, impacted on the ability of the AC/TC faecal ageing ratio to identify aged runoff from cowpats. As suggested by Brion (2005), it was noted that the AC/TC ratio, should always be evaluated in relation to the FIB concentration to determine its relevance in assigning faecal age. FIB levels below the water quality guidelines, in conjunction with high AC/TC ratios (>20.0) would indicate a reduced likelihood of recent faecal pollution. In addition, from the results of this study, the AC/TC ratio of water samples impacted by heavy rainfall will produce low AC/TC ratios indicative of recent pollution even when derived from cowpats that have been lying on the field for four months.
- Overall, after the first week post-defecation, the rainfall runoff samples from cowpats contained significantly lower concentrations of each of the FST markers compared with the re-suspended cowpats. This suggests that soil attenuation processes occurring during overland runoff may provide a further degradation barrier to microbes and FST markers derived from lighter rainfall cowpat runoff, leading to a reduction of the indicator signal entering waterbodies.

6.2 Recommendations for future research

- Conventional monitoring of F-RNA phage was identified as a useful adjunct to *E. coli* for detection of untreated human sewage discharges to waterways. Implementation of published genotyping methods using PCR markers for faecal source discrimination between animal and human-associated genotypes of F-RNA phage (Friedman et al., 2011; Wolf et al., 2008) need to be evaluated as an additional tool for FST PCR markers. Furthermore, epidemiological studies incorporating the genotypes of F-RNA phage are required to increase the understanding of health risks associated with identification of these species-specific phage markers.
- Although the faecal ageing ratio, AC/TC had significant negative correlations with *E. coli*, and FST markers, the maintenance/growth of the TC population in the cowpat contributed to the AC/TC ratio from cowpat runoff remaining below the level of 20 which would have suggested historical inputs. Detection of pathogens in ageing cowpats and their runoff would need to be investigated to see if the lower AC/TC ratios are indicating sources of faecal pollution that still present a health risk to humans.
- The urban river study was unable to validate the use of the AC/TC ratio as an indicator of fresh avian faecal inputs. Further investigations of waterways where avian faecal pollution is suspected as the dominant faecal input would need to be carried out to test if the AC/TC ratio is valid for avian inputs. Additional avian PCR markers would be needed to broaden the range of avian species detected. Avian steroid FST markers target a wider range of avian species but require the absence of human and herbivore sources to validate the AC/TC ratio.
- The steroid ratio, coprostanol/epicoprostanol identified untreated human sewage as the faecal source in river water. This is an important factor as untreated sewage has different health implications compared with treated sewage. In sediment, the utility of this ratio was suggested for discriminating between fresh and aged faecal inputs when derived from untreated human sewage. Further assessments are required to establish ratio thresholds for cop/epicop in these two matrices. This further analysis includes the conversion of coprostanol and cholesterol to epicoprostanol in sediment to establish criteria for differentiating fresh and aged untreated sewage inputs.
- The disconnect between aged faecal events in sediments, and the identification of persistent pathogens such as protozoa requires clarification to avoid the underestimation of health risk from re-suspended sediments containing aged faecal sources.

- Sources of faecal pollution from pigs have not been a notable concern in NZ because the prevalent livestock activities are sheep, cattle and dairy. NZ pig farms tend to be contained point sources; however, we do have feral pigs in the mountainous areas of NZ. Therefore, it is important to investigate both feral and livestock pig (porcine) sources for their FST signatures using PCR and steroid markers. It is necessary to clarify the ratios based on the stanols identified in mammalian faeces used by European studies to discriminate between human/bovine and porcine sources. Differences in the dominance of individual stanols (for example, 24-ethylcoprostanol) in bovine faeces has been noted between European and Australasian studies, which may affect ratio interpretation.
- Testing of farmed deer faeces for specificity of the CowM2 PCR marker noted 50% of individuals carried this PCR marker suggesting it would be better to call it a ruminant-associated marker rather than bovine-associated. Further testing of the specificity of PCR markers should be performed on feral deer faeces.
- The health risk associated with sheep farming has not been ascertained in international quantitative microbial risk assessments (QMRA). In NZ, the high levels of sheep farming suggests the requirement for a QMRA evaluation of sheep faeces using data published on pathogen levels in NZ sheep faeces. The increasing recognition of wildfowl as vectors of *Campylobacter* and other pathogens (Gorham and Lee, 2015; Moriarty et al., 2011b; Zhou et al., 2004) may also require a reassessment of the Soller et al. (2014) QMRA model for wildfowl and mixtures of wildfowl and human sources.
- There is a need for the further assessment of faecal pathogens in the farm cycle including the ageing of dairy effluent in animal wastewater ponds and its effect on the survival of microbes, incorporating this knowledge into mitigation strategies in the NZ context.
- Future studies based on metagenomic assays of water will be able to draw on the sequence information generated from cowpat runoff under simulated flood conditions in this rural study to compare with microbial river populations drawn from baseflow. The cowpat sequence data will be useful for the development of a faecal source library of bacteria, which could be applied to a metagenomic approach for FST. Furthermore, the metagenomic assay provided a unique perspective on the bacterial populations mobilised from decomposing cowpats. Further investigation is required to determine if potential bacterial candidates identified as targets of aged and fresh sources of bovine/ruminant faecal pollution are unique to the cowpat environment, or are ubiquitous in soil and aquatic environments.

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Appendix

Chapter Three: Urban river results

Table 30: Microorganisms and FST PCR markers in river water at the Boatsheds. Shading denotes active discharge period at KR and OT

WATER Boatsheds	CFU/ 100 mL		PCR markers gene copies/100 mL						CFU/ 100 mL	PFU/ 100 mL	MPN/ 100 mL	(oo)cysts/100 L	
	<i>E. coli</i>	AC /TC	GEN PCR	B.adol	HumBac	HumM3	Avian	Dog	<i>Clostridium</i>	Phage	<i>Campylobacter</i>	<i>Giardia</i>	<i>Cryptosporidium</i>
8-Mar-11	1000	1.52	*NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
23-Mar-11	700	2.65	25,081	1,900	NT	0	14,034	12,227	NT	NT	NT	NT	NT
30-Mar-11	1300	5.43	881,313	0	NT	0	18,134	7,240	NT	NT	NT	NT	NT
6-Apr-11	950	3.63	825,806	2,807	NT	0	880	16,267	NT	NT	NT	NT	NT
13-Apr-11	950	1.06	1,433,819	5,634	NT	0	21,368	667	NT	NT	NT	NT	NT
26-Apr-11	900	0.99	1,259,267	5,834	0	0	2,533	5,800	50	50	2.3	300	20
16-May-11	2400	0.92	3,221,094	48,502	38,835	1,500	0	6,366	0	1,050	0	180	20
28-Jun-11	2000	0.76	2,706,351	152,008	83,671	1,886	13,134	102,338	150	600	2.3	500	20
8-Sep-11	50	1.28	314,099	4,400	23,485	0	4,950	0	50	0	4.3	450	0
27-Sep-11	6200	1.01	6,908,206	355,018	146,674	6,400	6,267	0	50	NT	NT	NT	NT
11-Oct-11	1700	1.65	2,634,033	161,341	38,385	1,900	2,967	0	150	250	1.5	12	0
8-Nov-11	500	5.77	386,119	6,667	0	0	3,534	0	100	0	2.4	375	0
22-Nov-11	1100	1.70	724,619	4,667	0	0	37,835	0	150	0	0.4	30	0
6-Dec-11	900	0.61	994,028	4,534	0	0	2,001	0	100	0	2.3	36	0
20-Feb-12	450	0.42	1,488,041	98,838	48,502	0	5,517	3,472	100	150	0	45	0
6-Mar-12	550	1.29	1,148,824	0	0	0	26,435	1,623	50	0	9.3	18	0
11-Mar-13	1055	0.29	802,300	0	0	0	**633	0	NT	50	9.3	NT	NT
25-Mar-13	900	1.44	1,036,800	0	0	0	15,867	0	NT	0	2.3	NT	NT
8-Apr-13	1150	2.48	1,448,689	0	0	0	26,000	0	NT	0	4.3	NT	NT

*NT, Not tested; ** wildfowl PCR marker detection supported by steroid analysis

Table 31: Microorganisms and FST PCR markers in river water at Kerrs Reach. Shading denotes active discharge phase.

WATER	CFU/ 100 mL		PCR markers gene copies/100 mL						CFU/ 100 mL	PFU/ 100 mL	MPN/ 100 mL	(oo)cysts/100 L	
	<i>E. coli</i>	AC /TC	GenBac3	B.adol	HumBac	HumM3	Avian	Dog	<i>Clostridium</i>	Phage	<i>Campylobacter</i>	<i>Giardia</i>	<i>Cryptosporidium</i>
8-Mar-11	8,000	0.42	*NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
23-Mar-11	14,800	1.07	2,808,155	104,172	NT	3,622	1,517	2,653	NT	NT	NT	NT	NT
30-Mar-11	6,400	1.70	3,556,586	126,167	NT	4,000	8,667	7,254	NT	NT	NT	NT	NT
6-Apr-11	2,700	1.48	1,324,041	62,336	NT	2,767	0	14,901	NT	NT	NT	NT	NT
13-Apr-11	10,800	0.37	10,901,727	NT	NT	18,264	5,767	18,701	NT	NT	NT	NT	NT
26-Apr-11	9,400	0.63	6,376,867	348,684	92,338	5,800	16,750	0	550	3,950	9.3	300	20
16-May-11	4,200	0.80	6,832,408	433,355	238,179	6,634	0	18,250	150	3,450	110	240	20
28-Jun-11	3,200	1.00	2,094,753	108,505	110,839	2,267	3,258	87,504	350	1,200	0.7	250	20
8-Sep-11	1,600	0.47	5,090,293	551,694	161,841	3,934	4,834	1,720	200	450	9.3	750	0
27-Sep-11	5,200	1.04	8,556,135	645,032	170,509	5,467	0	0	350	NT	NT	NT	NT
11-Oct-11	1,700	2.31	868,891	20,901	12,717	0	0	0	300	250	0.4	54	0
8-Nov-11	1,600	1.39	1,244,962	82,004	80,671	1,167	0	0	250	200	9.3	375	0
22-Nov-11	2,300	1.76	1,700,118	31,568	0	900	6,384	0	350	0	0.7	60	0
6-Dec-11	700	1.40	428,621	3,632	0	0	0	0	50	0	1.5	12	0
20-Feb-12	850	2.98	469,657	5,484	5,967	0	0	0	0	250	0.4	6	0
6-Mar-12	900	0.67	589,458	15,634	4,917	**633	2,415	0	0	250	15	9	0
11-Mar-13	5,000	0.38	499,233	0	0	0	2,600	0	NT	100	46	NT	NT
25-Mar-13	1,070	1.11	516,433	8,883	0	0	2,033	0	NT	200	4.30	NT	NT
8-Apr-13	4,500	0.74	482,405	2,180	0	0	600	0	NT	0	46	NT	NT

*NT, Not tested; ** wildfowl PCR marker detection supported by steroid analysis

Table 32: Microorganisms and FST markers in river water at Owles Terrace. Shading denotes active discharge phase.

WATER	CFU/ 100 mL		PCR markers gene copies/100 mL						CFU/ 100 mL	PFU/ 100 mL	MPN/ 100 mL	(oo)cysts/100 L	
	<i>E. coli</i>	AC /TC	GenBac3	B.adol	HumBac	HumM3	Avian	Dog	<i>Clostridium</i>	Phage	<i>Campylobacter</i>	<i>Giardia</i>	<i>Cryptosporidium</i>
8-Mar-11	100,000	0.55	*NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
23-Mar-11	24,000	0.3	3,640,369	113,172	NT	3,233	0	813	NT	NT	NT	NT	NT
30-Mar-11	32,000	0.81	12,539,062	666,700	NT	14,974	1,067	88,404	NT	NT	NT	NT	NT
6-Apr-11	31,000	0.91	1,925,776	169,008	NT	9,019	0	4,267	NT	NT	NT	NT	NT
13-Apr-11	8,200	0.4	3,790,540	304,682	NT	4,878	733	16,367	NT	NT	NT	NT	NT
26-Apr-11	15,000	0.39	5,755,900	263,180	115,172	6,267	0	298,348	1,200	2,300	24	300	20
16-May-11	17,000	0.83	10,392,053	541,694	312,682	12,034	0	45,200	600	2,750	21	300	20
28-Jun-11	31,000	0.73	8,016,495	253,513	355,018	8,134	0	172,675	800	3,150	24	375	20
8-Sep-11	8,900	0.36	12,159,749	633,365	246,846	14,401	0	6,167	900	600	110	750	0
27-Sep-11	6,900	0.82	4,902,366	339,684	98,172	3,534	0	3,834	550	NT	NT	NT	NT
11-Oct-11	350	1.68	313,704	8,434	16,517	0	0	0	400	50	0.4	18	0
8-Nov-11	400	1.69	485,658	9,500	16,517	0	0	0	50	50	0.9	150	0
22-Nov-11	1,600	1.48	452,489	0	0	0	0	0	100	50	0	45	0
6-Dec-11	500	1.61	333,317	4,817	0	0	0	0	500	0	0	69	0
20-Feb-12	400	1.35	207,910	907	0	0	0	0	50	0	0	12	0
6-Mar-12	300	1.23	208,680	2,333	0	0	0	0	200	50	23	3	0
11-Mar-13	650	1.87	125,667	0	0	0	0	0	NT	0	4.30	NT	NT
25-Mar-13	750	15.75	311,000	0	0	0	0	0	NT	150	9.30	NT	NT
8-Apr-13	240	2.98	184,133	885	0	0	0	0	NT	0	4.30	NT	NT

*NT, Not tested;

Table 33: Microorganisms in sediment at the Boatsheds and Kerrs Reach. Shading denotes the active discharge phase at KR and OT

DATE	Boatshed sediments							Kerr's Reach sediments						
	<i>E. coli</i> CFU/g	AC /TC	<i>C. perfringens</i> CFU/g	Phage PFU/g	<i>Campylo- bacter</i> MPN/g	<i>Giardia</i> (oo)cysts/g	<i>Crypto- sporidium</i> (oo)cysts/g	<i>E. coli</i> CFU/g	AC /TC	<i>C. perfringens</i> CFU/g	Phage PFU/g	<i>Campylo- bacter</i> MPN/g	<i>Giardia</i> (oo)cysts/g	<i>Crypto- sporidium</i> (oo)cysts/g
8-Mar-11	3,746	3.9	*NT	NT	NT	NT	NT	13,024	0.6	NT	NT	NT	NT	NT
23-Mar-11	2,221	3.6	NT	NT	NT	NT	NT	2,020	1.9	NT	NT	NT	NT	NT
30-Mar-11	2,847	2.7	NT	NT	NT	NT	NT	23,383	1.6	NT	NT	NT	NT	NT
6-Apr-11	416	2.5	NT	NT	NT	NT	NT	21,426	2.9	NT	NT	NT	NT	NT
13-Apr-11	6,969	33.5	NT	NT	NT	NT	NT	1,896	0.9	NT	NT	NT	NT	NT
26-Apr-11	1,000	2.4	530	32	0	6.0	0.7	9,142	0.9	17,000	140	3.3	19.0	1.1
16-May-11	2,800	2.1	4,100	0	1.9	70.0	2.8	1,752	1.5	1,700	23	2.6	0.3	0.1
28-Jun-11	1,300	1.1	320	31	0	0.3	0.3	431	1.3	920	0	0.5	2.0	0.2
8-Sep-11	49	11.5	5,700	0	0	18.0	0.1	166	0.8	23,000	0	0	13.0	0.3
27-Sep-11	280	2.2	4,800	NT	NT	NT	NT	33,592	2.3	11,000	NT	NT	NT	NT
11-Oct-11	41	2.9	13,000	0	0	142	2.1	141	4.4	210,000	0	0	34.0	0
8-Nov-11	87	7.1	5,200	0	0	35.0	1.7	912	40.8	36,000	0	0	166	5.6
22-Nov-11	260	3.0	930	0	2.0	39.0	3.3	147	4.3	3,200	0	0	63.0	7.9
6-Dec-11	198	74.5	9,900	0	2.0	118	8.5	194	11.9	25,000	0	0	11.0	0
20-Feb-12	103	6.4	1,300	0	0.8	2,254	113	77	9.7	18,000	10	0	8.0	0.5
6-Mar-12	784	0.5	2,200	8	0	3.0	0.3	359	6.3	350	0	0	0.5	0
11-Mar-13	112	3.5	NT	0	0	0	0	19,740	0.6	NT	0	0	0	0
25-Mar-13	67	22.6	NT	0	0	0	0	**91,635	‡N/A	NT	0	11.1	0	0
8-Apr-13	219	12.7	NT	0	0	0	0	**19,609	3.5	NT	0	0.5	0.8	0

*NT, Not tested; **these two sampling events at KR occurred 50 m downstream of previous sampling site; ‡N/A, Total coliforms dominated plates and could not count atypical colonies

Table 34: Microorganisms in sediment at Owles Terrace. Shading denotes the active discharge phase

DATE	<i>E. coli</i> CFU/g	AC /TC	<i>C. perfringens</i> CFU/g	Phage PFU/g	<i>Campylobacter</i> MPN/g	<i>Giardia</i>	<i>Cryptosporidium</i> (oo)cysts/g
8-Mar-11	45,258	1.2	*NT	NT	NT	NT	NT
23-Mar-11	20,667	0.6	NT	NT	NT	NT	NT
30-Mar-11	3,739	1.2	NT	NT	NT	NT	NT
6-Apr-11	5,072	1.9	NT	NT	NT	NT	NT
13-Apr-11	2,633	0.6	NT	NT	NT	NT	NT
26-Apr-11	5,300	1.1	25,000	98	2.0	1.0	0.3
16-May-11	2,700	1.5	34,000	31	1.9	2.0	0.1
28-Jun-11	5,339	1.1	40,000	18	0	1.0	0.4
8-Sep-11	407	0.8	43,000	17	6.1	37.0	2.5
27-Sep-11	1,723	1.2	42,500	NT	NT	NT	NT
11-Oct-11	81	1.5	56,000	12	0	14.0	1.1
8-Nov-11	178	2.8	55,000	0	0	73.0	4.8
22-Nov-11	107	3.0	27,000	0	0	6.0	0.5
6-Dec-11	43	2.2	30,000	0	0	33.0	0
20-Feb-12	24	25.2	655	0	0	0.2	0
6-Mar-12	2,162	3.4	16,000	0	0	0.3	0
11-Mar-13	238	14.9	NT	0	0	0	0
25-Mar-13	176	18.6	NT	0	0	0	0
8-Apr-13	19	32.2	NT	0	0	0	0

*NT, Not tested;

Chapter Four: Rural study results

Table 35: Trial 1 - Mean concentration and gene copies (SD) of *E. coli* and PCR markers (respectively) in supernatant from irrigated and non-irrigated cowpats

Sampling Day	Irrigated supernatant					Non-irrigated supernatant				
	<i>E. coli</i> CFU/100 mL	PCR markers in irrigated supernatants GC/100 mL				<i>E. coli</i> CFU/100 mL	PCR markers in non-irrigated supernatants GC/100 mL			
		GenBac3	BacR	CowM2	%BacR/TotalBac		GenBac3	BacR	CowM2	%BacR/TotalBac
Day 0	3.8 x 10 ⁷ (7.7 x 10 ⁶)	7.0 x 10 ¹⁰ (2.8 x 10 ¹⁰)	1.1 x 10 ¹⁰ (3.8 x 10 ⁹)	2.4 x 10 ⁸ (7.1 x 10 ⁷)	17.0% (2.0)	3.8 x 10 ⁷ (7.7 x 10 ⁶)	7.0 x 10 ¹⁰ (2.8 x 10 ¹⁰)	1.1 x 10 ¹⁰ (3.8 x 10 ⁹)	2.4 x 10 ⁸ (7.1 x 10 ⁷)	17.0% (2.0)
Day 7	3.1 x 10 ⁷ (2.9 x 10 ⁶)	1.1 x 10 ¹⁰ (2.7 x 10 ⁹)	2.2 x 10 ⁹ (4.3 x 10 ⁸)	4.0 x 10 ⁷ (3.5 x 10 ⁶)	20.0% (1.3)	4.5 x 10 ⁷ (3.1 x 10 ⁶)	8.1 x 10 ⁹ (1.3 x 10 ⁹)	1.7 x 10 ⁹ (2.1 x 10 ⁸)	2.4 x 10 ⁷ (1.5 x 10 ⁷)	20.9% (0.9)
Day 14	1.4 x 10 ⁷ (9.4 x 10 ⁵)	1.8 x 10 ¹⁰ (4.6 x 10 ⁹)	2.5 x 10 ⁹ (4.5 x 10 ⁸)	4.8 x 10 ⁷ (1.2 x 10 ⁷)	14.5% (1.5)	1.3 x 10 ⁸ (6.6 x 10 ⁶)	5.2 x 10 ⁹ (6.7 x 10 ⁸)	7.0 x 10 ⁸ (1.1 x 10 ⁸)	1.4 x 10 ⁷ (3.1 x 10 ⁶)	13.4% (0.6)
Day 21	1.3 x 10 ⁸ (3.5 x 10 ⁶)	6.5 x 10 ⁹ (1.9 x 10 ⁹)	6.9 x 10 ⁸ (1.5 x 10 ⁸)	1.2 x 10 ⁷ (5.7 x 10 ⁶)	10.7% (0.7)	5.2 x 10 ⁸ (3.1 x 10 ⁷)	3.4 x 10 ⁹ (5.7 x 10 ⁸)	2.1 x 10 ⁸ (4.8 x 10 ⁸)	5.7 x 10 ⁶ (1.2 x 10 ⁶)	6.2% (0.8)
Day 28	1.7 x 10 ⁷ (6.3 x 10 ⁵)	6.6 x 10 ⁹ (1.1 x 10 ⁹)	2.2 x 10 ⁸ (7.2 x 10 ⁷)	3.1 x 10 ⁶ (5.2 x 10 ⁵)	3.2% (0.7)	1.2 x 10 ⁸ (5.9 x 10 ⁶)	7.4 x 10 ⁸ (2.8 x 10 ⁷)	4.2 x 10 ⁷ (1.3 x 10 ⁶)	9.3 x 10 ⁵ (1.3 x 10 ⁵)	5.7% (0.0)
Day 42	1.6 x 10 ⁷ (4.3 x 10 ⁵)	1.4 x 10 ⁸ (5.1 x 10 ⁷)	9.2 x 10 ⁶ (4.6 x 10 ⁶)	8.3 x 10 ⁵ (3.0 x 10 ⁵)	6.5% (1.2)	1.3 x 10 ⁶ (1.5 x 10 ⁵)	1.3 x 10 ⁸ (3.2 x 10 ⁷)	6.7 x 10 ⁶ (1.6 x 10 ⁶)	1.1 x 10 ⁶ (9.5 x 10 ⁵)	5.3% (0.2)
Day 77	4.8 x 10 ⁵ (1.8 x 10 ⁴)	4.9 x 10 ⁴ (1.6 x 10 ⁴)	9.1 x 10 ³ (3.2 x 10 ³)	ND	18.6% (3.5)	1.6 x 10 ⁶ (1.3 x 10 ⁵)	4.5 x 10 ⁶ (2.3 x 10 ⁶)	4.2 x 10 ⁴ (1.6 x 10 ⁴)	ND	1.0% (0.2)
Day 105	1.2 x 10 ⁵ (6.5 x 10 ³)	1.2 x 10 ⁴ (2.8 x 10 ³)	2.2 x 10 ³ (6.2 x 10 ²)	ND	17.5% (2.7)	3.4 x 10 ⁵ (4.1 x 10 ⁴)	4.8 x 10 ⁴ (1.9 x 10 ⁴)	8.7 x 10 ³ (4.5 x 10 ³)	ND	17.6% (2.7)
Day 133	1.6 x 10 ⁵ (2.6 x 10 ⁴)	2.7 x 10 ³ (3.8 x 10 ²)	ND*	ND	*N/A	1.8 x 10 ⁵ (1.4 x 10 ⁴)	2.8 x 10 ³ (5.7 x 10 ²)	ND	ND	N/A
Day 161	7.7 x 10 ⁴ (6.1 x 10 ³)	1.2 x 10 ³ (4.9 x 10 ²)	ND	ND	N/A	1.1 x 10 ⁵ (1.9 x 10 ⁴)	ND	ND	ND	N/A

*ND, not detected; †N/A, not applicable as no GenBac3 and BacR detected

Table 36: Trial 2 - Mean concentrations and ratios (SD) of microbes and PCR markers in re-suspended cowpat supernatant

Day of sampling	<i>E. coli</i> CFU/100 mL	AC/TC	GenBac3 GC/100 ML	BacR GC/100 ML	%BacR/GenBac3 (BacR/TotalBac)	CowM2 GC/100 ML
Day 1	1.6 x 10 ⁷ (2.4 x 10 ⁶)	0.10 (0.03)	3.5 x 10 ¹⁰ (3.0 x 10 ⁹)	8.0 x 10 ⁹ (1.1 x 10 ⁹)	23% (4)	1.3 x 10 ⁸ (1.3 x 10 ⁷)
Day 8	6.2 x 10 ⁶ (8.8 x 10 ⁵)	1.8 (0.1)	6.0 x 10 ⁹ (1.4 x 10 ⁹)	1.3 x 10 ⁹ (2.7 x 10 ⁸)	21% (1)	2.7 x 10 ⁷ (1.3 x 10 ⁷)
Day 15	8.6 x 10 ⁶ (3.4 x 10 ⁶)	1.4 (0.04)	7.1 x 10 ⁹ (2.8 x 10 ⁹)	1.1 x 10 ⁹ (3.5 x 10 ⁸)	16% (2)	2.7 x 10 ⁷ (1.0 x 10 ⁷)
Day 22	4.5 x 10 ⁶ (2.1 x 10 ⁶)	1.7 (0.6)	2.1 x 10 ⁸ (2.3 x 10 ⁸)	3.3 x 10 ⁶ (4.3 x 10 ⁶)	1% (0)	5.6 x 10 ⁴ (7.7 x 10 ⁴)
Day 29	1.6 x 10 ⁶ (4.1 x 10 ⁵)	6.8 (2.2)	2.4 x 10 ⁶ (9.0 x 10 ⁵)	4.6 x 10 ⁵ (2.6 x 10 ⁵)	18% (5)	1.5 x 10 ⁴ (8.6 x 10 ³)
Day 50	1.9 x 10 ⁶ (1.5 x 10 ⁶)	3.1 (0.9)	7.4 x 10 ⁵ (3.4 x 10 ⁵)	1.8 x 10 ⁵ (6.8 x 10 ⁴)	25% (4)	1.8 x 10 ³ (2.0 x 10 ³)
Day 71	5.5 x 10 ⁶ (1.5 x 10 ⁶)	3.8 (5.0)	6.6 x 10 ⁵ (2.5 x 10 ⁵)	1.0 x 10 ⁵ (2.7 x 10 ⁴)	16% (2)	*ND
Day 105	2.4 x 10 ⁵ (1.8 x 10 ⁵)	4.1 (4.9)	3.3 x 10 ⁴ (1.7 x 10 ⁴)	7.8 x 10 ³ (3.0 x 10 ³)	24% (3)	ND
Day 134	1.4 x 10 ⁴ (1.1 x 10 ⁴)	55 (20.4)	3.6 x 10 ⁴ (1.0 x 10 ⁴)	7.0 x 10 ³ (1.7 x 10 ³)	20% (1)	contaminated sample
Day 162	8.2 x 10 ³ (4.8 x 10 ³)	212 (254)	1.6 x 10 ⁴ (7.8 x 10 ³)	4.5 x 10 ³ (1.5 x 10 ³)	30% (9)	ND

Table 37: Trial 2 - Mean concentrations and ratios (SD) of microbes and PCR markers in cowpat rainfall runoff

Day of sampling	<i>E. coli</i> CFU/100 mL	AC/TC	GenBac3 GC/100 ML	BacR GC/100 ML	%BacR/GenBac3 (BacR/TotalBac)	CowM2 GC/100 ML
Day 1	1.1 x 10 ⁷ (2.6 x 10 ⁶)	0.11 (0.03)	3.6 x 10 ¹⁰ (9.8 x 10 ⁹)	7.9 x 10 ⁹ (3.3 x 10 ⁹)	21% (3)	9.4 x 10 ⁷ (5.3 x 10 ⁷)
Day 8	3.0 x 10 ⁴ (2.4 x 10 ⁴)	2.2 (0.5)	9.6 x 10 ⁶ (5.9 x 10 ⁶)	2.6 x 10 ⁶ (1.4 x 10 ⁶)	28% (3)	9.9 x 10 ⁴ (5.7 x 10 ⁴)
Day 15	3.4 x 10 ³ (1.1 x 10 ³)	5.8 (3.8)	1.4 x 10 ⁶ (1.1 x 10 ⁶)	4.1 x 10 ⁵ (3.1 x 10 ⁵)	34% (6)	1.6 x 10 ⁴ (1.2 x 10 ⁴)
Day 22	1.1 x 10 ³ (660)	2.9 (2.3)	1.0 x 10 ⁵ (1.3 x 10 ⁵)	2.4 x 10 ⁴ (2.5 x 10 ⁴)	33% (12)	1.1 x 10 ³ (1.3 x 10 ³)
Day 29	1.1 x 10 ⁴ (1.5 x 10 ⁴)	4.7 (2.0)	3.6 x 10 ³ (4.1 x 10 ³)	1.7 x 10 ³ (1.0 x 10 ³)	67% (39)	ND
Day 50	1.3 x 10 ³ (766)	56 (24)	380 (210)	160 (49)	45% (12)	ND
Day 71	5.5 x 10 ³ (8.2 x 10 ³)	5.8 (*N/A)	590 (290)	320 (100)	58% (15)	ND
Day 105	27 (21)	126 (95)	550 (610)	270 (330)	46% (9)	ND
Day 134	11 (10)	4.8 (0.4)	260 (110)	100 (17)	45% (25)	contaminated sample
Day 162	22 (8)	12.6 (4.3)	*ND	ND	*N/A	ND

*ND, not detected; **No standard deviation because based on a single sample; †N/A, not applicable as no GenBac3 and BacR detected

Table 38: Trial 1 - Mean Percentages of individual steroids/total sterols in (non-)irrigated cowpat supernatant over the five and a half month experimental period. Standard deviations are presented in italics. Percentages of coprostanol (H1) and 24-ethylcoprostanol (R1) can be found in the tables of FST ratios (Table 39 and Table 40 respectively).

IRR Supernatant	%Epicoprostanol	%Cholesterol	%Cholestanol	%24-M cholesterol	%24-E-epicop	%Stigmasterol	%24-Echolesterol	%24-Echolstanol
Day 0	0.6 <i>(0.3)</i>	8.6 <i>(0.8)</i>	1.2 <i>(0.2)</i>	2.4 <i>(0.2)</i>	6.6 <i>(3.0)</i>	0.4 <i>(0.3)</i>	6.3 <i>(0.7)</i>	6.1 <i>(1.5)</i>
Day 7	0.6 <i>(0.3)</i>	6.2 <i>(1.9)</i>	1.1 <i>(0.4)</i>	2.5 <i>(0.3)</i>	7.2 <i>(2.3)</i>	0.6 <i>(0.1)</i>	6.5 <i>(2.7)</i>	5.9 <i>(2.2)</i>
Day 14	1.2 <i>(0.3)</i>	8.5 <i>(0.1)</i>	1.6 <i>(0.2)</i>	2.6 <i>(0.4)</i>	8.5 <i>(1.3)</i>	0.7 <i>(0.2)</i>	7.5 <i>(3.3)</i>	6.8 <i>(2.2)</i>
Day 21	0.7 <i>(0.0)</i>	7.6 <i>(0.5)</i>	1.4 <i>(0.0)</i>	3.0 <i>(0.1)</i>	9.2 <i>(0.2)</i>	0.6 <i>(0.1)</i>	9.7 <i>(0.1)</i>	9.9 <i>(0.3)</i>
Day 28	0.8 <i>(0.1)</i>	8.4 <i>(0.3)</i>	1.6 <i>(0.1)</i>	3.1 <i>(0.1)</i>	10.1 <i>(0.1)</i>	0.8 <i>(0.1)</i>	8.9 <i>(1.1)</i>	8.9 <i>(1.3)</i>
Day 42	1.0 <i>(0.1)</i>	10.0 <i>(1.2)</i>	2.0 <i>(0.1)</i>	3.7 <i>(0.2)</i>	12.0 <i>(0.4)</i>	2.0 <i>(0.1)</i>	12.5 <i>(0.1)</i>	9.2 <i>(0.8)</i>
Day 77	0.9 <i>(0.1)</i>	8.0 <i>(0.9)</i>	2.4 <i>(0.1)</i>	3.9 <i>(0.2)</i>	12.4 <i>(0.4)</i>	1.0 <i>(0.1)</i>	9.8 <i>(0.2)</i>	15.7 <i>(2.0)</i>
Day 105	0.7 <i>(0.0)</i>	5.7 <i>(1.1)</i>	1.8 <i>(0.1)</i>	4.0 <i>(0.1)</i>	11.0 <i>(0.4)</i>	8.7 <i>(1.5)</i>	9.1 <i>(0.3)</i>	10.2 <i>(0.3)</i>
Day 133	0.9 <i>(0.1)</i>	7.3 <i>(0.9)</i>	1.7 <i>(0.2)</i>	4.1 <i>(0.2)</i>	10.3 <i>(0.4)</i>	1.5 <i>(0.2)</i>	11.2 <i>(4.5)</i>	8.7 <i>(1.8)</i>
Day 161	0.9 <i>(0.2)</i>	6.0 <i>(0.6)</i>	1.5 <i>(0.1)</i>	2.6 <i>(0.1)</i>	11.9 <i>(0.6)</i>	0.7 <i>(0.1)</i>	6.5 <i>(1.4)</i>	7.4 <i>(0.7)</i>
Overall mean	0.8 (0.2)	7.6 (1.3)	1.6 (0.4)	3.2 (0.7)	9.9 (2.0)	1.7 (2.5)	8.8 (1.5)	8.9 (2.8)
NIR supernatant								
Day 0	0.6 <i>(0.3)</i>	8.6 <i>(0.8)</i>	1.2 <i>(0.2)</i>	2.4 <i>(0.2)</i>	6.6 <i>(3.0)</i>	0.4 <i>(0.3)</i>	6.3 <i>(0.7)</i>	6.1 <i>(1.5)</i>
Day 7	0.9 <i>(0.3)</i>	9.2 <i>(0.8)</i>	1.6 <i>(0.2)</i>	3.4 <i>(0.2)</i>	9.1 <i>(3.0)</i>	0.9 <i>(0.3)</i>	8.9 <i>(0.7)</i>	9.1 <i>(1.5)</i>
Day 14	1.0 <i>(0.5)</i>	9.3 <i>(4.1)</i>	1.8 <i>(0.6)</i>	2.9 <i>(1.2)</i>	9.3 <i>(3.2)</i>	0.8 <i>(0.5)</i>	8.1 <i>(4.4)</i>	7.3 <i>(3.7)</i>
Day 21	0.8 <i>(0.2)</i>	8.2 <i>(1.2)</i>	1.7 <i>(0.2)</i>	3.1 <i>(0.2)</i>	10.3 <i>(0.5)</i>	0.9 <i>(0.1)</i>	9.8 <i>(1.1)</i>	11.5 <i>(1.6)</i>
Day 28	1.0 <i>(0.8)</i>	10.5 <i>(0.6)</i>	1.8 <i>(0.1)</i>	3.3 <i>(0.1)</i>	11.3 <i>(0.7)</i>	1.2 <i>(0.3)</i>	9.5 <i>(0.3)</i>	7.3 <i>(0.9)</i>
Day 42	0.9 <i>(0.1)</i>	12.7 <i>(1.8)</i>	2.0 <i>(0.1)</i>	4.3 <i>(0.2)</i>	9.1 <i>(0.7)</i>	4.3 <i>(0.3)</i>	23.3 <i>(1.6)</i>	7.4 <i>(1.6)</i>
Day 77	0.6 <i>(0.1)</i>	15.6 <i>(1.1)</i>	2.2 <i>(0.2)</i>	6.0 <i>(0.6)</i>	13.6 <i>(1.1)</i>	2.8 <i>(0.4)</i>	15.9 <i>(0.8)</i>	10.3 <i>(0.7)</i>
Day 105	0.8 <i>(0.1)</i>	5.1 <i>(0.9)</i>	2.0 <i>(0.1)</i>	3.2 <i>(0.4)</i>	12.2 <i>(3.7)</i>	4.3 <i>(0.4)</i>	7.4 <i>(3.0)</i>	10.4 <i>(1.7)</i>
Day 133	0.9 <i>(0.1)</i>	13.6 <i>(0.3)</i>	1.5 <i>(0.1)</i>	4.8 <i>(0.3)</i>	8.4 <i>(1.1)</i>	5.8 <i>(1.1)</i>	10.1 <i>(1.0)</i>	6.1 <i>(1.3)</i>
Day 161	0.8 <i>(0.2)</i>	5.3 <i>(0.9)</i>	1.7 <i>(0.1)</i>	3.5 <i>(0.3)</i>	10.5 <i>(0.3)</i>	1.1 <i>(0.3)</i>	5.6 <i>(1.2)</i>	7.8 <i>(0.2)</i>
Overall mean	0.8 (0.1)	9.8 (3.4)	1.7 (0.3)	3.7 (1.1)	10.1 (2.0)	2.2 (1.9)	10.5 (1.3)	8.3 (1.9)

Table 39: Trial 1 - Mean Sterol FST markers in irrigated and non-irrigated cowpat supernatants for detecting general faecal pollution (F1 and F2) and human/herbivore faecal contamination (H1-H6). Standard deviations are presented in italics.

Refer to Chapter One, Table 3 for interpretation of steroid ratios

Irrigated supernatant	Log₁₀ Total sterols adjusted (ng/ml)		F1 >0.5		F2 >0.5		H1 >5-6%		H2 >0.7		H3 >0.73		H4 >73%		H5 %		H6 >1.5	
Day 0	5.17	<i>(4.63)</i>	4.9	<i>(1.0)</i>	10.8	<i>(4.0)</i>	5.8%	<i>(2.1)</i>	0.83	<i>(0.03)</i>	0.1	<i>(0.1)</i>	8.9%	<i>(4.2)</i>	0	9.4	<i>(1.0)</i>	
Day 7	4.96	<i>(4.29)</i>	4.4	<i>(0.3)</i>	12.5	<i>(5.6)</i>	4.8%	<i>(2.1)</i>	0.81	<i>(0.01)</i>	0.1	<i>(0.1)</i>	7.3%	<i>(4.5)</i>	0	7.9	<i>(0.5)</i>	
Day 14	4.80	<i>(4.22)</i>	5.1	<i>(0.8)</i>	8.9	<i>(4.1)</i>	8.5%	<i>(2.1)</i>	0.83	<i>(0.02)</i>	0.2	<i>(0)</i>	13.4%	<i>(2.0)</i>	0	7.0	<i>(0.0)</i>	
Day 21	4.94	<i>(3.15)</i>	3.6	<i>(0.2)</i>	5.4	<i>(0.2)</i>	5.1%	<i>(0.0)</i>	0.78	<i>(0.01)</i>	0.1	<i>(0)</i>	8.9%	<i>(0.1)</i>	0	7.6	<i>(0.1)</i>	
Day 28	4.64	<i>(3.46)</i>	3.7	<i>(0.2)</i>	5.9	<i>(1.0)</i>	6.0%	<i>(0.9)</i>	0.79	<i>(0.01)</i>	0.1	<i>(0)</i>	10.4%	<i>(1.2)</i>	0	7.3	<i>(0.3)</i>	
Day 42	3.80	<i>(2.97)</i>	1.8	<i>(0.1)</i>	4.8	<i>(0.6)</i>	3.7%	<i>(0.2)</i>	0.65	<i>(0.01)</i>	0.1	<i>(0)</i>	7.7%	<i>(0.4)</i>	0	3.8	<i>(0.3)</i>	
Day 77	2.39	<i>(1.74)</i>	2.1	<i>(0.4)</i>	2.6	<i>(0.4)</i>	5.1%	<i>(0.7)</i>	0.68	<i>(0.04)</i>	0.1	<i>(0)</i>	11.2%	<i>(1.3)</i>	0	5.9	<i>(0.3)</i>	
Day 105	2.76	<i>(1.91)</i>	2.0	<i>(0.0)</i>	4.4	<i>(0.3)</i>	3.6%	<i>(0.1)</i>	0.67	<i>(0.00)</i>	0.1	<i>(0)</i>	7.5%	<i>(0.5)</i>	0	5.5	<i>(0.1)</i>	
Day 133	2.71	<i>(1.86)</i>	3.3	<i>(0.6)</i>	5.8	<i>(1.4)</i>	5.4%	<i>(1.1)</i>	0.76	<i>(0.03)</i>	0.1	<i>(0)</i>	9.9%	<i>(1.3)</i>	0	6.3	<i>(1.1)</i>	
Day 161	2.52	<i>(1.66)</i>	3.5	<i>(0.5)</i>	7.8	<i>(1.0)</i>	5.4%	<i>(0.8)</i>	0.78	<i>(0.02)</i>	0.1	<i>(0)</i>	8.7%	<i>(1.3)</i>	0	5.8	<i>(0.4)</i>	
Non-irrigated supernatant																		
Day 0	5.17	<i>(4.63)</i>	4.9	<i>(1.0)</i>	10.8	<i>(4.0)</i>	5.8%	<i>(2.1)</i>	0.83	<i>(0.03)</i>	0.1	<i>(0.1)</i>	8.9%	<i>(4.2)</i>	0	9.4	<i>(1.0)</i>	
Day 7	4.87	<i>(4.38)</i>	4.0	<i>(1.2)</i>	7.0	<i>(5.8)</i>	6.3%	<i>(2.9)</i>	0.79	<i>(0.05)</i>	0.1	<i>(0.1)</i>	12.2%	<i>(6.8)</i>	0	7.6	<i>(0.8)</i>	
Day 14	4.78	<i>(3.81)</i>	4.0	<i>(0.3)</i>	7.4	<i>(1.9)</i>	7.3%	<i>(1.1)</i>	0.80	<i>(0.01)</i>	0.1	<i>(0)</i>	12.2%	<i>(1.5)</i>	0	7.7	<i>(0.4)</i>	
Day 21	4.67	<i>(3.17)</i>	3.3	<i>(0.2)</i>	4.2	<i>(0.6)</i>	5.6%	<i>(0.3)</i>	0.77	<i>(0.01)</i>	0.1	<i>(0)</i>	10.4%	<i>(1.1)</i>	0	6.9	<i>(0.4)</i>	
Day 28	4.08	<i>(3.20)</i>	3.0	<i>(0.4)</i>	7.0	<i>(2.2)</i>	5.4%	<i>(0.8)</i>	0.75	<i>(0.03)</i>	0.1	<i>(0)</i>	9.9%	<i>(0.9)</i>	0	5.3	<i>(0.4)</i>	
Day 42	3.25	<i>(2.30)</i>	1.6	<i>(0.2)</i>	4.5	<i>(0.7)</i>	3.1%	<i>(0.1)</i>	0.61	<i>(0.03)</i>	0.1	<i>(0)</i>	8.6%	<i>(0.8)</i>	0	3.3	<i>(0.3)</i>	
Day 77	3.02	<i>(2.45)</i>	1.3	<i>(0.1)</i>	3.0	<i>(0.9)</i>	2.8%	<i>(0.4)</i>	0.56	<i>(0.02)</i>	0.1	<i>(0)</i>	8.5%	<i>(0.3)</i>	0	4.5	<i>(0.2)</i>	
Day 105	2.76	<i>(1.96)</i>	2.5	<i>(0.5)</i>	4.9	<i>(0.9)</i>	4.8%	<i>(0.8)</i>	0.71	<i>(0.04)</i>	0.1	<i>(0)</i>	8.8%	<i>(1.2)</i>	0	5.8	<i>(0.2)</i>	
Day 133	2.50	<i>(1.90)</i>	3.7	<i>(1.1)</i>	7.1	<i>(0.3)</i>	5.4%	<i>(1.4)</i>	0.78	<i>(0.06)</i>	0.1	<i>(0)</i>	11.0%	<i>(2.6)</i>	0	5.8	<i>(0.2)</i>	
Day 161	2.48	<i>(1.55)</i>	3.2	<i>(0.2)</i>	7.8	<i>(2.0)</i>	5.5%	<i>(0.5)</i>	0.76	<i>(0.01)</i>	0.1	<i>(0)</i>	8.6%	<i>(0.8)</i>	0	6.5	<i>(0.4)</i>	

Table 40: Trial 1 - Mean Sterol FST markers in irrigated and non-irrigated cowpat supernatants for detecting herbivore (R1 and R2, R3), Plant runoff (P1) and avian faecal contamination (Av1 and Av2). Standard deviations are presented in brackets and italics. Refer to Chapter One, Table 3 for interpretation of steroid ratios

Irrigated supernatant	R1 %	R2 %	R3	*R4	**P1 <1.0	Av1 ≥0.4	Av2 ≥0.5
Day 0	62.0% (8.8)	100	1.1 (0.2)	11.3 (6.9)	0.1 (0.0)	0.08 (0.02)	0.16 (0.03)
Day 7	64.7% (11.8)	100	1.2 (0.1)	11.9 (6.5)	0.1 (0.1)	0.08 (0.04)	0.17 (0.01)
Day 14	54.1% (4.4)	100	0.9 (0.5)	16.0 (3.6)	0.1 (0.1)	0.10 (0.03)	0.15 (0.02)
Day 21	52.9% (0.6)	100	1.9 (0)	17.4 (0.5)	0.2 (0.0)	0.14 (0.0)	0.20 (0.01)
Day 28	51.4% (0.7)	100	1.5 (0.4)	19.7 (0.3)	0.2 (0.0)	0.13 (0.02)	0.19 (0.01)
Day 42	44.0% (1.9)	100	2.5 (0.3)	27.2 (2.1)	0.3 (0.0)	0.14 (0.01)	0.30 (0.01)
Day 77	40.7% (1.7)	100	3.1 (0.9)	30.6 (1.8)	0.2 (0.0)	0.23 (0.03)	0.29 (0.04)
Day 105	45.3% (1.7)	100	2.8 (0.1)	24.3 (1.8)	0.2 (0.0)	0.15 (0.01)	0.30 (0.0)
Day 133	49.0% (3.8)	100	1.7 (0.5)	21.0 (2)	0.2 (0.1)	0.13 (0.02)	0.21 (0.03)
Day 161	57.0% (1.8)	100	1.4 (0.3)	20.9 (1)	0.1 (0.0)	0.10 (0.01)	0.20 (0.02)
Non-irrigated supernatant							
Day 0	62.0% (8.8)	100	1.1 (0.2)	11.3 (6.9)	0.1 (0.0)	0.08 (0.02)	0.16 (0.02)
Day 7	50.7% (19.0)	100	1.5 (0.5)	20.6 (11.2)	0.2 (0.2)	0.14 (0.08)	0.19 (0.08)
Day 14	52.3% (0.6)	100	1.0 (0.4)	17.7 (1.0)	0.2 (0.0)	0.11 (0.02)	0.18 (0.02)
Day 21	48.3% (2.7)	100	2.1 (0.1)	21.3 (2.6)	0.2 (0.0)	0.16 (0.02)	0.21 (0.02)
Day 28	48.6% (2.5)	100	1.4 (0.5)	23.4 (2.5)	0.2 (0.0)	0.11 (0.02)	0.22 (0.02)
Day 42	32.9% (2.1)	100	2.4 (0.1)	27.9 (4.0)	0.7 (0.1)	0.15 (0.02)	0.33 (0.02)
Day 77	30.1% (3.4)	100	3.8 (1.1)	46.2 (16.5)	0.5 (0.1)	0.19 (0.04)	0.39 (0.04)
Day 105	49.8% (3.1)	100	2.2 (0.6)	24.7 (3.7)	0.1 (0.0)	0.14 (0.02)	0.26 (0.02)
Day 133	43.3% (1.4)	100	1.2 (0.4)	19.5 (1.2)	0.2 (0.0)	0.11 (0.0)	0.19 (0.0)
Day 161	58.2% (2.3)	100	1.4 (0.4)	18.1 (1.3)	0.1 (0.0)	0.10 (0.03)	0.21 (0.03)

*R4 =24-ethylepicoprostanol/24-Ecop, a putative faecal ageing ratio

**P1 <1.0 indicative of herbivore pollution; 1.0-4.0 complex mix of sterols derived from plant runoff and herbivore;

>4.0 plant runoff; >7.0 may indicate avian contamination

Table 41: Trial 2 - Mean percentages of individual steroids/total steroids for each sampling event. Standard deviations are presented in italics. Percentages of coprostanol (H1) and 24-ethylcoprostanol (R1) can be found in the tables of FST ratios (Table 42 and Table 43, respectively).

Supernatant	%Epicoprostanol	%Cholesterol	%Cholestenol	%24-M cholesterol	%24-E-epicop	%Stigmasterol	%24- Echolesterol	%24- Echolstanol
Day 1	0.9 (0.3)	9.6 (3.1)	1.6 (0.9)	3.1 (0.4)	12.9 (4.1)	0.56 (0.05)	7.6 (1.4)	11.7 (2.8)
Day 8	1.6 (0.4)	11.4 (3.6)	2.8 (1.1)	3.1 (0.3)	16.1 (6.9)	0.59 (0.35)	10.5 (2.5)	12.2 (4.7)
Day 15	1.1 (0)	8.8 (0.5)	1.9 (0.1)	3.9 (0.2)	13.0 (0.2)	0.99 (0.06)	12.1 (1.7)	12.9 (0.9)
Day 22	1.1 (0)	11.4 (1.5)	2.4 (0.3)	4.1 (0.1)	13.5 (1.3)	0.99 (0.17)	9.8 (0.8)	12.0 (0.6)
Day 29	1.4 (0.2)	8.7 (0.9)	2.4 (0.3)	3.8 (0.4)	15.5 (1.9)	1.06 (0.13)	10.3 (1.7)	14.7 (1.0)
Day 50	1.2 (0.1)	7.2 (1.8)	2.8 (0.5)	3.9 (0.6)	13.4 (2.2)	0.85 (0.29)	10.3 (3.1)	14.1 (2.3)
Day 71	1.4 (0.3)	7.2 (0.7)	3.1 (0.4)	4.7 (0.3)	14.5 (4.0)	1.85 (1.12)	11.5 (1.2)	13.1 (1.8)
Day 105	1.1 (0)	4.6 (0.2)	2.1 (0)	4.1 (0.6)	15.1 (2.7)	1.63 (1.05)	10.3 (1.1)	13.9 (1.6)
Day 134	1.3 (0.1)	5.2 (1.2)	2.1 (0.3)	3.8 (0.2)	12.2 (1.1)	1.00 (0.10)	10.5 (0.5)	18.3 (0.9)
Day 162	1.5 (0.4)	9.8 (5.0)	3.1 (0.8)	4.4 (0.6)	14.4 (2.0)	0.72 (0.11)	10.0 (3.6)	15.7 (3.7)
Overall mean	1.3 (0.2)	8.4 (2.4)	2.4 (0.5)	3.9 (0.5)	14.1 (1.3)	1.00 (0.40)	10.3 (1.2)	13.9 (2.0)
Rainfall Runoff								
Day 1	1.3 (0.1)	10.9 (2.7)	2.6 (0.7)	3.9 (0.5)	13.1 (2.8)	0.46 (0.13)	6.7 (1.1)	9.7 (4.0)
Day 8	0.9 (0.1)	14.1 (0.5)	2.4 (0.4)	6.8 (1.0)	11.8 (1.3)	2.43 (1.16)	13.1 (1.7)	10.6 (0.7)
Day 15	0.8 (0.3)	17.0 (1.6)	2.5 (0.3)	8.6 (0.2)	9.7 (2.2)	3.38 (1.55)	16.3 (6.2)	9.8 (0.2)
Day 22	0.9 (0.1)	13.2 (2.6)	2.8 (1.0)	11.6 (0.2)	10.3 (1.5)	2.71 (0.91)	21.3 (5.6)	8.8 (2.1)
Day 29	0.8 (0)	9.9 (1.4)	2.1 (0.3)	8.0 (0.7)	7.9 (0.7)	2.99 (0.71)	26.7 (3.8)	11.8 (1.6)
Day 50	0.7 (0.1)	9.6 (1.3)	1.7 (0.2)	10.5 (4.4)	6.3 (1.8)	4.16 (1.65)	28.0 (4.4)	8.6 (1.4)
Day 71	1.0 (0.4)	10.5 (2.9)	3.3 (0.6)	8.5 (1.7)	12.6 (4.4)	4.55 (0.95)	16.0 (5.4)	11.2 (4.0)
Day 105	0.9 (0.1)	8.7 (2.0)	1.7 (0.1)	9.6 (3.0)	8.0 (2.1)	8.20 (3.93)	28.2 (2.1)	9.0 (2.8)
Day 134	1.4 (0.3)	6.9 (2.3)	2.1 (0.7)	7.0 (1.3)	9.6 (0.8)	2.68 (0.15)	21.6 (3.3)	12.8 (1.0)
Day 162	0.6 (0.1)	6.3 (1.8)	1.2 (0.3)	51.1 (10.5)	5.0 (0.8)	0.93 (0.21)	10.7 (3.7)	5.9 (2.4)
Overall mean	0.9 (0.3)	10.7 (3.3)	2.2 (0.6)	12.6 (13.7)	9.4 (2.7)	3.30 (2.10)	18.9 (7.5)	9.8 (1.9)

Table 42: Trial 2 - Mean steroid ratios for FST analysis in cowpat supernatant and rainfall impacted runoff from cowpats for detecting general faecal pollution (F1 and F2) and human/herbivore faecal contamination (H1-H6). Standard deviations are presented in italics. Refer to Chapter One, Table 3 for interpretation of steroid ratios

Supernatant	Log₁₀ Total steroids adjusted (ng/ml)	F1 >0.5	F2 >0.5	H1 >5-6%	H2 >0.7	H3 >0.73	H4 >73%	H5 %	H6 >1.5
Day 1	5.43 (4.80)	3.8 (2.1)	4.1 (1.0)	5.0% (1.5)	0.77 (0.09)	0.1 (0)	9.9% (4.0)	0	5.9 (0.8)
Day 8	4.99 (4.38)	3.9 (2.3)	3.0 (1.3)	9.5% (2.7)	0.77 (0.09)	0.3 (0.1)	23.5% (8.8)	0	6.0 (0)
Day 15	5.10 (4.31)	3.1 (0.2)	3.1 (0.3)	5.9% (0.3)	0.76 (0.01)	0.2 (0)	13.1% (0.2)	0	5.4 (0.2)
Day 22	3.82 (3.53)	2.5 (0.1)	3.2 (0.1)	6.0% (0.4)	0.71 (0.01)	0.2 (0)	13.5% (1.2)	0	5.2 (0.1)
Day 29	3.51 (2.94)	2.6 (0.5)	2.4 (0.3)	6.3% (0.9)	0.72 (0.04)	0.2 (0)	15.1% (3.1)	0	4.6 (0.1)
Day 50	3.20 (2.93)	2.0 (0.1)	3.2 (0.7)	6.2% (0.7)	0.67 (0.02)	0.2 (0)	13.1% (0.6)	0	5.1 (0)
Day 71	3.51 (3.07)	2.0 (0.5)	2.8 (0.5)	5.3% (0.3)	0.66 (0.06)	0.1 (0)	12.2% (0.2)	0	4.4 (0.2)
Day 105	3.26 (2.81)	2.4 (0.3)	2.6 (0.7)	6.7% (3.1)	0.70 (0.03)	0.2 (0.2)	16.5% (10.6)	0	4.8 (0.7)
Day 134	3.18 (2.20)	2.7 (0.5)	2.8 (0.8)	5.4% (0.6)	0.73 (0.03)	0.1 (0)	11.2% (1.9)	0	4.2 (0.1)
Day 162	3.16 (2.09)	2.2 (0.3)	2.7 (0.8)	5.6% (0.5)	0.68 (0.03)	0.1 (0)	12.2% (0.5)	0	4.3 (0.2)
Rainfall runoff	Log (10)Total steroids ng/L								
Day 1	8.05 (7.66)	3.1 (0.4)	5.2 (2.6)	7.7% (1.7)	0.75 (0.02)	0.2 (0)	15.1% (3.0)	0	6.0 (0.6)
Day 8	4.80 (4.21)	2.3 (0.3)	3.1 (0.2)	5.3% (0.4)	0.69 (0.03)	0.2 (0)	14.1% (1.2)	0	5.7 (0.2)
Day 15	4.80 (4.60)	1.7 (0.7)	2.8 (0.5)	4.2% (1.4)	0.62 (0.11)	0.1 (0)	12.8% (2.3)	0	4.9 (0.2)
Day 22	4.54 (4.45)	1.7 (1.1)	2.8 (0.7)	4.2% (1.1)	0.60 (0.15)	0.2 (0)	14.8% (2.8)	0	4.6 (0.9)
Day 29	3.80 (3.36)	2.4 (0.8)	2.1 (0.2)	4.8% (0.7)	0.69 (0.06)	0.2 (0)	16.2% (2.3)	0	5.9 (0.8)
Day 50	3.83 (3.65)	2.2 (0.1)	3.1 (0.3)	3.8% (0.4)	0.69 (0.01)	0.1 (0)	12.8% (1.3)	0	5.4 (0.4)
Day 71	4.17 (3.50)	1.3 (0.7)	2.7 (1.3)	4.2% (1.6)	0.55 (0.11)	0.2 (0.1)	14.0% (7.9)	0	4.2 (0.1)
Day 105	4.20 (3.74)	2.0 (0.5)	2.5 (0.2)	3.3% (1.0)	0.65 (0.07)	0.1 (0)	12.9% (0.5)	0	3.7 (1.0)
Day 134	3.79 (3.37)	2.9 (0.6)	2.4 (0.3)	5.8% (1.2)	0.74 (0.03)	0.2 (0.1)	16.6% (5.2)	0	4.1 (0.1)
Day 162	3.95 (2.81)	2.4 (0.4)	2.9 (1.1)	2.8% (0.4)	0.70 (0.03)	0.2 (0)	15.6% (2.4)	0	5.1 (0.7)

Table 43: Trial 2 - Mean steroid FST markers in cowpat supernatant and rainfall impacted runoff from cowpats for detecting herbivore (R1 and R2, R3), Plant runoff (P1) and avian faecal contamination (Av1 and Av2). Standard deviations are presented in italics.

Refer to Chapter One, Table 3 for interpretation of steroid ratios

Supernatant	R1	R2	R3	*R4	**P1	Av1	Av2	Stig/24-Ecop
Day 1	47.0% (7.2)	100	2.5 (1.2)	29 (12)	0.2 (0.0)	0.16 (0.03)	0.21 (0.08)	0.01 (0.0)
Day 8	32.3% (9.2)	100	1.4 (0.6)	52 (25)	0.4 (0.2)	0.21 (0.11)	0.21 (0.08)	0.02 (0.01)
Day 15	39.3% (1.6)	100	2.2 (0.3)	33 (1)	0.3 (0.1)	0.20 (0.02)	0.21 (0.01)	0.03 (0.0)
Day 22	38.5% (1.6)	100	2.0 (0.2)	35 (5)	0.3 (0.0)	0.19 (0.01)	0.25 (0.01)	0.03 (0.01)
Day 29	35.8% (4.0)	100	2.4 (0.5)	44 (11)	0.3 (0.1)	0.22 (0.01)	0.24 (0.03)	0.03 (0.01)
Day 50	41.3% (2.6)	100	2.1 (0.6)	32 (3)	0.2 (0.1)	0.19 (0.04)	0.29 (0.02)	0.02 (0.01)
Day 71	38.1% (3.2)	100	2.7 (0.5)	39 (13)	0.3 (0.0)	0.21 (0.04)	0.29 (0.05)	0.05 (0.03)
Day 105	36.8% (12.3)	100	2.4 (1.3)	45 (22)	0.3 (0.2)	0.22 (0.02)	0.26 (0.02)	0.05 (0.05)
Day 134	42.9% (3.8)	100	3.0 (0.2)	29 (5)	0.2 (0.0)	0.23 (0.04)	0.23 (0.03)	0.02 (0.0)
Day 162	40.1% (2.0)	100	2.9 (0.9)	36 (3)	0.2 (0.1)	0.22 (0.05)	0.27 (0.02)	0.02 (0.0)
Rainfall runoff								
Day 1	43.6% (3.2)	100	1.3 (0.7)	30 (9)	0.2 (0.0)	0.14 (0.05)	0.22 (0.08)	0.01 (0)
Day 8	32.5% (2.7)	100	2.0 (0.1)	37 (6)	0.4 (0.1)	0.19 (0.0)	0.27 (0.08)	0.08 (0.04)
Day 15	27.8% (4.9)	100	2.5 (0.9)	35 (2)	0.6 (0.4)	0.21 (0.03)	0.34 (0.01)	0.13 (0.09)
Day 22	24.1% (1.2)	100	2.3 (1.2)	43 (8)	0.9 (0.2)	0.20 (0.04)	0.36 (0.01)	0.11 (0.04)
Day 29	25.0% (2.0)	100	2.5 (0.6)	32 (4)	1.1 (0.2)	0.26 (0.02)	0.27 (0.03)	0.12 (0.04)
Day 50	26.5% (5.2)	100	2.2 (0.3)	24 (3)	1.1 (0.4)	0.21 (0.02)	0.28 (0.02)	0.17 (0.1)
Day 71	28.1% (8.3)	100	3.0 (1.9)	49 (22)	0.6 (0.4)	0.21 (0.05)	0.40 (0.05)	0.18 (0.08)
Day 105	22.3% (6.1)	100	2.7 (0.2)	39 (20)	1.3 (0.4)	0.23 (0.04)	0.29 (0.02)	0.42 (0.28)
Day 134	30.2% (5.9)	100	2.3 (0.6)	32 (7)	0.7 (0.2)	0.24 (0.02)	0.22 (0.03)	0.09 (0.02)
Day 162	15.5% (2.1)	100	2.1 (0.9)	32 (1)	0.7 (0.2)	0.22 (0.06)	0.26 (0.02)	0.06 (0.01)

*R4 =24-ethylepicoprostanol/24-Ecop, a putative faecal ageing ratio

**P1 <1.0 indicative of herbivore pollution; 1.0-4.0 complex mix of sterols derived from plant runoff and herbivore;

>4.0 plant runoff; >7.0 may indicate avian contamination