The sources of “natural” microorganisms in streams

Client Report

Prepared with support from Envirolink

PREPARED FOR:	**Environment Southland and West Coast Regional Council**
CLIENT REPORT No:	**CSC 15004**
PREPARED BY:	**Megan Devane**
REVIEWED BY:	**Brent Gilpin and Elaine Moriarty**

ACKNOWLEDGEMENTS

ESR would like to acknowledge Envirolink for funding this report and the support of Roger Hodson (ES) and Jonny Horrox (WC) in the application.

DISCLAIMER

CONTENTS

EXECUTIVE SUMMARY

Faecal Indicator Bacteria (FIB), which include *Escherichia coli* and enterococci, live primarily in the gut of humans, animals and birds, and are almost always present in faeces. Testing water for the presence of FIBs is a low cost way of evaluating the potential presence of faecal pollution in water, and therefore also the potential presence of disease-causing microbes (pathogens). It is too expensive and time-consuming to test water for all the individual pathogens that could be circulating in a local animal or human community at the time of sampling. To protect human health, guidelines have been established based on the levels of FIB. For example, drinking water must have <1 *E. coli*/100 mL, and for swimming and other full-immersion activities, rivers should have <260 *E. coli*/100 mL (Ministry for the Environment, 2003) .

In recent years, with the advent of sophisticated microbial and chemical testing methods, the ability to identify sources of faecal contamination has arisen. In most situations these tools allow the identification of a source of contamination such as septic tanks, dairy farms or wildfowl. However, in some rivers there can be elevated levels of *E. coli* which cannot be attributed to a particular faecal source(s). It may be that those high numbers of FIB are related to faecal inputs that the current source tracking tools cannot identify (such as certain wild animals). However, it is now recognised that some FIB in water may not be associated with faecal contamination, and therefore their presence may overestimate health risk.

Over the previous decade, evidence of environmental reservoirs as sources of FIB has arisen, negating the dogma that these bacterial indicators are only viable when isolated from the enteric environment of warm-blooded animals. It is now established that FIB, such as *E. coli* can persist and potentially multiply in tropical and temperate environments far removed from their natural reservoir of the animal gut. FIB isolated from environmental reservoirs have been termed “naturalised” FIB. The environmental reservoirs recognised as sources of FIB include sediments (Anderson et al., 2005; Davies et al., 1995; Devane et al., 2014), macrophytes and plants (Badgley et al., 2010a; Badgley et al., 2010c; Whitman et al., 2005), macroalgae (Badgley et al., 2011), periphyton (Ksoll et al., 2007), soils (Ishii et al., 2006a) and microinvertebrates (Neogi et al., 2014).

“Naturalised” FIB may be derived from faecal deposition whereby the microbe has subsequently adapted to reproduce and maintain its population within a non-host environment such as sediment. However, there is another theory that some “naturalised” FIB diverged from faecally-associated FIB many thousands of years ago and are natural

inhabitants of the environment; or in the case of enterococci, enteric strains may be derived from environmental strains (Weigand et al., 2014). In this alternative supposition, it is assumed that “naturalised” FIB have not been recognized as different, because they are identified by traditional testing methods as the same bacterial species as enteric FIB (Luo et al., 2011). With the advent of new Deoxyribonucleic acid (DNA) sequencing technologies, however, these “naturalised” strains have been documented as containing different genes to faecal FIB while still maintaining the core genes ascribed to their bacterial species (Luo et al., 2011). Furthermore, this lack of recognition of environmental sources of FIB may have been exacerbated by the focus on strains of FIB isolated from hosts rather than the environment, because the greatest majority of FIB strains in culture collections are derived from clinical settings (Walk et al., 2009).

Environmental reservoirs of FIB are of concern to water managers when faecal indicator bacteria and pathogenic microorganisms are re-suspended from such reservoirs as stream bed sediments, macrophytes and beach sand into the overlying water column (Mulugeta et al., 2012; Obiri-Danso and Jones, 2000). Disturbances of reservoirs can occur during heavy rainfall events. From the aspect of water quality monitoring, the problem of re-suspension can be limited by water managers restricting sampling to flow periods where rainfall has been absent in the preceding days. Recreational water activities such as wading, can also mix sediments etc. into the water column allowing potential pathogens to come into contact with recreational users during swimming and boating activities (Pettibone et al., 1996). Recent evidence has suggested entrainment of FIB from sediment to water column during base flow due to hyporrheic exchange leading to a continuous “bleed” of microorganisms into the water which impacts on water quality (Grant et al., 2011; Litton et al., 2010). Whether this bleed of microorganisms from the sediment includes pathogens has yet to be investigated.

The persistence of FIB in the environment and their subsequent naturalisation as part of the environment calls into question the efficiency of FIB to perform as indicators of faecal contamination in aquatic environments (Perchec-Merien and Lewis, 2013). These “naturalised” sources of FIB could be contributing to apparent microbial indicator load through re-suspension from sediments, washing from macrophytes and other vegetation (especially decaying vegetation, which may provide ideal conditions for microbial growth), and soil run-off. The presence of naturalised FIB is likely to confound the correlation between indicator and pathogen and make it difficult to determine the health risk represented by elevated FIB. These findings raise complex questions, and answers may depend on location, type of environment and types of faecal pollution source.

New techniques are being developed to address the issue of "naturalised" *E. coli* contributing to FIB monitoring. These methods range from simple, cost-effective tests to more sophisticated tools based on the Polymerase Chain Reaction (PCR). Novel PCR assays have been developed to target "naturalised" *E. coli*. It has been postulated that these *E. coli* can be differentiated from enteric *E. coli* because they lack some of the genes required for host colonisation, and acquired other genes which aid survival outside of the host. The aim of the new methods would be to quantitatively determine the contribution of "naturalised" *E. coli* to concentrations of *E. coli* in a water sample, and understand the human health risk associated with those "naturalised" *E. coli*. Currently however, the recommendation remains that whenever FIB are elevated above the Water Quality standards it should be assumed that there is a public health risk until further investigation has demonstrated otherwise.

The relationship between FIB and pathogens may be clarified by determining the age of the faecal pollution event in the water body. The faecal ageing ratio, Atypical colonies/Total coliforms (AC/TC) is a cost-effective test that monitors the dynamic population in a waterway when the background microbial population in that water body is dominated by an influx of microbes from a recent faecal input. During a faecal contamination event, the faecal ageing ratio is dominated by the bacteria derived from the faeces, but this gradually changes as the faecal bacteria (represented by TC) die-off in the aquatic environment and the river microbial population (AC) re-establishes itself as the dominant microflora. The faecal ageing ratio is a simple culture test that can be performed at the same time as the FIB tests, with the AC and TC colonies differentiated on the same chromogenic media. If a problem with fresh elevated sources of *E. coli* is recognised using the combined FIB and faecal ageing test, then additional testing requiring more sophisticated faecal source tracking (FST) tools can be employed.

For the site under investigation, it is important that sanitary surveys and FST studies are carried out to determine the likely faecal and non-faecal inputs of FIB. In addition, research is required that questions whether environmental reservoirs are acting as sources for pathogen transmission. This requires comprehensive testing of environmental samples for the pathogens of concern, and correlation with concurrent detection of the faecal indicators.

Investigation of water quality suspected of faecal contamination, therefore, should follow a multi-tiered approach, initiated by identification of elevated FIB levels such as *E. coli*. The cost-effective faecal ageing ratio (AC/TC) could be included in this first tier with *E. coli*. Identification of *E. coli* at levels suggesting a potential health risk should lead to a second tier of testing which would include assays to track down the sources of faecal contamination. Levels of FIB suspected of having a dominant contribution from environmental populations, could initiate a third tier of investigation, including subtyping of FIB species to determine

clonal relatedness, which would indicate a naturalised population. Alternatively, novel PCR assays targeting FIB strains thought to be environmentally-associated could be applied in conjunction with other PCR markers for faecal source tracking

1. INTRODUCTION

Elevated levels of faecal indicator bacteria (FIB) have been detected in waterways throughout New Zealand (NZ) where, despite extensive faecal source tracking (FST), no source has been positively identified. It could be that some of these FIB are associated with faecal sources such as feral animal populations that currently are not identifiable using the existing FST tools. In addition, it has been proposed that these bacteria may represent "naturalised" sources of *Escherichia coli*/enterococci and other FIB, in that they have adapted to persist and reproduce in aquatic and terrestrial environments post-defecation. There is concern that these "naturalised" sources may lead to high counts of *E. coli*/enterococci in recreational water in the absence of a faecal source. Currently, a number of Regional Councils routinely analyse water samples for FST to identify sources of pollution that are impacting on waterways. Faecal source tracking has been carried out at a number of sites in NZ. While a source has been established for many of the sites, this has not been possible for all. This results in uncertainty of source, and Councils are unsure of the next step in the process. This report reviews current literature in the area of environmental sources of FIB which do not represent sources of recent faecal deposition. We will provide this report to Regional Councils and present the findings of the literature review to the Surface Water Integrated Management (SWIM) meeting in autumn, 2015 and discuss the findings with all Regional Council Scientists present.

The human health and environmental benefit of receiving this advice is building on the knowledge and understanding of factors that impact water quality. It will allow Councils to assess which factors they can manage to improve water quality such as overland flow, and others which they may not be able to alter, including "naturalised" sources of *E. coli* in a stream. This advice will allow Councils to target the sources they can manage and produce real positive improvements in water quality. It will be invaluable to all Councils when reviewing the quality of their water and identifying sources as currently the concept of bacteria from a non-faecal source in a waterway is not considered.

1.1 Report Structure

Section 2 The role of the faecal indicator bacterial in identifying a public health risk from faecal contamination and implications for recreational water quality standards and public health risk

Section 3 Limitations of faecal indicator bacteria and whether they always represent a health risk when identified in a water body

Section 4 Environmental reservoirs of non-host FIB which include sediment, sand, soil, macrophytes and terrestrial plants

Section 5 The relevance of determining the age of a faecal input to a waterway

Section 6 Developments in novel methods for differentiating between the enteric FIB and “naturalised” FIB strains in water samples

Section 7 Frequently asked questions with answers

Section 8 Glossary of Terms

2. THE ROLE OF THE BACTERIAL INDICATOR

The role of the indicator in water quality assessment is to identify substances that could be of potential risk to human health. In the case of microbial water quality, the indicator is a substance that is strongly associated with faecal contamination and therefore, indicates risk of human infectious disease.

Factors that determine the ideal microbial indicator include (Standridge, 2008):

- 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 disease-causing organisms (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 present in the faeces of individuals of 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 usually present in much lower concentrations compared to the indicators. 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.

1.1 Recreational Water Quality Guidelines

The dramatic reduction in waterborne disease over the last 100 years in developed countries such as New Zealand, owed much to the simple detection of faecal indicator bacteria (FIB) in water as sentinels of faecal contamination. Recreational water quality criteria (RWQC) are based on scientific conclusions from the relationship between concentrations of culturable faecal indicator bacteria and rates of gastrointestinal illness (GI). In epidemiological studies of recreational waterways, the coliform bacterium, *Escherichia coli*, has shown a strong correlation with the rates of 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 *E. coli*

in freshwater and enterococci levels in marine water quality guidelines established by the United States Environmental Protection Agency (USEPA, 1996). In the *New Zealand Microbiological Water Quality Guidelines for Marine and Freshwater Recreational Areas* (Ministry for the Environment, 2003) it states that fresh water containing less than 260 *E. coli* per 100 mL (Alert Level) is acceptable for recreation such as swimming, but that concentrations higher than 550 *E. coli* per 100 mL are not acceptable (Action Level). In marine waters, the levels of enterococci for the Alert level are 140 most probable number (MPN)/100 mL and for the Action level are 280 MPN/100 mL.

Recently, 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 USEPA believes that RWQC are protective of human health irrespective of the source of the faecal contamination. However, section 6 of USEPA, 2012 describes site specific protocols for determining health hazards based on faecal sources specific to a location, because not all biological sources have been reported as having the same health hazard attributed to their 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. The lower risk from birds is attributed to the lower level of pathogen carriage by bird species.

1.1.1 Correlations between FIB and pathogens

Detection of pathogens is partly dependent on the type of faecal input, be it animal, human or bird, treated or raw faecal waste, and whether the pathogen is circulating in the animal/human community at the time of the faecal contamination event. Pathogens can be present in low concentrations in a waterway but because of their low infectious dose this can still represent a human health risk. The low concentration of pathogens makes detection difficult and requires identification of indicators of faecal inputs as a surrogate to pathogen detection.

Detection of pathogens is partly dependent on the type of faecal input, be it animal, human or bird, treated or raw faecal waste, and whether the pathogen is circulating in the animal/human community at the time (Soller et al., 2010; Wu et al., 2011). Pathogens such as the protozoan *Cryptosporidium* may be present in low concentrations eg 1 oocyst/10 L but because of its low infectious dose may represent a significant risk to primary contact recreation or drinking water supplies. McBride et al. (2012) determined the median infectious dose (ID_{50}) for *Cryptosporidium* to be ≈35 oocysts.

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). It is recognised 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 (Harwood et al., 2005).

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, PCR and qPCR markers of human pollution. Large numbers of people (n = 1000-21,000) were surveyed but few correlations were observed with bathers compared with control groups. The studies employing qPCR markers for enterococci have shown the potential to predict the case numbers of swimming-related illnesses (Wade et al., 2008; Wade et al., 2006; Wade et al., 2010).

Work on the partitioning behaviour of the pathogenic protozoans, *Cryptosporidium* and *Giardia* species has suggested that *E. coli* and other microbial faecal indicators such as *Clostridium perfringens* have similar settling velocities when microbes associate with particulate matter in waterways (Cizek et al., 2008). This suggests that these microbial indicators may display similar transport behaviour in receiving waters as the protozoans. The Cizek et al. (2008) study, however, did not identify any strong correlations between the concentrations of one particular indicator and the protozoan pathogens. *E. coli* and enterococci were noted to be the best overall indicators of *Giardia* and evidence from a major human sewage input to a urban river supports this finding for *E. coli* (Devane et al., 2014). In Cizek et al. (2008), *C. perfringens* was the one of the better indicators of the presence of *Cryptosporidium*, which was consistent with the findings of previous studies (Ferguson et al., 1996; Payment and Franco, 1993). In contrast, the study of Devane et al. (2014) identified *C. perfringens* as a ubiquitous inhabitant of water in an urban river impacted by major human sewage discharges. There was no relationship, therefore, detected between *C. perfringens* and either protozoan, *Cryptosporidium* or *Giardia* in water in that study. The study of Cizek et al. (2008) also noted that a single day of storm inputs could be equivalent to several months of dry weather inputs and they suggested that the most productive focus for improving water quality would focus on reducing storm related inputs, rather than dry weather events such as leaking septic tanks.

2 Microbial faecal indicators and their limitations

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. 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). Environmental factors that affect the FIB-pathogen relationship include wastewater treatment, river dilution, movement within a river, storage in sediments, and the intrinsic characteristics of the microorganisms.

2.1.1 What is the problem for recreational water managers?

In general, **point sources of faecal contamination** such as wastewater from municipal treatment plants and slaughterhouses result in very high levels of microbial indicators, and therefore, there is a good correlation between FIB and faecal input.

The role of faecal microbes, such as *Escherichia coli* and enterococci, as indicators of water quality has been publicly questioned due to research revealing the growth and/or persistence of faecal microbial indicators in the environment (McLellan et al., 2001; Mulugeta et al., 2012; Solo-Gabriele et al., 2000; Whitman et al., 2006). The debate has arisen in the developed world as the source of faecal microbes in water has moved from high levels of contamination related to human faecal inputs, to lower levels of diffuse pollution from a range of non-human sources. Point sources of faecal contamination such as wastewater from municipal treatment plants generally result in very high levels of microbial indicators, which are more readily identified compared with diffuse pollution sources. As an example, a study of an urban river impacted by large volumes of human wastewater observed that *E. coli*, was a reliable indicator of human health risk due to its correlation with detection of *Campylobacter* and pathogenic protozoan (Devane et al., 2014). Non-point sources of diffuse pollution include leaking sewer pipes or septic tanks, wildlife sources and agricultural land runoff. This diffuse pollution may be reflected in lower but persistent concentrations of traditional microbial indicators, which are difficult to trace and require more sophisticated faecal source tracking tools (Field and Samadpour, 2007; Gilpin et al., 2008; Hagedorn et al., 2011; Santo Domingo et al., 2007).

Diffuse pollution sources can result in lower but persistent concentrations of traditional microbial indicators, compared with point sources. Therefore diffuse sources such as leaking sewer pipes or septic tanks, wildlife sources and agricultural land runoff, can be difficult to trace and require sophisticated faecal source tracking tools.

Moderate to low levels of *E. coli* in freshwater that are close to the NZ recreational guidelines between 260-550 *E. coli*/100 ml are problematic for water quality managers tasked with recommending site closures for recreational activities. At levels of *E. coli* above 550 *E. coli*/100 ml, water managers are required to take action to mitigate the sources of pollution. Faecal sources of contamination, however, may not be readily apparent during routine surveys of waterways. In addition, the concerns raised about the potential environmental sources of FIB further confound water management practices for eliminating the sources of faecal pollution (Anderson et al., 2005; Byappanahalli et al., 2003a; Whitman et al., 2006).

FIB are no longer considered to be exclusively found in the gut environment of warm-blooded hosts with a short survival time once excreted into the environment. **Environmental reservoirs**, including soils, beach sand, sediment, algae, macrophytes and plants, have been identified where FIB can not only survive but proliferate.

When the faecal coliforms were first proposed as a method of assessing water quality, it was thought that faecal coliforms 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 FIB in water environments was considered unlikely because ambient temperatures ranged from 4-25°C and nutrient status was in continual flux. 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; Desmarais et al., 2002; Solo-Gabriele et al., 2000). These investigations have been extended to temperate environments where FIB have been shown to persist and then in subsequent research shown to have the ability to replicate in the environment under nutrient regimes and temperatures not previously thought credible by scientists (Byappanahalli et al., 2003a; Ishii et al., 2006a; McLellan, 2004; Whitman and Nevers, 2003).

Laboratory experiments of *E. coli* cultured in broth under different temperature regimes noted that strains of *E. coli* were unable to replicate at 2°C and most strains

required temperatures of 11°C for replication (Ingle et al., 2011). However, *E. coli* belonging to the putative "naturalised" Clades replicated at 5°C. Beversdorf et al. (2007) conducted temperature experiments for growth of *E. coli* in sandplots. All sandplots inoculated with *E. coli* (~10^7 cells /100 g sand) demonstrated an initial increase in *E. coli* concentration within the first 24 hours as has been observed in other environmental experiments on water (Gilpin et al., 2013) and faeces (Moriarty et al., 2012; Sinton et al., 2007b). The sandplots exposed to environmental conditions with fluctuating temperatures (23-32°C) showed the highest *E. coli* cell increases of 100–fold compared with temperature-controlled sandplots. The environmental sandplots maintained cell numbers up to 6 days followed by a gradual decrease in cell density. The temperature-controlled sandplots showed maximum increases of twenty-fold *E. coli* with ambient temperature (23-25°C) >4°C and 37°C >44.5°C and no recovery of cells from either 50°C or 55°C sandplots. Cell densities from sandplots of ambient, 4°C and 37°C after initial increases remained stable up to 2 days before die-off. Temperatures of 37°C and 44.5°C showed the faster rates of cell density decline compared with lower temperatures. Over the course of the 19 day experiment, the environmental and ambient temperature sandplots showed the longest persistence of *E. coli* followed by 4°C conditions.

Temperature inactivation of *E. coli* in marine and lake waters (approximate temperature range 10-30°C and 4-40°C, respectively) was noted to increase with increasing water temperature with higher inactivation of *E. coli* in marine water compared with fresh (Pachepsky et al., 2014). In addition to temperature effects, the survival rates of FIB in the environment will be dependent on salinity, sunlight inactivation, organic matter and the impact of predators on the bacterial population (Geldreich, 1966; Gilpin et al., 2013; Rozen and Belkin, 2001; Sinton et al., 2002). Effects of UV and visible light in sunlight 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). The lower survival rates of *E. coli* in marine waters compared with fresh has generally been attributed to the higher ionic (osmotic) stress on bacterial cell membranes in seawater making them more vulnerable to sunlight inactivation (Moss and Smith, 1981; Sinton, 2005). However, salinity can also reduce bacterial predator populations which can have a positive impact on FIB survival as seen for PCR markers that target bacteria in marine waters (Okabe and Shimazu, 2007). High organic matter content such as sewage inputs can increase survival of *E. coli* in aquatic environments (Rozen and Belkin, 2001). The characteristics and abundance of reservoirs for FIB such as sediment and sand in or near a waterway, will also affect FIB persistence and/or replication.

There have been discrepancies between research studies on factors affecting survival of FIB and Korajkic et al. (2014) has suggested that decay factors may be impacted by the length of the experimental period. Korajkic et al. (2014) suggest that sunlight may only be important in the early stages (first few days) of decay, after which predator/competitor relationships in aquatic environments become 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 to 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). Based on all these factors it would be expected that populations of naturalised FIB in a specific location would be in flux and this would impact on the levels measured in water.

Genotypic and phenotypic studies in various climates have shown that populations of *E. coli* and enterococci isolated from soils and sand cluster by location into distinct but diverse groups. Such clustering, especially at the genotypic level, suggests that *E. coli* in the natural environment come from multiple sources but have sufficiently diverged from animal and bird sources in the same geographical location for them to no longer be considered of recent faecal origin. (Byappanahalli et al., 2012e; Fujioka and Byappanahalli, 2001; Ishii et al., 2006a; Perchec-Merien and Lewis, 2013). This has led to the term "naturalised" *E. coli* populations which are capable of replication in the environment (Perchec-Merien and Lewis, 2013; Whitman et al., 2014b).

3 Environmental reservoirs of non-host FIB

3.1 Sediments, soils and sand as environmental reservoirs for FIB

Studies have shown that even in the absence of recent faecal inputs, the faecal indicators *E. coli* and enterococci can occur as part of the microflora in sediments, soil, sand, terrestrial and aquatic plants, and algal mats (Byappanahalli et al., 2003a; Byappanahalli et al., 2012a; Byappanahalli et al., 2003d; Perchec-Merien and Lewis, 2013; Whitman et al., 2005).

3.1.1 FIB in soils and sediments

Research has identified populations of FIB in tropical and sub-tropical soil environments (Byappanahalli et al., 2012b; Desmarais et al., 2002; Solo-Gabriele et al., 2000) and temperate climates (Byappanahalli et al., 2006a). In a subtropical study by Desmarais et al.

(2002), soil samples taken up to 90 cm from the edge of river banks recorded low levels of enterococci $<10^2$ MPN/gram (g) dry weight (dw) soil and *E. coli* concentrations were less than 700 MPN/g dw with lowest concentrations $<10^2$ MPN/g dw at the furthest site from the river bank edge. In another subtropical environment, Solo-Gabriele (2000) identified soil concentrations of *E. coli* collected from river banks in the range of 14-300 MPN/g dw. Similar concentrations of *E. coli* were identified in Hawaiian soils; with average enterococci levels of 977 MPN/g soil (Byappanahalli et al., 2012b). In the temperate soils of a coastal forest on the Great Lakes, Byappanahalli et al. (2006a) noted average soil concentrations of *E. coli* of 16 MPN/g dw with up to 1659 MPN/g dw. In these studies, soil run-off during rainfall events and high tide influenced the re-suspension of microbes, which were identified as contributing to the elevated FIB in the streams and rivers under study.

Studies have shown that even in the absence of recent faecal inputs, the faecal indicators *E. coli* and enterococci can occur as part of the microflora in **sediments, soil, sand, terrestrial and aquatic plants, and algal mats. Clonal populations of FIB** have been detected in these environments suggesting the ability of FIB to survive and grow outside of the animal host.

Sediments in a freshwater lake in Switzerland were evaluated for FIB concentrations near the outlet of a wastewater treatment plant (Haller et al., 2009). FIB levels ranged between 10^5-10^7 colony forming units (CFU)/100 g dry sediment. Intact sediment cores were collected and stored at 4ºC for 90 days, and reported persistence of *E. coli* and enterococci up to 90 days in the top surface layer (2 cm depth) at concentrations between 10^4 -10^5 CFU/100 g dry sediment.

There is increasing evidence that FIB not only persist outside intestinal environments, but also actively grow in soil and sand environments and algal mats across the tropical to temperate climate spectrum (Byappanahalli and Fujioka, 2004; Byappanahalli et al., 2012b; Byappanahalli et al., 2003d; Byappanahalli et al., 2006c; Desmarais et al., 2002; Staley et al., 2014). Mesocosm experiments using sediment and water collected from a river system in Florida were performed by Desmarais et al. (2002) and revealed growth of enterococci and *E. coli* under conditions of simulated tidal influence with wetting and drying phases. Soil moisture, nutrient conditions and indigenous soil bacteria were identified by Byappanahalli and Fujioka (2004) as limitations on the growth of *E. coli*, but to a lesser extent on enterococci in Hawaiian soils. They still observed, however, slow and sporadic replication of FIB which allowed the FIB to establish as a minor portion of the soil microflora. Pote et al. (2009) observed growth and persistence of FIB in sediment microcosms derived from a

freshwater lake in Switzerland. Low temperature (10ºC) and low organic matter content of sediment were seen to impact negatively on FIB concentrations in the sediment. Other studies have confirmed the importance of organic matter content in sediment, with higher levels of organics facilitating the survival of FIB in sediment and soil (Desmarais et al., 2002; Lee et al., 2006).

3.1.2 FIB in beach sands

The occurrence of *E. coli* populations in beach sand has also been reported. In a study of California marine beach sands, Lee et al. (2006) observed levels of enterococci >10^4 MPN/100 g wet sediment in enclosed beaches that were not subject to intense wave activity. This level was two to three orders of magnitude higher compared with enterococci levels at eleven open beaches, where the authors postulate that wave activity led to the increase of re-suspension and seaward transportation of enterococci. The relationship between *E. coli* levels in sediment at the enclosed beaches compared with open beaches was less clear as levels of *E. coli* in sediment generally ranged between 10^2-10^4 MPN/100g wet sediment. In microcosm experiments, Lee et al. (2006) also observed that the presence of (autoclaved) beach sediment enhanced the levels of *E. coli* in the overlying water by two to three orders of magnitude. In contrast, in microcosms lacking sediment, the *E. coli* levels in the water decreased over the first two days by three orders of magnitude. The researchers suggest that enhancement of *E. coli* levels in water was due to formation of biofilms on sand surfaces and higher organic matter associated with the sediment. Whitman and Nevers (2003) performed a comprehensive spring-summer survey of *E. coli* concentrations in water, and foreshore and submerged sand at a freshwater lake beach. Mean counts of *E. coli* were consistently highest in foreshore sand (10^4) > submerged sand (10^3) > water (10^2) (all counts reported as CFU/100 mL). They noted a disconnect between *E. coli* counts in water between consecutive days, which calls in to question, for example, the reliance on Day 1 FIB results determining beach closures on Day 2. Annual replacement of foreshore sand for the swimming season reported a rapid re-colonisation of *E. coli* concentrations in the imported sand. A synchronous trend of decreasing and increasing *E. coli* counts in sand and water was observed over the spring-summer study, with a gradual increase in *E. coli* concentrations in both media, which they attributed to physical parameters such as air and water temperature increases. The authors provided evidence that the water was not concentrating *E. coli* to the sand, including the higher concentrations in sand compared with water, and the steady equilibrium between counts in sand and water over time. They concluded that beach sand plays an important role in water quality FIB levels and that the sand is acting as a source of *E. coli* for the water rather than as a sink. In another study, freshwater, temperate beach sand at up to 40 metres inshore (backshore sand) was shown

to be a reservoir of *E. coli* and enterococci with seasonal persistent populations observed (Byappanahalli et al., 2006c). Growth studies of *E. coli* and enterococci in beach sand noted persistence in non-sterile sand but not growth. Lack of growth was attributed to the presence of predators restricting FIB numbers and competition with indigenous bacteria for limited nutrient resources (Hartz et al., 2008).

Multiple *E. coli* and enterococci virulence genes including Shiga toxin genes and antibiotic resistant genes have been identified in marine sediments in Italy including at recreational beaches where the overlying seawater complied with RWQC (Di Cesare et al., 2014; Luna et al., 2010; Vignaroli et al., 2013; Vignaroli et al., 2012). These findings have prompted the researchers to recommend including the sediment environments in sampling strategies when monitoring water quality. A recent workshop that brought together international experts discussed the issues relating to the microbial quality of beach sands in regards to public health risk with a view to developing the standardisation of routine evaluation of sand at coastal sites of recreational significance (Sabino et al., 2014). One of the recommendations was that beach sand should be screened for a variety of pathogens known to impact human health, and monitoring of sand microbial quality should occur in conjunction with routine water monitoring.

3.1.3 Transport of FIB between the sediment and overlying water column

Many rivers, particularly those in catchments prone to erosion, have the potential to store many microorganisms in their sediments. Transport and transfer of microbes between the sediment and water column may be a dynamic process that is likely to occur during base river flow as well as high flow events (Litton et al., 2010; Yakirevich et al., 2013). One hypothesis proposes that continual input and entrainment of microbes into the water column is due to exchange of water across the sediment-water interface in the region beneath and alongside a river bed, where there is mixing of shallow groundwater and surface water (Grant et al., 2011). However, 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 different flow conditions. Characterisation of *E. coli* populations in streambeds was investigated by Piorkowski et al. (2014). These researchers determined that sampling to define *E. coli* concentrations required different strategies during baseflow and storm flow conditions. During baseflow, effective particle sediment size and water velocity were the most important parameters impacting on *E. coli* re-suspension. In contrast, during storm flow, although water velocity was again an important factor, organic carbon in the sediment and median particle diameter of sediment were also important explanatory variables. DNA fingerprinting of *E. coli* populations revealed that areas of deposition (e.g. pools) within a stream bed had different populations of *E. coli* strains

compared with the high velocity features (riffles and runs). Therefore sampling strategies should account for morphological variability in the streambed rather than only sampling sediment directly underlying the collection of a water sample. They also advise that population characterisation of *E. coli* isolates requires up to 120 isolates per streambed feature to capture the inherent high diversity.

Transport and transfer of microbes between the sediment and water column may be a dynamic process that is likely to occur during base river flow as well as high flow events.

3.2 Macrophytes and terrestrial plants as reservoirs for FIB

3.2.1 The alga Cladophora as a reservoir for FIB

A study of FIB association with macrophytes has mainly focused on the green alga *Cladophora* which inhabits freshwater and marine aquatic environments. *Cladophora* grows on hard substrates in shallow waters where it colonises rocks and piers (Dodds and Gudder, 1992). It can break free of these substrate surfaces and form large free floating mats and it is these mats that were first identified as sources of FIB when they washed up on freshwater beaches in the Great Lakes area of North America (Byappanahalli et al., 2003d; Olapade et al., 2006; Whitman et al., 2003). The *Cladophora* are thought to provide protection to microorganisms from predation and the harmful effects of UV irradiation, and also provide nutrient sources from algal surface exudates (Beckinghausen et al., 2014; Marks and Power, 2001; Zulkifly et al., 2012). *Cladophora,* therefore*,* is thought to provide a haven for a variety of organisms including diatoms, bacteria and cyanobacteria. An examination using 16S rRNA sequencing of the epiphytic bacterial community of actively growing *Cladophora* did not identify pathogens or *E. coli* associated with the alga (Zulkifly et al., 2012), however another study using culturable methods and PCR did identify pathogens (Ishii et al., 2006d).

Microcosm studies of the impact of UV irradiation on the interaction of *E. coli* and *Salmonella* with *Cladophora* noted extended survival of the two microorganisms when in association with the alga, suggesting a protective effect by the *Cladophora* mats (Beckinghausen et al., 2014). Initial concentrations of *E. coli* and *Salmonella* were 10^5 CFU/100 mL and *E. coli* reported a 7 log removal after six hours in water with no algae present compared with 16 hours for the same log removal when algae were present in the microcosm. In comparison to *E. coli*, *Salmonella* showed a higher resistance to die-off with and without algal presence, though its persistence was greater in the presence of the algal mats.

In another study, focused on *in situ* free-floating algal mats, the *E. coli* concentrations associated with *Cladophora* mats were investigated by sampling water underlying the mats, on either side of the mats (extending out from the left and right sides), and at regularly spaced intervals within the mats (Heuvel et al., 2010). Concentrations of *E. coli* in the water beneath the algal mats was statistically significantly higher (3.0-4.0 Log_{10} MPN/100 mL) than in the surrounding water (1.0- 3.5 Log_{10} MPN/100 mL). Significant positive correlations were identified between *E. coli* attached to the algae (>4.0 Log_{10} CFU/g dw, on all sampling occasions) and *E. coli* in the water underlying the algal mat. The concentration gradient of *E. coli* at regularly spaced intervals within and extending from the algal mat, showed that there were higher *E. coli* concentrations at the centre of the mat compared to the edges presumably due to the mat's protective influence from the effects of wave and wind.

3.2.2 Clonal relationships within FIB species in algal mats

A follow-up study on the *Cladophora* mats (Badgley et al., 2011) confirmed that algal mats may be a source of *E. coli* to nearby water. The population structure of over 4000 *E. coli* isolates from the mats collected over a three year timeframe and during multiple three day sampling periods was determined by DNA fingerprint analyses. Overall, genetic analysis revealed a high degree of diversity between isolates over spatial and temporal (annual) periods, although 33% of isolates were represented by multiple clonal groups. Badgley et al. (2011) suggest that these clonal populations may represent "naturalised" groups of *E. coli* that are persisting in the environment and have adapted to survive and replicate within the algal mats. When the finer detail of diversity was examined between mats and over the 3 day periods for individual mats, then *E. coli* isolates were highly related within individual mats. In addition, there was temporal genetic clustering of isolates when the same mat was examined over 3 consecutive days, suggesting transient populations that changed on a daily basis. Together these results reflect a complex dynamic of *E. coli* strains from a variety of faecal inputs with concurrent populations of "naturalised" *E. coli* populations. The authors conclude that the mosaic pattern of *E. coli* strains makes it difficult to use them as targets for tracking the source of faecal pollution.

3.2.3 Submerged aquatic vegetation as reservoirs of FIB

Badgley et al. (2010a) conducted a survey of the concentration and genetic make-up of enterococci populations associated with sediment, water and a variety of submerged aquatic vegetation (SAV) in freshwater and estuarine subtropical environments. They noted a decreasing mean density of enterococci concentrations in SAV > sediment > water with a range of genetic diversity from high to low, with the lowest diversity noted in lake water during summer. They also showed evidence for the persistence of certain strains which might represent naturalised populations of enterococci.

Badgley et al. (2010c) performed mesoscosm studies on the effects of submerged macrophytes on enterococci populations. They seeded the mesocosms with freshwater populations of water, sediment and SAV (mostly the plant *Hydrilla verticillata,* known as Esthwaite Waterweed). All of the matrices, at the time of sampling, had chronically elevated levels of enterococci. Concentrations of enterococci were monitored over 14 days in mesocosms of sediment and water both with and without SAV. As in Badgley et al. (2010a), the highest densities were observed in the macrophytes compared with sediments and water. However, when comparing their unit mass to the other two matrices, the contribution of macrophyte-associated enterococci was determined to be a minor portion of the entire system. However, the presence of SAV facilitated the persistence of enterococci in the vegetated mesocosms compared with non-vegetated mesocosms. Badgley et al. (2010c) emphasise that the importance of FIB-associated substrates on water quality concentrations will be location dependent, in regards to the total mass of the bacterial reservoirs and the volume of water available for re-suspension. Genotypic typing of the enterococci strains at the beginning and during the study, revealed that the population of enterococci was dominated by a single strain of *Enterococcus casseliflavus. E. casseliflavus* has commonly been associated with waterfowl which were identified as inhabitants of the lake from which the matrices for these experiments were collected. This "naturalised" strain of *E. casseliflavus* persisted over the ten month duration of the study, suggesting its ability to persist and reproduce in the environment.

Leewis (2006) studied the impact of the non-algal aquatic macrophyte species of *Sagittaria* and *Myriophyllum* on *E. coli* concentrations in freshwater. Similar to the *Cladophora* mat experiments of Heuvel et al. (2010), Leewis (2006) noted an increase in concentration of *E. coli* associated with macrophyte mats in comparison to *E. coli* concentrations in the water at distances of 2, 5 and 10 metres from the mats.

3.2.4 Periphyton as reservoirs for FIB

Biofilms of periphyton form on submerged substrates and consist of diatoms, green algae, protozoa and a complex community of microbes including cyanobacteria and fungi. Ksoll et al. (2007) investigated the contribution of periphyton as reservoirs of faecal coliforms and *E. coli* in a freshwater temperate lake. Periphyton-associated concentrations of faecal coliforms peaked at 10^5 CFU/cm^2 in the summer. Using DNA fingerprinting analyses, Ksoll et al. (2007) determined that the major faecal sources of the *E. coli* contributing to water samples overlying the periphyton were waterfowl, sewage and periphyton. They also

identified some temporally persistent strains of *E. coli* that could not be attributed to a source and postulated that these strains may belong to a group of “naturalised” *E. coli*.

3.2.5 Microinvertebrates as an environmental reservoir of microorganisms

The identification of microorganisms in microinvertebrates adds another dimension to the persistence of faecally-derived microbes in the environment, with the microinvertebrate postulated to act as a Trojan horse. Neogi et al. (2014) defines microinvertebrates as organisms mostly in the range of 50-500 µm with an upper limit of <5 mm, which includes rotifers, copepods, protozoa, nematodes, crustacean larvae and some insect larvae. Microinvertebrates can have a negative influence on the numbers of bacteria in an ecosystem by the forces of predation, whereby the grazing rates of some *in vitro* protozoa have been estimated at 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; Thomas, 2013). These microorganisms 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). Nematodes have been observed to feed on and produce viable and infective forms of the oocysts of *Cryptosporidium*, and cells of bacterial pathogens, thus acting as vectors of these disease-causing organisms (Anderson et al., 2003; Huamanchay et al., 2004). Ingestion of microinvertebrates by avian species can also provide a transmission route for dissemination of pathogens (Neogi et al., 2014).

3.2.6 Other environmental reservoirs of FIB

Additional environmental reservoirs such as cattle water troughs and pitcher plants have also been suggested for *E. coli* (LeJeune et al., 2001; Whitman et al., 2005). Enterococci have been identified in low numbers (at concentrations 10^1-10^4 CFU/g) as part of the epiphytic population on grasses (Muller et al., 2001; Ott et al., 2001). Isolates from grass included *Enterococcus* species of *E. faecalis* and *E. faecium* which are associated with faecal environments but also a majority of the isolates could not be definitively defined to a specific species of *Enterococcus* (Muller et al., 2001). Enterococci species present on plant material would be available for mobilisation into waterways during rainfall events that generate overland runoff. Harwood et al. (2004) suggested genomic methods are required to differentiate the sources of enterococci and investigated a PCR method that identified 100% of *E. faecalis* isolates from polluted waters and other environmental sources which were likely derived from recent faecal inputs.

3.3 Microbial targets for FST PCR markers occurring as "naturalised" populations in the environment

Bacterial communities belonging to the *Cytophaga-Flavobacteriales-Bacteroides* group have been identified in association with *Cladophora* (Olapade et al., 2006). These bacterial groups have been used as the targets for identifying the animal/human sources of faecal contamination. A study by Whitman et al. (2014a) identified free-living *Bacteroides* species associated with *Cladophora* mats, however genotypic sequencing suggested they were not closely related to enteric *Bacteroides* species. This finding again questions the dogma that water quality indicators reside solely in the enteric environment of animal hosts. This would have implications for the PCR markers that target the *Bacteroidetes* order as indicators of general faecal pollution and suggest that detection of these general markers may overestimate faecal contamination events. However, it may not impact the assessment of those markers that specifically detect a particular animal or human source based on a specific subset of the *Bacteroidetes* order. Further genomic studies of environmental *Bacteroidetes* will increase the understanding of the relevance of these specific PCR markers to identification of faecal sources.

4 Faecal ageing

4.1.1 AC/TC faecal ageing ratio

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 (TC) derived from sewage, which displace the background microflora normally associated with the waterway. During routine microbial plate enumeration of TC it was noted that atypical red and pink colonies might be detected alongside the distinctive green metallic sheen of total coliforms on the same agar medium. These red and pink atypical colonies were considered to be a nuisance, however it has been hypothesized that a large proportion of atypical colonies (AC) are indigenous to nutrient rich waterways (Brion and Mao, 2000) and have been shown to be relatively stable in comparison to TC levels in rivers during both high and low river flows (Nieman and Brion, 2003). 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*.

AC/TC ratios in fresh animal manure start at values <1 and increase with faecal aging. For fresh human sewage the AC/TC values are <1.5. Domestic sewage is a composite of faecal material of varying age and therefore may have higher AC/TC ratios. In water, AC/TC ratios of <5.0 suggest the input of fresh faecal material (Brion, 2005) due to high numbers of TC. The ratio again rises over time

as the faecal material ages and total coliforms die-off in the aquatic environment. The aged faecal material produces a higher AC/TC ratio (>20) than for fresh faecal material indicating the passage of time. After a rainfall event, it has been observed that the AC/TC ratios decrease as runoff from land carrying fresher faecal material is washed into the river system (Brion et al., 2002). The AC/TC ratio is a quick and cheap plate count assay similar to FIB tests and could prove to be a useful frontline addition to the microbial indicator toolbox alongside *E. coli* enumeration, prior to applying more sophisticated tools to water quality monitoring.

4.1.2 Sterol faecal ageing ratio

A second faecal ageing tool based on quantification of faecal sterols uses a sterol ratio comparing the human stanol, coprostanol and its isomer epicoprostanol. Epicoprostanol is present in very low levels in fresh human sewage but increases in concentration with ageing of the faecal material as cholesterol and/or coprostanol are converted to epicoprostanol by microbes in the sewage/sludge (Leeming et al., 1998; McCalley et al., 1981). A high coprostanol/epicoprostanol ratio, therefore, is suggestive of a recent faecal event. Combining the two faecal aging ratios of AC/TC and coprostanol/epicoprostanol has shown promise in detecting a difference between fresh and historical human faecal input to water (Devane et al., submitted "Relationships between chemical and microbial faecal source tracking markers in urban river water and sediments after continuous discharge of human sewage").

5 Developments in the differentiation of environmental and faecally derived *E. coli*, with implications for water quality monitoring

The identification of "naturalised" FIB in the environment that may be confounding routine water quality monitoring of FIB has recently stimulated research into the possibility of differentiating between FIB directly related to recent faecal inputs and those that are persisting and growing in the environment. The advent of whole genome sequencing has opened another world to researchers where they can drill down into the finer detail of a bacterial species and their concomitant strains - strains that were previously understood to be indistinguishable using the traditional biochemical testing regimes and 16S rDNA sequencing analyses (Luo et al., 2011; Walk et al., 2009; Weigand et al., 2014).

Research has raised questions as to whether the "naturalised" FIB are in fact made up of two groups: those strains of recent faecal origin that have adapted to persist/grow in the environment and also "true" environmental strains of FIB diverged from the faecally–derived

strains over long time frames of thousands to millions of years. These latter strains may only now be recognised as different to enteric FIB due to new sequencing technologies.

A study byWalk et al. (2009) identified five novel clades of *E. coli* isolates. Clades are a phylogenetically distinct group derived from a common ancestor. The *E. coli sensu stricto* ("strictly belonging to the species *Escherichia coli")* are known to fall into seven main phylogenetic groups (A, B1, B2, C, D, E and F) and virulent extra-intestinal strains belong mainly to groups B2 and D. Group E includes the pathogenic *E. coli* O157:H7, the most well-recognised member of *E. coli* associated with food and waterborne outbreaks of gastrointestinal disease (Gilbert et al., 2008; Samadpour et al., 2002). Most commensal strains belong to group A (Clermont et al., 2000; Clermont et al., 2013).

E. coli have been isolated from non-enteric environments such as soil, freshwater and freshwater sediments which are phenotypically and taxonomically indistinguishable to the enteric *E. coli.* Therefore, during routine water quality monitoring, both the environmental and the enteric isolates would be identified as contributing to the *E. coli* concentration in the water.

The novel clades of *E. coli* have been designated as I-V and include three clades (III-V) represented by strains of *E. coli* that have adapted to survival outside of the intestinal environment of an animal or bird host. The environmental clades of the *Escherichia* genus have been termed cryptic clades by Walk et al. (2009) as they are phenotypically indistinguishable but genetically divergent from typical *E. coli* (Deng et al., 2014). Clade V has, to date, been the most abundant of the environmental clades isolated (Vignaroli et al., 2014). Genome sequencing of representatives of these five "environmental" clades and additional enteric *E. coli* isolates from clinical specimens, food and avian species was performed by Luo et al. (2011). The *E. coli* isolated from the non-enteric environments included soil, freshwater and freshwater sediments and were identified as phenotypically and taxonomically indistinguishable to the enteric *E. coli*, which included isolates of both pathogenic and commensal strains. Therefore, during routine water quality monitoring, both the environmental and the enteric isolates would be identified as contributing to the *E. coli* concentration in the water. Genomic sequencing of the *E. coli* isolates revealed that while they all shared a core set of genes; there were genes that were either specific to, or highly represented in, either the environmental or enteric groups. For example, enteric *E. coli* had specific functional genes related to the transport and acquisition of nutrients abundant in the gut (e.g. gluconate). In contrast, the functions of genes specific to the environmental groups were related to survival in a non-enteric environment, such as enzymes which aid the hydrolysis of bacterial cell walls. These findings have been supported by a comparative microarray DNA-DNA hybridisation study by Oh et al. (2012, which identified bacterial

adhesion genes in enteric *E. coli* which were important for host intestinal colonisation by both pathogenic and commensal isolates. These adherence associated genes were absent in environmental strains of *E. coli*.

An important improvement for water managers would be development of biomarkers that differentiate between environmental and faecally-derived strains of FIB based on genetic differences that distinguish the two groups as they are indistinguishable by the current culturing and biochemical methods used for water quality monitoring.

Luo et al. (2011) and Oh et al. (2012) have suggested that an important consideration for water managers would be development of biomarkers based on specific genes that differentiate between environmental and faecally-derived strains of *E. coli*. A study investigating the virulence potential of the putative environmental *E. coli* clades observed that they were poor competitors in gut colonisation compared with the enteric strains (Ingle et al., 2011). Furthermore, although these environmental clades carried a varied range of virulence factors they were reported to be uncommon isolates from vertebrate hosts, indicating they could be opportunistic pathogens (Clermont et al., 2011). The researchers suggest that the low pathogen potential of the environmental strains means their identification in water environments during routine water quality monitoring may be overestimating the public health risk. Other important ecological characteristics of these environmental clades which differentiate them from enteric isolates include enhanced ability to form biofilms, lower temperature growth tolerance (as low as 5ºC) whilst maintaining an optimal growth temperature of 41ºC similar to all other members of the *E. coli* genus, and low levels of antibiotic resistance (Ingle et al., 2011). These factors increase their fitness for exploiting non-host environments, and suggest reduced exposure to the antibiotic rich environments of agricultural animals and humans. A study by Vignaroli et al. (2014) investigated the virulence features of Clades III-V which had been isolated from coastal marine sediments. Twenty of the 138 isolates from the sediments were classified as belonging to the environmental (termed cryptic) clades. Seventeen of these 20 environmental *E. coli* were susceptible to antimicrobials, they also all lacked the enteroaggregative *E. coli* pathotype virulence genes. In addition, 60% were positive for a gene encoding an enterotoxin (EAST1), and 70% for group II capsule production genes. Adhesion and invasion assays were performed on eight of the environmental *E. coli*, each varying in their gene content. Clade V displayed the highest adhesion properties similar to intestinal strains but no Clades showed the ability to invade the intestinal cell lines tested. The authors have suggested a dual role for Clade V, where it has adapted to the environment but still retains the ability for an intestinal lifestyle with the potential for

expression of virulence traits. The study also identified a potential environmental biomarker, *pduC*, which encodes a propanediol hydratase, and was statistically significantly associated with, but not exclusive to, the environmental clades.

Additional genomic-based studies may identify whether these novel clades remain in the species *E. coli* or the genus *Escherichia* requires formation of a new species to incorporate these environmental isolates. Unlike *Escherichia fergusonii* (Farmer et al., 1985) and *E. alberti* (Oaks et al., 2010), the putative environmental clades of *E. coli* have been shown to be phenotypically and taxonomically indistinguishable to the enteric *E. coli* isolates using the standardised API20E Identification System (BioMerieux, Inc.) and the BBL Crystal Identification System (Becton, Dickinson and Company) (Walk et al., 2009). Clermont et al. (2011) characterised and compared them with the phenotype of the majority of enteric *E. coli*, which are positive for the catabolism of lysine and ornithine. Overall with the exception of Clade 1 (the majority were positive for catabolism of both ornithine and lysine), the environmental clades were identified as being either negative for lysine and/or ornithine catabolism, distinguishing them from the enteric *E. coli*. Strains of C1 clade were also shown to be genotypically very close to enteric *E. coli* and therefore, could be treated as enteric bacteria (Deng et al., 2014; Luo et al., 2011).

PCR markers have been designed that target genes specific to either enteric or environmental "naturalised" *E. coli*. Genes have been identified in enterococci which may be targets for differentiation of the enteric enterococci from "naturalised" enterococci. Further research is required to validate these PCR biomarkers and determine the public health risk associated with the so-called "naturalised" FIB.

Genetic screening methods may be a more efficient method for differentiating between environmental and enteric *E. coli*. Clermont et al. (2000) designed a simple triplex PCR method for assigning *E. coli* to four phylogenetic groups. This PCR system was updated in 2013 to include four new phylogenetic groups identified by genomic data, and it also recognised the novel environmental clades (II-V) (Clermont et al., 2013). Clermont et al. (2011) designed an allele specific endpoint PCR to discriminate within the environmental clades of *E. coli* and exclude amplification of the enteric *E. coli* strains more often associated with infectious disease in humans. This allele specific PCR targeted the *aes* gene and a DNA fragment *chu*A. Other researchers are investigating PCR methods for differentiating between environmental and enteric *E. coli,* as PCR would provide the most efficacious genetic classification system for water managers. (Deng et al., 2014) identified a gene that is likely to be important for the enteric population of *E. coli* to survive the intestinal environment

and they proposed that identification of the putative glucosyltransferase gene (*ycj*M) in *E. coli* may confirm *E. coli* derived from a recent faecal origin. However, there was not a clear delineation, because although *ycj*M was identified in >955 of clinical and faecal isolates from animals and birds, some members of the environmental clades (III-V) were also positive at 23% of the isolates.

An important question is whether the identification of microbial indicators in aquatic environments represents a potential health risk from faecal contamination or if biomarkers designed to exclusively detect “environmental “ strains can reduce concern of a health hazard associated with their presence in a waterway. In the search for biomarkers that distinguish between FIB adapted to environmental versus enteric habitats, it is important to determine whether the habitat associated strains have the ability to switch between their environmental reservoirs and the enteric environment by turning on genes required for either lifestyle or whether those genes are absent from their genome. Weigand et al. (2014) have investigated the genome of *Enterococcus* species. They identified putative habitat-specific strains of enterococci where the environmental and enteric strains could not be differentiated by traditional phenotypic tests or by genetic tests using DNA-DNA hybridisation or 16S rDNA sequencing. Evaluation of genome sequencing of enterococci isolates from enteric environments identified specific gene signatures for carbohydrate utilisation of sugars which are prevalent in the gut such as xylose. In addition, pathogenicity islands (PAI) were identified as a common feature in the genome of enteric enterococci but not environmental isolates. PAI contain sequences which allow insertion/deletion into chromosomes and thus horizontal gene transfer between bacterial genomes. PAI are known to encode virulence genes and aid host colonisation. Genomic evidence presented by Weigand et al. (2014) suggests that the extra-enteric strains may predate the enteric strains, meaning that enterococci originated as environmental strains which then adapted to an enteric environment by acquisition of accessory metabolic functions, for example, via pathogenicity islands. A potential biomarker for environmental enterococci was the nickel uptake operon which was almost exclusively identified in environmental enterococci. The authors suggest that deletion of this operon in the enteric environment may not be disadvantageous, because nickel is present in much higher concentrations in the intestine compared with freshwater environments. Di Cesare et al. (2014) noted that virulence and antibiotic resistance genes were more commonly identified in enterococci derived from clinical settings compared with strains isolated from marine sediments. In particular, the virulence gene *esp* important in host colonisation and biofilm formation (Fisher and Phillips, 2009) was only identified in clinical isolates. They also identified the gene conferring resistance to the heavy metal, copper, in the environmental enterococci and suggested its presence may be related to

industrial pollution outputs in the Adriatic Sea environment from which the enterococci were isolated.

Identification of Clades III-V in Australia, United States and Puerto Rico (Walk et al., 2009) suggest a wide geographic representation suggesting they may also be represented in NZ. Analysis of sequence databases of *E. coli* strains has infrequently turned up members of Clades I-V. The databases have a major focus on strains isolated from human faecal samples and therefore are representative of pathogenic and commensal *E. coli* to the exclusion of other habitats which remain under sampled (Walk et al., 2009). The lack of occurrence in clinical settings also suggests Clades I-V are not highly pathogenic strains.

PCR markers have been designed that target genes specific to both enteric and environmental "naturalised" *E. coli*, and genes have been identified in enterococci which may be targets to differentiate the enteric enterococci from "naturalised" enterococci. However, a lot of research is required to determine the usefulness of these biomarkers and their relevance to water quality monitoring whose basic function is to determine a public health risk. It is unknown whether identification of these "naturalised" sources of FIB will represent a health risk in themselves or because they are associated with pathogens which have persisted in the environment. There is also a need to clarify if there are two groups of "naturalised" FIB consisting of those recently defecated into the environment and able to persist under favourable conditions; and those that diversified from the *E. coli* or enterococci lineages a long time ago, which have no relevance to faecal inputs but have the ability to confound water quality monitoring.

It is unknown whether identification of "naturalised" sources of FIB will represent a health risk in themselves or because they are associated with pathogens which have persisted in the environment. There is also a need to clarify if there are two groups of "naturalised" FIB consisting of those recently defecated into the environment and able to persist under favourable conditions; and those that diversified from the *E. coli* or enterococci lineages a long time ago, and therefore, may have no relevance to faecal inputs but do have the ability to confound water quality monitoring.

6 Frequently asked questions (FAQ)

1) Would nutrient status of a river/freshwater body affect the *E. coli* levels? Yes, nutrients associated with natural or foreign sources such as faecal inputs, provides additional resources to support persistence and growth of FIB in the water column and sediment, and in addition can increase the background river microbial population as well.

2) Would soil type/catchment influence FIB? Yes, these are physical parameters that will influence the persistence of FIB in the environment. For example, *E. coli* is known to have a greater affinity for soil particles larger than 2 μm, with preferential attachment to particles sized 16-30μm in size (Oliver et al., 2007). Differences in sediment type in a stream bed can influence whether sediments act as a net sink or a net source of microbes during base and storm flows, and will impact stand-down periods before a stream can be used again for recreation. The topography in a hilly catchment would increase runoff of faecal sources and FIB into waterways in comparison to a flat terrain. Also the types of activity carried out in the catchment will impact on FIB reservoirs in a catchment. For example, a catchment where agricultural practices involve livestock will probably increase sources of faecal pollution compared with forestry or native bush.

3) What should I do if elevated *E. coli* is detected? You need to establish the faecal source of the FIB by conducting a sanitary survey of the catchment, and then confirm this by carrying out faecal source tracking assays to differentiate between animal, bird and human faecal contamination or mixed sources of pollution.

4) What if FST has no answer? Repeat water sampling with multiple samples at different times to confirm persistent elevated FIB. Follow up those water samples which have high FIB by applying FST markers, including additional FST markers such as PCR markers that target different animal/bird hosts.

5) How do we know the FIB are not from a source that a test is not available for? Not all of the different faecal sources in the environment such as feral animals, can be tested with the current suite of FST tools. As an example, the avian PCR markers will not include all species of birds, especially native birds. However, the faecal sterol assay can indicate the presence of avian pollution from all bird species when assayed in the absence of human and animal faecal contamination.

6) There have been occasions where I had high *E. coli* readings but no source identified despite very high general faecal markers found. (Refer to answer No. 5) If there is no suspected faecal contamination source, then it may be appropriate to investigate

“naturalised” sources of FIB. From our experience and that of international researchers, the bacteria targeted by the general PCR marker may be prevalent in the water environment (refer to section 4.3). The assumption that all of the members of this large *Bacteroidetes* group of bacteria cannot grow outside of the animal host appears to be false. These bacteria require an environment devoid of oxygen for growth, but there is increasing evidence to suggest they both persist and grow in the environment. Therefore identification of high levels of the general PCR marker may be another indicator of a naturalised population. However, this does not invalidate the host-specific PCR markers based on the *Bacteroidetes*, which are less likely to be identified in the environment.

7) How do you use this information to guide my compliance with National Objectives Framework (NOF). The NOF allows for community decisions on setting objectives and limits for water quality that suit the local environment and are in line with the cultural values determined by the community. A thorough investigation of a particular location is required before nominating the Attribute state for a particular waterway. Investigation of water quality suspected of faecal contamination should follow a multi-tiered approach, initiated by identification of elevated FIB levels such as *E. coli*. Identification of *E. coli* at levels suggesting a potential health risk should lead to the second tier of testing which would include assays to track down the sources of faecal contamination. Levels of FIB suspected of having a dominant contribution from “naturalised” populations, could initiate a third tier of investigation including subtyping of FIB species to identify clonal naturalised populations. If a high level of “naturalised” E. coli were identified in a catchment or water body, we would suggest proceeding with caution as these identified “naturalised “FIB populations may still represent a health risk from those pathogens which are able to persist in the environment. Currently, there is also a lack of clarification around the types of “naturalised” FIB and whether “naturalised” FIB fall into two categories of faecally-derived “naturalised” FIB and those that diverged from faecal FIB thousands to millions of years previously. However, if the multi-tier testing evidence suggests a “naturalised” FIB population is the main contributor to the waterway of interest, then pathogen testing may be a necessary step to help clarify public health risk.

8a) Can all FIB grow in the environment? Not all strains of *E. coli* (or any other FIB) can persist or grow in the environment. Experiments have shown a significant die-off of FIB when discharged to water bodies, and subtyping studies have indicated that the remaining *E. coli*/enterococci are characterised by a few dominant strains. Survival and growth of those strains will be dependent on a multitude of interacting factors including the physical characteristics of the receiving water, the type of pollution source e.g. raw sewage versus

treated wastewater, the climate (especially sunlight), presence of microinvertebrates which can acts as both predators of microbes and as environmental host reservoirs.

8b) Can pathogens become naturalised? Viruses are not known to grow in the environment as they require a specific host organism, however they are known to persist in environmental reservoirs. One of the limitations ascribed to FIB in correlating with health risks, is that they do not persist as long as viruses and protozoa in aquatic environments.

Bacterial and protozoan pathogens have been recorded as persisting in the environment depending on physical and climatic conditions. Another important factor in their survival and dissemination in waterways is their apparent ability to evade digestion after ingestion by microinvertebrates such as amoeba (bacteria) and nematodes (bacteria and protozoa such as *Cryptosporidium*). Typically microinvertabrates are predators of microorganisms, however some, including pathogens, can evade the microinvertebrates immune system and not only persist but actually grow within the amoeba. This is exemplified by the Trojan horse aspect of amoeba and bacterial pathogens like *Legionella*, where the amoeba shelters the *Legionella* within and allows it to survive and proliferate, even through the routine chlorination of drinking water (King et al., 1988; Neogi et al., 2014).

8c) Are naturalised populations of bacteria in the sediment, sand or other matter not always suitable for source tracking? The bacteria that ESR target as PCR markers for FST are not based on the FIB: *E. coli* and enterococci. The ESR PCR markers, in general, target bacterial members of the order *Bacteroidales*, which are an anaerobic group of bacteria less likely to grow in the environment. The sterols and fluorescent whitening agents (FWA only identify human sources) used for faecal source tracking are particularly useful for this purpose as they have a long term signature in sediments and soil.

9) What does ID of "naturalised" *E. coli* mean for water managers? Refer to Section 6 of this document which discusses developments in identifying *E. coli* subytpes that may persist in the environment and in fact have genetically diverged from strictly enteric faecal habitats.
9b) Can they detect "naturalised" *E. coli* using routine methods? No, current methods do not differentiate between true enteric FIB and those strains that have become "naturalised" to the environment. ESR is currently investigating new genetic methods to identify and differentiate "naturalised" *E. coli* strains from faecally derived *E. coli*.

9c) Are they present in the NZ environment? ESR is currently investigating new genetic methods to identify and differentiate "naturalised" *E. coli* strains in the NZ environment to determine their prevalence and whether they are confounding water quality monitoring.

9d) What are the health risks? Pathogenicity of these strains has not been fully determined, however some of the "naturalised" *E. coli* strains, still retain genes that allow them to colonise animal and human hosts. However, whether they carry virulence factors which allow them to cause infection and disease in humans, is a secondary question to the fact that they are not indicating a recent faecal input to the environment. Therefore, these FIB strains do not potentially correlate with the pathogens associated with faeces -that is, they are not acting as reliable indicators of a public health risk.

9e) Would you always get naturalised *E. coli* and enterococci together in the environment? This is unknown and requires additional research, particularly in the NZ environmental context.

9f) How long can they live in the environment? Can they all grow? These are questions awaiting detailed research, which will be aided by the development of improved assays to identify these "naturalised" FIB strains. Research has suggested that these *E. coli* clades diverged from the true enteric *E. coli* a long time ago (thousands to millions of years). If true this indicates that these *E. coli* strains are a natural part of the microflora of soil etc. and persist for long periods in the environment. We hope to study these populations in the NZ environment and also monitor international research on this subject.

Answers to questions from Jonny Horrox, jh@wcrc.govt.nz

10) Is it possible to get a more tangible idea of the likely significance of naturalised, or adaptive *E. coli* in our waterways? 1% 20% 40 %?? Is it right to assume that naturalised and lingering *E. coli* is more prevalent when *E. coli* is at lower (dare I say 'background') levels ie < 270 /100 ml? At this stage we do not have a good understanding of the prevalence of naturalised *E. coli* in our environment including the waterways. Therefore, we cannot assume that lower levels of *E. coli* are "naturalised" strains and are therefore OK from a health risk perspective. That is a new area of research that we are beginning to address by trialling some PCRs developed overseas that the international researchers who designed them believe identify *E. coli* that could be "naturalised", because they are not commonly identified in the massive stock cultures of *E. coli* around the world (that have been largely isolated from clinical cases). This factor suggests that these "naturalised" *E. coli* are not highly virulent. Sequencing of their genomes has revealed that some have lost important genes required for host colonisation of the intestine and have gained other genes to allow better adaptation to environmental conditions. If these PCRs for "naturalised" *E. coli* work well and are not identified in our faecal DNA libraries of animals, birds and humans then we can start looking for "naturalised" *E. coli* in the environment.

I suspect that every location with chronic levels of *E. coli* from unknown sources will have different characteristics and need to be investigated individually as to whether they represent "naturalised" populations or unidentified faecal sources. Therefore. they would require a third tier (beyond, FIB and FST) of investigation to look at whether the *E. coli* represent a range of diverse subtypes as usually seen in fresh faecal inputs or are clonal and therefore likely to be "naturalised".

11) **What effect does temperature have on survival? Does cold water reduce the likelihood of naturalised contributions, or post gut survival?** The bad news is that research, in general, suggests that microbes actually survive better at lower environmental temperatures of <10°C. Optimal temperatures for replication, however, are in the range of 20-30°C but persistence is reduced at higher temperatures.

12) **Conducting FST on samples with low *E. coli* eg < 500 /100 ml, seems to be inconclusive. Could there be more guidance on when it is and isn't worthwhile using FST? Because its expensive and often yields inconclusive results. This will also have a bearing on differentiating naturalised from faecal source.** FST works well on samples with low *E. coli* if they are from a fresh, single source. For example we have had very definitive results from samples with 50 *E. coli*/100ml due to leaking sewer pipes. However, we generally recommend targeting higher levels of contamination as those are the ones of most concern. There is also an increased probability of definitive results with higher levels of contamination. Also when *E. coli* levels are low, it would be advisable to speak directly to a scientist to discuss which one of the FST tools are applicable to your particular location. For example, FWA are the least expensive tool, but from our experience are not suited to high dilution environments like rivers, however, they work well for urban stormwater drains for tracking leaking sewer pipes.

13) **If conditions are favourable for naturalised or long surviving *E. coli*, would this mean the same for pathogens, hence it remains a relevant indicator?**

This could be a wise conclusion at present, as we have no information to suggest that persistent/ "naturalised" *E. coli* **is or is not** correlated with pathogens, especially, obviously pathogenic *E. coli*, and viruses and the (oo)cysts of protozoa like *Cryptosporidium*, which are able to survive long term in the environment. Therefore, the *E. coli* is acting as a sentinel that there has been a past faecal event and pathogens could be present in the sediments, etc. We believe *E. coli* will continue to be a good, cheap, frontline tool for identifying problematic recreational areas which need further investigation.

14) **Green lipped mussels – *E. coli* analysis of tissue: could longer survival/naturalisation be significant in tissue/living mussels?** Based on what we have said above we believe the answer is likely to be a yes, but again what is the relevance to pathogens and health (as in question 13)?

GLOSSARY

Biofilm

Biofilms are composed of microbial communities made up of different species that are able to aggregate together and adhere to surfaces because they produce extracellular polymers which enhance their adherence properties. Biofilms form on living tissue and non-living surfaces (e.g. rocks).

Biomarkers

Biomarkers are a signal for a particular biological property, and are based on biological entities such as the PCR markers that target bacteria associated with faecal material.

Clade

A clade is a group of (micro)organisms derived from a single ancestor and therefore termed monophyletic, thus representing a single "branch" on the “tree of life”.

FIB

Faecal indicator bacteria (FIB) are used for water quality monitoring because they occur in high numbers in the individual faecal material of most animals and birds. FIB include; total coliforms (TC); faecal coliforms (FC), *Escherichia coli* (*E. coli*) and enterococci.

Gastrointestinal illness (GI)

Is an illness that occurs specifically in the gastrointestinal tract (stomach and upper and lower intestine of mammals). Common symptomology of GI are fever, diarrhoea, vomiting and stomach cramps, however not all of these need to be present to define GI.

Genome

The genome is the entire genetic material of an organism, which is encoded either by DNA or, in the case of some viruses by RNA

Naturalised

Naturalisation is any process by which a non-native (micro)organism spreads into a foreign environment and is able to reproduce and maintain its population within that novel environment.

Pathogen

A pathogen is any infectious agent such as a virus, bacterium, fungus, protozoan, prion or parasite that can cause disease in its host organism.

Opportunistic pathogen

This microorganism does not generally live and reproduce by causing disease in a host unless the opportunity arises where the immune defences of the host are compromised. These normally, non-pathogenic microorganisms can be found in either non-host environments (aquatic and soil) or associated with a host as normal microflora, eg on the skin or in the intestine.

Sensu stricto

Sensu stricto in relation to microbial taxonomy, groups those strains of a bacterial species that strictly adhere to all the biological definitions characterising that particular species. In the case of *E. coli* it is those strains that fall within the defined phylogroups of *E. coli* and do not include the novel “naturalised”clades of *E. coli*.

REFERENCES

Anderson GL, Caldwell KN, Beuchat LR, Williams PL. Interaction of a free-living soil nematode, Caenorhabditis elegans, with surrogates of foodborne pathogenic bacteria. J Food Prot 2003; 66: 1543-9.

Anderson KL, Whitlock JE, Harwood VJ. Persistence and Differential Survival of Fecal Indicator Bacteria in Subtropical Waters and Sediments. Appl. Environ. Microbiol. 2005; 71: 3041-3048.

Badgley BD, Nayak BS, Harwood VJ. The importance of sediment and submerged aquatic vegetation as potential habitats for persistent strains of enterococci in a subtropical watershed. Water Res 2010a; 44: 5857-66.

Badgley BD, Thomas FI, Harwood VJ. The effects of submerged aquatic vegetation on the persistence of environmental populations of Enterococcus spp. Environ Microbiol 2010c; 12: 1271-81.

Badgley BD, Thomas FI, Harwood VJ. Quantifying environmental reservoirs of fecal indicator bacteria associated with sediment and submerged aquatic vegetation. Environ Microbiol 2011; 13: 932-42.

Beckinghausen A, Martinez A, Blersch D, Haznedaroglu BZ. Association of nuisance filamentous algae Cladophora spp. with E. coli and Salmonella in public beach waters: impacts of UV protection on bacterial survival. Environ Sci Process Impacts 2014; 16: 1267-74.

Beversdorf LJ, Bornstein-Forst SM, McLellan SL. The potential for beach sand to serve as a reservoir for *Escherichia coli* and the physical influences on cell die-off. J Appl Microbiol 2007; 102: 1372-81.

Booth J, Brion GM. The utility of the AC/TC ratio for watershed management: a case study. Water Science and Technology 2004; 50: 199-203.

Brion GM. The AC/TC bacterial ratio: a tool for watershed quality management. Journal of Water and Environment Technology 2005; 3: 271- 277.

Brion GM, Mao HH. Use of total coliform test for watershed monitoring with respect to atypicals. Journal of Environmental Engineering 2000; 128: 175-181.

Brion GM, Neelakantan TR, Lingireddy S. A neural-network-based classification scheme for sorting sources and ages of fecal contamination in water. Water Res 2002; 36: 3765-74.

Buse HY, Ashbolt NJ. Differential growth of Legionella pneumophila strains within a range of amoebae at various temperatures associated with in-premise plumbing. Letters in Applied Microbiology 2011; 53: 217-24.

Byappanahalli M, Fowler M, Shively D, Whitman R. Ubiquity and persistence of *Escherichia coli* in a Midwestern coastal stream. Appl Environ Microbiol 2003a; 69: 4549-55.

Byappanahalli M, Fujioka R. Indigenous soil bacteria and low moisture may limit but allow faecal bacteria to multiply and become a minor population in tropical soils. Water Sci Technol 2004; 50: 27-32.

Byappanahalli MN, Nevers MB, Korajkic A, Staley ZR, Harwood VJ. Enterococci in the environment. Microbiol Mol Biol Rev 2012a; 76: 685-706.

Byappanahalli MN, Roll BM, Fujioka RS. Evidence for occurrence, persistence, and growth potential of Escherichia coli and enterococci in Hawaii's soil environments. Microbes Environ 2012b; 27: 164-70.

Byappanahalli MN, Shively DA, Nevers MB, Sadowsky MJ, Whitman RL. Growth and survival of Escherichia coli and enterococci populations in the macro-alga Cladophora (Chlorophyta). FEMS Microbiol Ecol 2003d; 46: 203-11.

Byappanahalli MN, Whitman RL, Shively DA, Sadowsky MJ, Ishii S. Population structure, persistence, and seasonality of autochthonous *Escherichia coli* in temperate, coastal forest soil from a Great Lakes watershed. Environ Microbiol 2006a; 8: 504-13.

Byappanahalli MN, Whitman RL, Shively DA, Ting WT, Tseng CC, Nevers MB. Seasonal persistence and population characteristics of *Escherichia coli* and enterococci in deep backshore sand of two freshwater beaches. J Water Health 2006c; 4: 313-20.

Byappanahalli MN, Yan T, Hamilton MJ, Ishii S, Fujioka RS, Whitman RL, et al. The population structure of Escherichia coli isolated from subtropical and temperate soils. Sci Total Environ 2012e; 417-418: 273-9.

Cizek AR, Characklis GW, Krometis LA, Hayes JA, Simmons OD, 3rd, Di Lonardo S, et al. Comparing the partitioning behavior of *Giardia* and *Cryptosporidium* with that of indicator organisms in stormwater runoff. Water Res 2008; 42: 4421-38.

Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the Escherichia coli phylogenetic group. Appl Environ Microbiol 2000; 66: 4555-8.

Clermont O, Christenson JK, Denamur E, Gordon DM. The Clermont Escherichia coli phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environmental Microbiology Reports 2013; 5: 58-65.
Clermont O, Gordon DM, Brisse S, Walk ST, Denamur E. Characterization of the cryptic Escherichia lineages: rapid identification and prevalence. Environmental Microbiology 2011; 13: 2468-2477.
Codony F, Perez LM, Adrados B, Agusti G, Fittipaldi M, Morato J. Amoeba-related health risk in drinking water systems: could monitoring of amoebae be a complementary approach to current quality control strategies? Future Microbiol 2012; 7: 25-31.
Davies-Colley RJ, Bell RG, Donnison AM. Sunlight Inactivation of Enterococci and Fecal Coliforms in Sewage Effluent Diluted in Seawater. Appl Environ Microbiol 1994; 60: 2049-2058.
Davies CM, Long JA, Donald M, Ashbolt NJ. Survival of fecal microorganisms in marine and freshwater sediments. Appl Environ Microbiol 1995; 61: 1888-96.
Deng D, Zhang L, Mustapha A, Xu D, Wuliji T, Farley M, et al. Differentiating enteric Escherichia coli from environmental bacteria through the putative glucosyltransferase gene (ycjM). Water Research 2014; 61: 224-231.
Desmarais TR, Solo-Gabriele HM, Palmer CJ. Influence of soil on fecal indicator organisms in a tidally influenced subtropical environment. Applied and Environmental Microbiology 2002; 68: 1165-72.
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; 485-486C: 666-680.
Di Cesare A, Pasquaroli S, Vignaroli C, Paroncini P, Luna GM, Manso E, et al. The marine environment as a reservoir of enterococci carrying resistance and virulence genes strongly associated with clinical strains. Environ Microbiol Rep 2014; 6: 184-90.
Dick LK, Stelzer EA, Bertke EE, Fong DL, Stoeckel DM. Relative decay of Bacteroidales microbial source tracking markers and cultivated Escherichia coli in freshwater microcosms. Applied and Environmental Microbiology 2010; 76: 3255-62.
Dodds WK, Gudder D. The ecology of *Cladophora*. Journal of Phycology 1992; 28: 415-427.
Farmer JJ, 3rd, Fanning GR, Davis BR, O'Hara CM, Riddle C, Hickman-Brenner FW, et al. Escherichia fergusonii and Enterobacter taylorae, two new species of Enterobacteriaceae isolated from clinical specimens. J Clin Microbiol 1985; 21: 77-81.
Ferguson CM, Coote BG, Ashbolt NJ, Stevenson IM. Relationships between indicators, pathogens and water quality in an estuarine system. Water Research 1996; 30: 2045.
Field KG, Samadpour M. Fecal source tracking, the indicator paradigm, and managing water quality. Water Res 2007; 41: 3517-38.
Fisher K, Phillips C. The ecology, epidemiology and virulence of Enterococcus. Microbiology 2009; 155: 1749-57.
Fujioka RS, Byappanahalli M. Microbial ecology controls the establishment of fecal bacteria in tropical soil environment. In: Matsuo T, Hanaki K, Takizawa S, Satoh H, editors. Advances in Water and Wastewater Treatment Technology: Molecular Technology, Nutrient Removal, Sludge Reduction and Environmental Health. Elsevier, Amsterdam, the Netherlands, 2001, pp. 273-83.
Geldreich EE. Sanitary significance of Faecal Coliforms in the environment. Federal Water Pollution Control administration, Publication WP-20-3. 1966.
Gilbert M, Monk C, Wang HL, Diplock K, Landry L. Screening policies for daycare attendees: lessons learned from an outbreak of E. coli O157:H7 in a daycare in Waterloo, Ontario. Canadian Journal of Public Health. Revue Canadienne de Sante Publique 2008; 99: 281-5.
Gilpin B, Sinton L, Devane M. Where's it coming from? Faecal source tracking - the current state of play. Water and wastes in NZ 2008: 48-52.
Gilpin BJ, Devane M, Robson B, Nourozi F, Scholes P, Lin S, et al. Sunlight inactivation of human polymerase chain reaction markers and cultured fecal indicators in river and saline waters. Water Environ Res 2013; 85: 743-50.
Grant SB, Litton-Mueller RM, Ahn JH. Measuring and modeling the flux of fecal bacteria across the sediment-water interface in a turbulent stream. Water Resources Research 2011; 47.
Greub G, Raoult D. Microorganisms resistant to free-living amoebae. Clin Microbiol Rev 2004; 17: 413-33.
Hagedorn C, Blanch AR, Harwood VJ. Microbial source tracking: methods, applications, and case studies. New York: Springer, 2011.
Haller L, Poté J, Loizeau J-L, Wildi W. Distribution and survival of faecal indicator bacteria in the sediments of the Bay of Vidy, Lake Geneva, Switzerland. Ecological Indicators 2009; 9: 540-547.
Hartz A, Cuvelier M, Nowosielski K, Bonilla TD, Green M, Esiobu N, et al. Survival potential of Escherichia coli and Enterococci in subtropical beach sand: implications for water quality managers. J Environ Qual 2008; 37: 898-905.

Harwood VJ, Delahoya NC, Ulrich RM, Kramer MF, Whitlock JE, Garey JR, et al. Molecular confirmation of *Enterococcus faecalis* and *E. faecium* from clinical, faecal and environmental sources. Lett Appl Microbiol 2004; 38: 476-82.
Harwood VJ, Levine AD, Scott TM, Chivukula V, Lukasik J, Farrah SR, et al. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Appl Environ Microbiol 2005; 71: 3163-3170.
Harwood VJ, Staley C, Badgley BD, Borges K, Korajkic A. Microbial source tracking markers for detection of fecal contamination in environmental waters: relationships between pathogens and human health outcomes. Fems Microbiology Reviews 2014; 38: 1-40.
Heuvel AV, McDermott C, Pillsbury R, Sandrin T, Kinzelman J, Ferguson J, et al. The green alga, Cladophora, promotes Escherichia coli growth and contamination of recreational waters in Lake Michigan. Journal of Environmental Quality 2010; 39: 333-344.
Huamanchay O, Genzlinger L, Iglesias M, Ortega YR. Ingestion of Cryptosporidium oocysts by Caenorhabditis elegans. J Parasitol 2004; 90: 1176-8.
Ingle DJ, Clermont O, Skurnik D, Denamur E, Walk ST, Gordon DM. Biofilm formation by and thermal niche and virulence characteristics of Escherichia spp. Appl Environ Microbiol 2011; 77: 2695-700.
Ishii S, Ksoll WB, Hicks RE, Sadowsky MJ. Presence and Growth of Naturalized Escherichia coli in Temperate Soils from Lake Superior Watersheds. Appl. Environ. Microbiol. 2006a; 72: 612-621.
Ishii S, Yan T, Shively DA, Byappanahalli MN, Whitman RL, Sadowsky MJ. Cladophora (Chlorophyta) spp. harbor human bacterial pathogens in nearshore water of Lake Michigan. Appl Environ Microbiol 2006d; 72: 4545-53.
Jimenez L, Muniz I, Toranzos GA, Hazen TC. Survival and activity of Salmonella typhimurium and Escherichia coli in tropical freshwater. The Journal of Applied Bacteriology 1989; 67: 61-9.
Kapoor V, Smith C, Santo Domingo JW, Lu T, Wendell D. Correlative assessment of fecal indicators using human mitochondrial DNA as a direct marker. Environ Sci Technol 2013; 47: 10485-93.
King CH, Shotts EB, Jr., Wooley RE, Porter KG. Survival of coliforms and bacterial pathogens within protozoa during chlorination. Appl Environ Microbiol 1988; 54: 3023-33.
Kinzelman J, McLellan SL, Daniels AD, Cashin S, Singh A, Gradus S, et al. Non-point source pollution: determination of replication versus persistence of Escherichia coli in surface water and sediments with correlation of levels to readily measurable environmental parameters. J Water Health 2004; 2: 103-14.
Korajkic A, McMinn BR, Shanks OC, Sivaganesan M, Fout GS, Ashbolt NJ. Biotic interactions and sunlight affect persistence of fecal indicator bacteria and microbial source tracking genetic markers in the upper Mississippi river. Appl Environ Microbiol 2014; 80: 3952-61.
Ksoll WB, Ishii S, Sadowsky MJ, Hicks RE. Presence and sources of fecal coliform bacteria in epilithic periphyton communities of Lake Superior. Appl Environ Microbiol 2007; 73: 3771-8.
Lee CM, Lin TY, Lin CC, Kohbodi GA, Bhatt A, Lee R, et al. Persistence of fecal indicator bacteria in Santa Monica Bay beach sediments. Water Res 2006; 40: 2593-602.
Leeming R, Nichols PD, Ashbolt NJ. Distinguishing sources of faecal pollution in Australian Inland and coastal water using sterol biomarkers and microbial faecal indicators. Water services association of Australia, 1998, pp. 1-45.
Leewis M-CCE. Impact of aquatic macrophytes on Escherichia coli concentrations at recreational inland beaches. 1439816. Northern Michigan University, Ann Arbor, 2006, pp. 107-107 p.
LeJeune JT, Besser TE, Hancock DD. Cattle water troughs as reservoirs of *Escherichia coli* O157. Appl Environ Microbiol 2001; 67: 3053-7.
Litton RM, Ahn JH, Sercu B, Holden PA, Sedlak DL, Grant SB. Evaluation of chemical, molecular, and traditional markers of fecal contamination in an effluent dominated urban stream. Environ Sci Technol 2010; 44: 7369-75.
Luna GM, Vignaroli C, Rinaldi C, Pusceddu A, Nicoletti L, Gabellini M, et al. Extraintestinal Escherichia coli carrying virulence genes in coastal marine sediments. Appl Environ Microbiol 2010; 76: 5659-68.
Luo C, Walk ST, Gordon DM, Feldgarden M, Tiedje JM, Konstantinidis KT. Genome sequencing of environmental Escherichia coli expands understanding of the ecology and speciation of the model bacterial species. Proc Natl Acad Sci U S A 2011; 108: 7200-5.
Macek M, Carlos G, Memije P, Ramı´rez P. Ciliate-*Vibrio cholerae* interactions within a microbial loop: an experimental study. Aquat Microb Ecol 1997; 13: 257-266.
Marks JC, Power ME. Nutrient induced changes in the species composition of epiphytes on Cladophora glomerata Kütz. (Chlorophyta). Hydrobiologia 2001; 450: 187-196.
McBride G, Ross T, Dufour A. Comparative risk analysis. In: Dufour A, Bartram J, Bos R, Gannon V, editors. Animal waste, water quality and human health. Published on behalf of WHO by IWA Publishing, 2012.

McCalley DV, Cooke M, Nickless G. Effect of sewage treatment on faecal sterols. Water Research 1981; 15: 1019-1025.
McLellan SL. Genetic Diversity of Escherichia coli Isolated from Urban Rivers and Beach Water. Appl. Environ. Microbiol. 2004; 70: 4658-4665.
McLellan SL, Daniels AD, Salmore AK. Clonal populations of thermotolerant Enterobacteriaceae in recreational water and their potential interference with fecal Escherichia coli counts. Appl Environ Microbiol 2001; 67: 4934-8.
Ministry for the Environment. Microbiological Water Quality Guidelines for Marine and Freshwater Recreational Areas. Ministry for the Environment (MfE) and Ministry of Health (MoH), Wellington, New Zealand, 2003, pp. 155.
Moriarty EM, Weaver L, Sinton LW, Gilpin B. Survival of *Escherichia coli*, enterococci and *Campylobacter jejuni* in Canada goose faeces on pasture. Zoonoses Public Health 2012; 59: 490-7.
Moss SH, Smith KC. Membrane damage can be a significant factor in the inactivation of Escherichia coli by near-ultraviolet radiation. Photochemistry and photobiology 1981; 33: 203.
Muller T, Ulrich A, Ott EM, Muller M. Identification of plant-associated enterococci. J Appl Microbiol 2001; 91: 268-78.
Mulugeta S, Hindman R, Olszewski AM, Hoover K, Greene K, Lieberman M, et al. Contamination level and location of recreational freshwater influence the ability to predict *Escherichia coli* concentration by qPCR targeting *Bacteroides*. J Environ Manage 2012; 103: 95-101.
Neogi SB, Yamasaki S, Alam M, Lara RJ. The role of wetland microinvertebrates in spreading human diseases. Wetlands Ecology and Management 2014; 22: 469-491.
Nieman J, Brion GM. Novel bacterial ratio for predicting faecal age. Water Sci Technol 2003; 47: 45-9.
Nshimyimana JP, Ekklesia E, Shanahan P, Chua LH, Thompson JR. Distribution and abundance of human-specific *Bacteroides* and relation to traditional indicators in an urban tropical catchment. Journal of Applied Microbiology 2014; 116: 1369-83.
Oaks JL, Besser TE, Walk ST, Gordon DM, Beckmen KB, Burek KA, et al. Escherichia albertii in wild and domestic birds. Emerg Infect Dis 2010; 16: 638-46.
Obiri-Danso K, Jones K. Intertidal sediments as reservoirs for hippurate negative campylobacters, salmonellae and faecal indicators in three EU recognised bathing waters in North West England. Water Research 2000; 34: 519-527.
Oh S, Buddenborg S, Yoder-Himes DR, Tiedje JM, Konstantinidis KT. Genomic diversity of Escherichia isolates from diverse habitats. PLoS One 2012; 7: e47005.
Okabe S, Shimazu Y. Persistence of host-specific Bacteroides-Prevotella 16S rRNA genetic markers in environmental waters: effects of temperature and salinity. Appl Microbiol Biotechnol 2007; 76: 935-44.
Olapade OA, Depas MM, Jensen ET, McLellan SL. Microbial communities and fecal indicator bacteria associated with Cladophora mats on beach sites along Lake Michigan shores. Appl Environ Microbiol. 2006; 72: 1932-8.
Oliver DM, Clegg CD, Heathwaite AL, Haygarth PM. Preferential Attachment of Escherichia coli to Different Particle Size Fractions of an Agricultural Grassland Soil. Water, air, and soil pollution 2007; 185: 369-375.
Ott EM, Muller T, Muller M, Franz CM, Ulrich A, Gabel M, et al. Population dynamics and antagonistic potential of enterococci colonizing the phyllosphere of grasses. J Appl Microbiol 2001; 91: 54-66.
Pachepsky YA, Blaustein RA, Whelan G, Shelton DR. Comparing temperature effects on *Escherichia coli*, *Salmonella*, and *Enterococcus* survival in surface waters. Lett Appl Microbiol 2014; 59: 278-83.
Payment P, Franco E. *Clostridium perfringens* and somatic coliphages as indicators of the efficiency of drinking water treatment for viruses and protozoan cysts. Appl Environ Microbiol 1993; 59: 2418-24.
Perchec-Merien A-M, Lewis GD. Naturalized Escherichia coli from New Zealand wetland and stream environments. FEMS microbiology, ecology 2013; 83: 494-503.
Pettibone GW, Irvine KN, Monahan KM. Impact of a ship passage on bacteria levels and suspended sediment characteristics in the Buffalo River, New York. Water Research 1996; 30: 2517-2521.
Piorkowski GS, Gregory SP, Rob CJ, Lisbeth Truelstrup H, Greg SB. Characterizing spatial structure of sediment E. coli populations to inform sampling design. Environmental monitoring and assessment 2014; 186: 277-291.
Pote J, Haller L, Kottelat R, Sastre V, Arpagaus P, Wildi W. Persistence and growth of faecal culturable bacterial indicators in water column and sediments of Vidy Bay, Lake Geneva, Switzerland. Journal of Environmental Sciences 2009; 21: 62-69.
Rozen Y, Belkin S. Survival of enteric bacteria in seawater. FEMS Microbiol Rev 2001; 25: 513-29.
Sabino R, Rodrigues R, Costa I, Carneiro C, Cunha M, Duarte A, et al. Routine screening of harmful microorganisms in beach sands: implications to public health. Sci Total Environ 2014; 472: 1062-9.

Samadpour M, Stewart J, Steingart K, Addy C, Louderback J, McGinn M, et al. Laboratory investigation of an *E. coli* O157:H7 outbreak associated with swimming in Battle Ground Lake, Vancouver, Washington. J Environ Health 2002; 64: 16-20, 26, 25.
Santo Domingo JW, Bambic DG, Edge TA, Wuertz S. Quo vadis source tracking? Towards a strategic framework for environmental monitoring of fecal pollution. Water Res 2007; 41: 3539-52.
Savichtcheva O, Okabe S. Alternative indicators of fecal pollution: relations with pathogens and conventional indicators, current methodologies for direct pathogen monitoring and future application perspectives. Water Research 2006; 40: 2463-76.
Savichtcheva O, Okayama N, Okabe S. Relationships between Bacteroides 16S rRNA genetic markers and presence of bacterial enteric pathogens and conventional fecal indicators. Water Res 2007; 41: 3615-28.
Sinclair RG, Rose JB, Hashsham SA, Gerba CP, Haas CN. Criteria for selection of surrogates used to study the fate and control of pathogens in the environment. Appl Environ Microbiol 2012; 78: 1969-77.
Sinton L, Hall C, Braithwaite R. Sunlight inactivation of Campylobacter jejuni and Salmonella enterica, compared with Escherichia coli, in seawater and river water. J Water Health 2007a; 5: 357-65.
Sinton LW. Biotic and abiotic effects. In: Belkin S, Colwell RR, editors. Oceans and Health: Pathogens in the Marine Environment. Springer Science + Business Media, New York, 2005, pp. 69-92.
Sinton LW, Braithwaite RR, Hall CH, Mackenzie ML. Survival of indicator and pathogenic bacteria in bovine feces on pasture. Appl Environ Microbiol 2007b; 73: 7917-7925.
Sinton LW, Finlay RK, Lynch PA. Sunlight inactivation of fecal bacteriophages and bacteria in sewage-polluted seawater. Appl Environ Microbiol 1999; 65: 3605-13.
Sinton LW, Hall CH, Lynch PA, Davies-Colley RJ. Sunlight inactivation of fecal indicator bacteria and bacteriophages from waste stabilization pond effluent in fresh and saline waters. Appl Environ Microbiol 2002; 68: 1122-31.
Sobsey MD. Inactivation of Health-Related Microorganisms in Water by Disinfection Processes. Water Science & Technology 1989; 21: 179-195.
Soller JA, Schoen ME, Bartrand T, Ravenscroft JE, Ashbolt NJ. Estimated human health risks from exposure to recreational waters impacted by human and non-human sources of faecal contamination. Water Research 2010; 44: 4674-4691.
Solo-Gabriele HM, Wolfert MA, Desmarais TR, Palmer CJ. Sources of *Escherichia coli* in a Coastal Subtropical Environment. Appl. Environ. Microbiol. 2000; 66: 230-237.
Staley C, Dunny GM, Sadowsky MJ. Environmental and animal-associated enterococci. Adv Appl Microbiol 2014; 87: 147-86.
Standridge J. E. coli as a public health indicator of drinking water quality. Journal: American Water Works Association 2008; 100: 65-75.
Strachan NJ, MacRae M, Thomson A, Rotariu O, Ogden ID, Forbes KJ. Source attribution, prevalence and enumeration of Campylobacter spp. from retail liver. International Journal of Food Microbiology 2012; 153: 234-6.
Sun S, Kjelleberg S, McDougald D. Relative Contributions of Vibrio Polysaccharide and Quorum Sensing to the Resistance of Vibrio cholerae to Predation by Heterotrophic Protists. PLoS ONE 2013; 8: e56338.
Thomas MC. The temporal distribution of planktonic protists in southwestern Alberta and their role in the persistence of the human pathogen Campylobacter jejuni. 1569511. University of Lethbridge (Canada), Ann Arbor, 2013, pp. 157.
USEPA. Environmental Indicators of Water Quality in the United States. United States Environmental Protection Agency (USEPA), Washington, DC, 1996.
USEPA. Recreational Water Quality Criteria U.S. Environmental Protection Agency, Office of Water 820-F-12-058, Washington, DC, 2012.
Vignaroli C, Di Sante L, Magi G, Luna GM, Di Cesare A, Pasquaroli S, et al. Adhesion of marine cryptic Escherichia isolates to human intestinal epithelial cells. ISME J 2014.
Vignaroli C, Luna GM, Pasquaroli S, Di Cesare A, Petruzzella R, Paroncini P, et al. Epidemic Escherichia coli ST131 and Enterococcus faecium ST17 in coastal marine sediments from an Italian beach. Environ Sci Technol 2013; 47: 13772-80.
Vignaroli C, Luna GM, Rinaldi C, Di Cesare A, Danovaro R, Biavasco F. New sequence types and multidrug resistance among pathogenic Escherichia coli isolates from coastal marine sediments. Appl Environ Microbiol 2012; 78: 3916-22.
Wade TJ, Calderon RL, Brenner KP, Sams E, Beach M, Haugland R, et al. High sensitivity of children to swimming-associated gastrointestinal illness: results using a rapid assay of recreational water quality. Epidemiology 2008; 19: 375-83.

Wade TJ, Calderon RL, Sams E, Beach M, Brenner KP, Williams AH, et al. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environ Health Perspect 2006; 114: 24-8.
Wade TJ, Pai N, Eisenberg JN, Colford JM, Jr. Do U.S. Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis. Environmental Health Perspectives 2003; 111: 1102-9.
Wade TJ, Sams E, Brenner KP, Haugland R, Chern E, Beach M, et al. Rapidly measured indicators of recreational water quality and swimming-associated illness at marine beaches: a prospective cohort study. Environ Health 2010; 9: 66.
Walk ST, Alm EW, Gordon DM, Ram JL, Toranzos GA, Tiedje JM, et al. Cryptic lineages of the genus *Escherichia*. Appl Environ Microbiol 2009; 75: 6534-44.
Wanjugi P, Harwood VJ. The influence of predation and competition on the survival of commensal and pathogenic fecal bacteria in aquatic habitats. Environ Microbiol 2013; 15: 517-26.
Weigand MR, Ashbolt NJ, Konstantinidis KT, Santo Domingo JW. Genome sequencing reveals the environmental origin of enterococci and potential biomarkers for water quality monitoring. Environ Sci Technol 2014.
Whitman R, Byappanahalli M, Spoljaric AM, Przybyla-Kelly K, Shively DA, Nevers MB. Evidence for free-living Bacteroides in Cladophora aong the shores of the Great Lakes. Aquatic Microbial Ecology 2014a; 72: 117-126.
Whitman RL, Byers SE, Shively DA, Ferguson DM, Byappanahalli M. Occurrence and growth characteristics of Escherichia coli and enterococci within the accumulated fluid of the northern pitcher plant (Sarracenia purpurea L.). Can J Microbiol 2005; 51: 1027-37.
Whitman RL, Harwood VJ, Edge TA, Nevers MB, Byappanahalli M, Vijayavel K, et al. Microbes in beach sands: Integrating environment, ecology and public health. Reviews in Environmental Science and Biotechnology 2014b; 13: 329-368.
Whitman RL, Nevers MB. Foreshore sand as a source of *Escherichia coli* in nearshore water of a Lake Michigan beach. Appl Environ Microbiol 2003; 69: 5555-62.
Whitman RL, Nevers MB, Byappanahalli MN. Examination of the watershed-wide distribution of *Escherichia coli* along Southern Lake Michigan: an integrated approach. Applied and Environmental Microbiology 2006; 72: 7301-10.
Whitman RL, Shively DA, Pawlik H, Nevers MB, Byappanahalli MN. Occurrence of Escherichia coli and enterococci in Cladophora (Chlorophyta) in nearshore water and beach sand of Lake Michigan. Appl Environ Microbiol 2003; 69: 4714-9.
Wu J, Long SC, Das D, Dorner SM. Are microbial indicators and pathogens correlated? A statistical analysis of 40 years of research. J Water Health 2011; 9: 265-78.
Yakirevich A, Pachepsky YA, Guber AK, Gish TJ, Shelton DR, Cho KH. Modeling transport of Escherichia coli in a creek during and after artificial high-flow events: three-year study and analysis. Water Res 2013; 47: 2676-88.
Zulkifly S, Hanshew A, Young EB, Lee P, Graham ME, Graham ME, et al. The epiphytic microbiota of the globally widespread macroalga Cladophora glomerata (Chlorophyta, Cladophorales). Am J Bot 2012; 99: 1541-52.

INSTITUTE OF ENVIRONMENTAL SCIENCE AND RESEARCH LIMITED

Kenepuru Science Centre
34 Kenepuru Drive, Kenepuru, Porirua 5022
PO Box 50348, Porirua 5240
New Zealand
T: +64 4 914 0700 **F:** +64 4 914 0770

Mt Albert Science Centre
120 Mt Albert Road, Sandringham, Auckland 1025
Private Bag 92021, Auckland 1142
New Zealand
T: +64 9 815 3670 **F:** +64 9 849 6046

NCBID – Wallaceville
66 Ward Street, Wallaceville, Upper Hutt 5018
PO Box 40158, Upper Hutt 5140
New Zealand
T: +64 4 529 0600 **F:** +64 4 529 0601

Christchurch Science Centre
27 Creyke Road, Ilam, Christchurch 8041
PO Box 29181, Christchurch 8540
New Zealand
T: +64 3 351 6019 **F:** +64 3 351 0010

www.esr.cri.nz