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The role of estuarine sediments as a reservoir for pathogenic microorganisms

Tracy Louise Perkins

September 2015



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A thesis submitted to Bangor University in candidature
for the degree

Philosophiae Doctor

School of Biological Sciences

Bangor University, Bangor, Gwynedd, LL57 2UW

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Summary

Estuarine environments are biologically productive ecosystems that are both economically and socially important. Consequently, a decline in the microbiological water quality can pose a risk to human health and have severe socioeconomic consequences, especially for areas that rely on tourism and shellfisheries for income. The enumeration of faecal indicator bacteria (FIB) in water samples has been the paradigm for estimating water quality in coastal zones, but there is an emerging view that sediments are a poorly studied and yet a significant reservoir of FIB. The aims of this thesis were: (I) to investigate the role of sediments as a reservoir for FIB and other potentially pathogenic bacteria; (II) to examine the spatial ecology of FIB in relation to sediment composition (grain size and organic matter content); (III) to investigate the influence of point and diffuse pollution sources on the abundance of bacteria in marine and estuarine sediments; (IV) to investigate the abundance of human pathogenic bacteria in the Conwy estuary, North Wales, UK, and (V) to investigate the influence of suspended particulate matter (SPM) on the survival of FIB in both fresh and brackish water. Culturable *E. coli*, total coliforms, enterococci (FIB), *Salmonella*, *Campylobacter*, *Vibrio* spp. and heterotrophic bacteria were enumerated in sediments and water from the Conwy estuary that is subject to various point and diffuse sources of pollution. FIB counts were three orders of magnitude greater in sediments compared with the overlying water column, demonstrating that estuarine sediments are a significant reservoir for FIB and other potential pathogens. In addition, sediment grain size analysis and organic matter content determinations revealed that finer sediments such as clay, silt and very fine sand contained significantly higher concentrations of all bacterial groups enumerated. The enumeration of FIB in marine sediments surrounding an offshore sewage outfall pipe revealed that spatial variations in FIB abundance reflected the course of the sewage effluent plume as predicted by a hydrodynamic model, demonstrating the impact of point sources of microbial pollution on the underlying sediments. To address the actual pathogen content of sediments in the Conwy estuary (rather than only indicator bacteria), PCR and qPCR were utilized to detect and quantify known pathogen virulence genes, revealing that estuarine sediments are a reservoir for pathogenic bacteria. Furthermore, qPCR suggested greater concentrations of FIB compared with culture counts from the same sample, indicating the possible presence of viable but non-culturable (VBNC) bacteria. Consequently, sediment-associated bacteria pose a risk to human health if they are resuspended into the water column under certain hydrodynamic processes, as tide-dominated estuaries usually contain large areas of fine sand that are easily mobilized. To investigate the influence of SPM concentration on FIB survival, fresh and brackish water containing low (~16 mg/l), high (~160 mg/l) and extreme (~1650 mg/l) SPM concentrations were inoculated with crude sewage and sheep faeces. FIB were enumerated every 24 hrs for 5 days, revealing that SPM concentrations influence FIB survival in brackish water but had minimal influence in freshwater over time. In general, FIB concentrations increased with a decrease in SPM concentration. These data add to a limited body of evidence on the role of sediments as a reservoir for pathogenic bacteria, with implications for routine monitoring protocols that assess the microbial pollution of environmental waters. In addition, these data suggest that catchment-based risk assessments of microbial pollution in aquatic systems should consider the source of FIB, the hydrodynamics of the environment, and the subsequent influence of SPM concentrations, all of which determine the survival of FIB in aquatic environments.

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Dedication

This PhD is dedicated to Barry, Sandra and Steven Collins

Who always believed in me and supported me for the duration of this PhD

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I would also like to thank my friends for being understanding and putting up with me particularly Hazel, Katie and Carys.

Last but definitely not least, I would like to thank my husband to be Simon for supporting me, never complaining and cooking my dinners, I couldn't have done it without you.

Abbreviations

bp	base pairs
cDNA	Complimentary Deoxyribonucleic Acid
CFU	Colony Forming Units
CSO	Combined Sewer Overflow
CTAB	Cetyltrimethyl Ammonium Bromide
ddH₂O	Double distilled water
DNA	Deoxyribonucleic Acid
DOM	Dissolved Organic Matter
EC	Electrical Conductivity
EC	Electronic Commerce
EPS	Extracellular Polymeric Substances
ESF	European Social Fund
EU	European Union
FAO	Food and Agricultural Organization
FB	Faecal Bacteria
FC	Faecal Coliform
FIB	Faecal Indicator Bacteria
GESAMP	Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection
GI	Gastrointestinal
HAV	Hepatitis A Virus
HEV	Hepatitis E Virus
KESS	Knowledge Economy and Skills Scholarship
LOI	Light On Ignition
PAIs	Pathogenicity-Associated Islands
PCR	Polymerase Chain Reaction

p.e.	Population Equivalent
PIB	Pathogen Indicator Bacteria
PSU	Practical Salinity unit
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNA-seq	Ribonucleic Acid-sequencing
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SEM	Standard Error Margin
SPM	Suspended Particulate Matter
spp.	Species
SPSS	Statistical Package for the Social Sciences
T0	Time at point “0”
TOC	Total Organic Carbon
UK	United Kingdom
USA	United States of America
UV	Ultra Violet
VBNC	Viable But Non-Culturable
WHO	World Health organisation
w/v	Weight to Volume ratio
WWTP	Waste Water Treatment Plant

CHAPTER 1

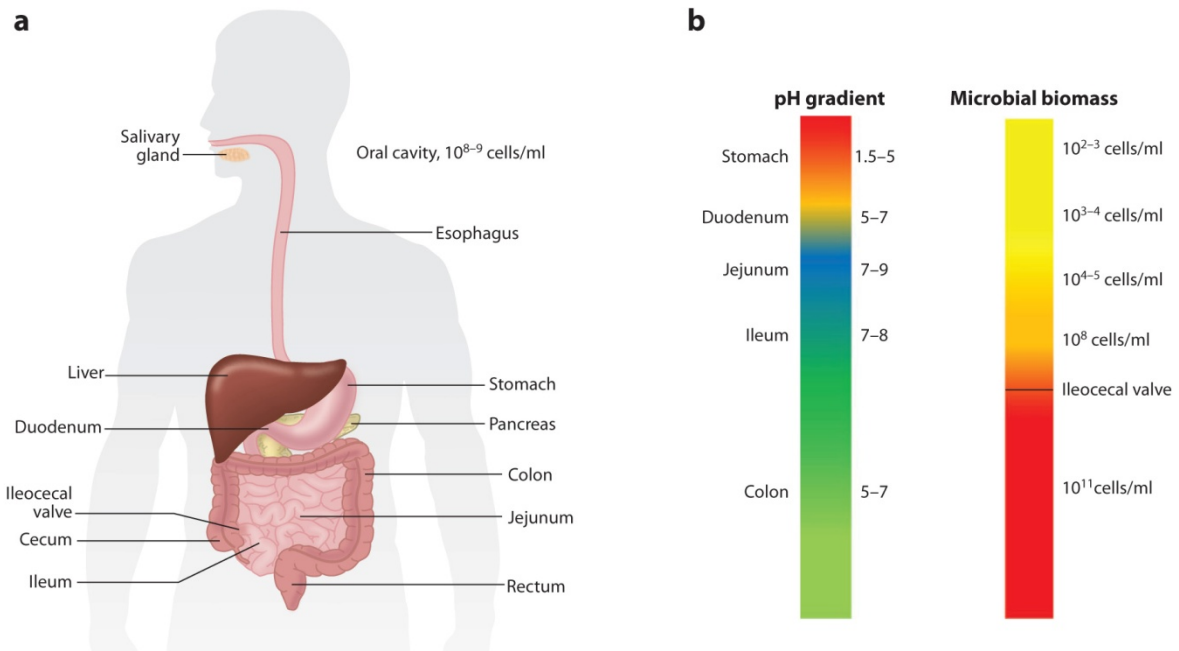
Introduction

1.1 The nature of the problem

The gut of warm blooded animals is a unique environment that provides a nutrient rich habitat for millions of microbes (Ley *et al.*, 2006). The human colon contains extremely high densities of microbes, where quantities can reach 10^{11} cells/ml (the human colon has a volume of 0.5 l) (Fig. 1.1) (Whitman *et al.*, 1998). The family Enterobacteriaceae, of the phylum Proteobacteria, consists of many coliform and faecal coliform bacteria. *Escherichia coli* is included in the faecal coliform group and although a common commensal member of the gut microbiome of humans and animals, some strains have the potential to be pathogenic (Adlerberth & Wold, 2009). *E. coli* types are described as either commensal, extraintestinal or diarrheagenic, as various strains have the ability to cause intestinal and extraintestinal disease. (Kaper, 2005).

E. coli O157:H7 has been identified as a major public health concern, as infection can cause haemorrhagic colitis which can lead to serious and life threatening complications such as haemolytic uraemic syndrome (Ferens & Hovde, 2011). Cattle have been identified as an important reservoir of O157:H7 (Ferens & Hovde 2011) and infection is often associated with the consumption of contaminated meat (Dean-Nystrom *et al.*, 1998). *E. coli* is an extremely diverse species, which in addition to the gut, has been isolated from both terrestrial and aquatic habitats such as sediment (Perkins *et al.*, 2014), soil (Byappanahalli *et al.*, 2006) and water (Barcina, 1995).

In addition to members of the Enterobacteriaceae, intestinal enterococci (members of the phylum Firmicutes) are also found in the gastrointestinal (GI) tract of humans and other animals and can be found at levels between 10^6 - 10^8 CFU/g of faeces (Adlerberth & Wold, 2009). They are widely distributed among the animal kingdom and are part of the gut microbiome of mammals, birds, reptiles and insects (Yost *et al.*, 2011) They are Gram-positive Firmicutes that are able to grow at a wide range of temperatures ranging between 10°C to 45°C (Domig *et al.*, 2003). Enterococci are associated with millions of infections in animals and humans every year (Byappanahalli *et al.*, 2012). *E. faecium* and *E. faecalis* are the two most widely reported pathogens that have the ability to cause a variety of infections, which include urinary tract infection, wound infections and endocarditis (inflammation around the heart) (Domig *et al.*, 2003). *Enterococcus* spp. have been isolated from many different environments including freshwater (Thevenon *et al.*, 2012), soil and vegetables (Abriouel *et al.*, 2008), seawater and beach sand (Goodwin *et al.*, 2012).



C Bacterial population present

Oral cavity:

Gemella (e.g., *G. haemolysans*), *Granulicatella*, *Streptococcus* (e.g., *S. mitis*), *Veillonella*, *Prevotella*, *Porphyromonas*, *Rothia*, *Neisseria*, *Fusobacterium*, *Lactobacillus*

Allochthonous microbes are generally outnumbered by autochthonous microbes.

Stomach:

Helicobacter pylori

Allochthonous: *Gemella* (e.g., *G. haemolysans*), *Granulicatella*, *Streptococcus* (e.g., *S. mitis*), *Veillonella*, *Prevotella*, *Porphyromonas*, *Rothia*, *Neisseria*, *Fusobacterium*, *Lactobacillus*

Small intestine:

Escherichia coli, *Klebsiella*, *Enterococcus*, *Bacteroides*, *Ruminococcus*, *Dorea*, *Clostridium*, *Coprococcus*, *Weissella*, *Lactobacillus* (some species)

Allochthonous: *Granulicatella*, *Streptococcus* (e.g., *S. mitis*), *Veillonella*, *Lactobacillus*

Large intestine:

Five major phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia*, and *Proteobacteria*. Hundreds of species.

Allochthonous microbes are generally outnumbered by autochthonous microbes.

Fig. 1.1 The human GI tract and its microbial community. (a) The main sections of the human gastrointestinal tract. (b) pH gradient from the stomach to distal gut (left bar) and microbial biomass (right bar). (c) Each box details the dominant microbes in each habitat (allochthonous or autochthonous). Image from (Walter & Ley, 2011).

Coliforms have long been used as indicator bacteria to monitor faecal pollution of environmental waters. However, they are a large and complex group of bacteria which includes many bacteria that may not have originated from faeces (Carrero-colon *et al.*, 2011). More recently, coliforms have been replaced by alternate indicators such as enterococci and *E. coli*, which are considered to be a more precise indicator of pollution (WHO, 2006). Pathogens contained in faecal matter typically occur at low levels in the environment, and therefore, their detection can be time-consuming and costly (Meays *et al.*, 2004). Subsequently, it is much more practical to enumerate non-pathogenic Faecal Indicator Bacteria (FIB), which are more abundant, as a proxy for the potential risk of pathogenic bacteria (Meays *et al.*, 2004). However, as technology evolves and detection is becoming increasingly more efficient and accurate, the detection of other faecal bacterial species and their associated pathogens is possible.

The genus *Salmonella*, like *E. coli*, belongs to the family Enterobacteriaceae. They are commensal members of the gut microbiota of many animals including birds, mammals, reptiles, livestock and insects (Diez-Gonzalez, 2011). More than 2,500 serovars of *Salmonella* have been identified, but it is the subspecies of *S. enterica* that has been predominantly linked with disease in humans and animals (Timme *et al.*, 2013). *S. enterica* is considered to be one of the main causes of foodborne infections worldwide (Timme *et al.*, 2013). The pathogenic serotypes within this group can cause multiple clinical conditions such as enteritis (inflammation of the small intestine causing swelling fever and diarrhoea) septicaemia and abortion in humans and other animals (Uzzau *et al.*, 2000). Information collected from population studies and national surveillance systems revealed that from 1996 to 2000 *Salmonella* were the leading cause of death related to the consumption of contaminated foods in England and Wales (Adak *et al.*, 2005). In addition, a study by Scallan *et al.*, (2011) estimated that each year in the USA Salmonellosis is the leading cause of hospitalization and death from a foodborne illness. *Salmonella* have also been isolated from various different environments including beach sand (Bolton *et al.*, 1999), estuarine water and sediments (Bolton *et al.*, 1999).

Campylobacter are Gram-negative, microaerophilic bacteria. When compared to other enteric bacteria they have a rather restricted temperature range for growth, which is between ca. 30 to 46°C (Humphrey *et al.*, 2007). Similar to *Salmonella*, *Campylobacter* spp. are able to colonise the GI tract of different animals including livestock, birds, cats and dogs (Blaser, 1997). *C. lari* and *C. coli* are recognised as important zoonotic pathogens, but *C. jejuni* is one of the most familiar, with most infections thought to be caused by food poisoning (Blaser, 1997). In addition, *Campylobacter* infections in cattle and sheep can have reproductive implications (Thompson *et al.*, 1988). In developed and industrialized countries, pathogenic strains of

Campylobacter are thought to be one of the main causes of enteric illnesses in humans (Levin, 2007). Numerous studies have isolated *Campylobacter* spp. from secondary environments such as rivers (Bolton *et al.*, 1987) and coastal environments (Alonso & Alonso, 1993). Furthermore, *Salmonella* and *Campylobacter* have been isolated from shellfish, harvested for human consumption posing a risk to human health (Bakr *et al.*, 2011; Wilson & Moore, 1996). Despite this, the monitoring of *Salmonella* and *Campylobacter* presence in bathing waters is not a requirement of EU legislation. *E. coli* has been defined in the past as a good indicator of faecal pollution, but its use for all ecosystems is under question (Desmarais *et al.*, 2002). For example, certain studies have shown that *Salmonella* spp. can survive for longer in soil than *E. coli* (Temple *et al.*, 1980), and enterococci have been shown to survive as well as *E. coli* (Desmarais *et al.*, 2002) and even for longer periods in sediments (Haller *et al.*, 2009). In addition, results from the DNA fingerprinting of *E. coli* in beach sand indicated that some *E. coli* strains may have become naturalised to the environment (Ishii *et al.*, 2007). Furthermore, growing evidence suggests that strains of FIB can proliferate in environmental waters independent of a host (Power *et al.*, 2005). Consequently, microorganisms derived from the GI tract of humans and other animals enter terrestrial and aquatic natural environments where they have the potential to re-enter the host and cause infection by a number of routes.

1.2 Transmission routes of pathogens in the catchment

Pathogens that are present in faecal matter can contaminate water bodies via a number of point and non-point or “diffuse” sources. A point source of pollution occurs at one geographical location, such as waste-water effluent entering the environment via a sewage outfall pipe. The treatment of waste water is essential for protecting water resources, wildlife and public health. The traditional solution to control the release of microbial contaminants from the release of sewage effluent is addressed by sewage treatment plants (Kay *et al.*, 2008). However, many studies have revealed that Faecal Bacteria (FB) can still enter the environment despite treatment (Loutit & Lewis, 1985; Rittenberg *et al.*, 1958). Furthermore, under heavy rainfall and storm conditions, wastewater can be discharged into the sea untreated through combined sewer overflows (CSO’s) (Passerat *et al.*, 2011). In comparison, a diffuse source of pollution is the widespread input of contaminants, a typical example is microbial contamination from agricultural runoff, particularly from land where excreted waste is re-applied for crop cultivation (Topp *et al.*, 2009). In addition, many rural areas discard domestic wastewater through on-site septic tanks. These tanks are often linked with drainage fields where soil adsorption filters out many potentially harmful substances

such as bacteria and phosphates (Cheung & Venkitachalam, 2000), but surface and ground waters can become polluted. Site inspections in North Carolina revealed that faulty septic tanks in highly developed areas were a source of high faecal coliform counts found in surrounding estuarine waters (Cahoon *et al.*, 2006). A diffuse source of pollution is often much more difficult to quantify. Furthermore, identifying the source of contaminants can be extremely problematic compared to a point source of pollution (Meays *et al.*, 2004). In addition, extreme weather conditions such as storms and floods can result in the increased transportation of contaminants from the land to the sea (Wetz & Yoskowitz, 2013). As the transition point where rivers meet the sea estuarine environments are particularly susceptible to pollution as they filter large proportions of water from riverine inputs (GESAMP, 2001).

1.3 Socioeconomic impacts of microbial pollution in coastal waters

Estuarine and coastal environments are of major socioeconomic importance; these regions not only provide areas of recreation, but also provide many resources for economic activities (Costanza *et al.*, 1998). Fisheries and aquaculture provide jobs for 0.5 million people in the EU, and maritime tourism provides approximately 3 million jobs, which in 2005 resulted in a turnover of €72 billion (COM(2008) 534 final). It is estimated that 60% of the world's population reside in areas around the coast and this figure is set to increase, resulting in a rise in the interactions between the ocean and humans. (Knap *et al.*, 2002). Consequently, an increase in anthropogenic activities exerts environmental pressures on these coastal environments. Furthermore, climate observations have recorded an increase in extreme weather conditions over recent years which further impacts upon the health of coastal systems (Wetz & Yoskowitz, 2013). Faecal contamination of environmental waters can therefore have significant negative impacts on human health, causing problems on a world wide scale.

1.4 Infection relating to bacterial contamination of seafood

Seafood is an important food source for many parts of the world and represents a major source of protein for over two billion people (FAO, 1999). Estuarine filter feeding shellfish such as mussels, scallops and oysters are especially susceptible to bacterial contamination if grown in sewage contaminated waters (Bakr *et al.*, 2011). Eating shellfish that are harvested from polluted waters can cause human infectious diseases (Wittman & Flick, 1995). Every year over 800 million meals are consumed that potentially consist of contaminated shellfish and other sea foods

(Shuval, 2003). In addition, over 4 million people are estimated to be infected with the potential debilitating or even lethal hepatitis A and E (HAV/ HEV) every year from eating contaminated shellfish (Shuval, 2003).

Whilst not a genus of bacteria originating from the gut, *Vibrio* spp. are ubiquitous in aquatic environments. They belong to the phylum Proteobacteria and are Gram-negative facultative anaerobes (Oliver *et al.*, 1983). They are commonly found in marine environments, particularly brackish and estuarine waters (Singleton *et al.*, 1982). Several *Vibrio* spp. cause disease in humans, however, the most important are *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae* (Gopal *et al.*, 2005). *V. vulnificus* can cause septicemia which can be fatal (Howard & Bennett, 1993), *V. parahaemolyticus* is mainly responsible for mild gastroenteritis (Daniels *et al.*, 2000), whilst *V. cholerae* can cause acute gastrointestinal infection which can lead to death if left untreated (Heidelberg *et al.*, 2000). *V. cholerae* has been responsible for several pandemics throughout the 19th and 20th Centuries resulting in high mortality rates (Dziejman *et al.*, 2002). Approximately 200 serotypes of *V. cholerae* are known but only two types 01 and 0139 are thought to relate to “true” cholera, with most cases of infection thought to be brought on by eating contaminated sea foods (Cabral, 2010).

1.5 Factors affecting pathogen survival in water

The survival of FIB can vary between bacterial types and can differ among different water bodies (Anderson *et al.*, 2005). For example, in coastal waters, salinity plays an important role in survival; it has been shown that FIB survival is lower in high salinities and light also has a deleterious effect, especially when combined with a high salinity content (Bordalo *et al.*, 2002). It has been suggested that the survival of FIB is determined by the amount of exposure to sunlight, but in freshwater bodies FIB are more resistant to the effects of UV than in seawater (Fujioka *et al.*, 1981) and thus should be taken into consideration when setting the criteria for water quality measures. The lack of nutrients has also been reported several times as a limiting factor on the survival of allochthonous bacteria in environmental waters (Barcina *et al.*, 1997; Hendricks, 1972), but certain water bodies will be susceptible to regular deposits of nutrients such as nearshore waters from ground water runoff, which will aid in the persistence of FIB concentrations (Boehm *et al.*, 2009). Predation by protozoa and competition are also factors affecting their survival rates in environmental waters (Korhonen & Martikainen, 1991).

1.6 Factors affecting pathogen survival in sediments

The survival of FIB within an environment is controlled by the interactions of a complex combination of physical, chemical and biological parameters. For sediments, previous studies have demonstrated that nutrient availability (Haller *et al.*, 2009), organic matter content (Pote *et al.*, 2009), sediment grain size (Haller *et al.*, 2009; Howell *et al.*, 1996), clay content (Burton *et al.*, 1987), heavy metal content (Jones, 1964), predation by protozoa (Davies *et al.*, 1995; Garcia-Lara *et al.*, 1991; Enzinger & Cooper, 1976), competition with other microorganisms (Marino & Gannon, 1991), temperature (Pote *et al.*, 2009), salinity, sunlight intensity (Pommepuy *et al.*, 1992; Davies & Evison, 1991) and seasonal variation (Faust *et al.*, 1975) can all influence the survival and persistence of FIB. It is now largely accepted that sediments may provide favourable conditions for bacteria to survive and grow (Chandran *et al.*, 2011; Craig *et al.*, 2004; Burton *et al.*, 1987; LaLiberte & Grimes, 1982). Comparative studies have led to the conclusion that survival of FIB is greater in sediments compared to the water column (Sherer *et al.*, 1992; Shiaris *et al.*, 1987; Hood & Ness, 1982; Grimes, 1975), which can be attributed to the higher organic content (Craig *et al.*, 2004; Gerba & McLeod, 1976). Stephenson and Rychert, (1982) evaluated bottom sediments in six streams and found that concentrations of *E. coli* were as much as 760-fold greater than that of *E. coli* concentrations in the overlying water. It was also proposed that organic matter content had a significant impact on survival. Faecal coliform (FC) concentrations were evaluated in the sediments and the overlying waters of a freshwater environment, demonstrating that FC concentrations were 100-fold greater in the mud (Van Donsel & Geldreich, 1971). Faust *et al.*, (1975) performed *in situ* experiments to investigate the physical parameters effecting the survival of *E. coli* in estuarine water, revealing that persistence varied with the season and salinity concentrations, and survival was extended with the addition of montmorillonite. It was also proposed that the most important factor influencing the survival of *E. coli* was temperature, with low temperatures prolonging existence. Montmorillonite (a group of minerals that form a clay) is usually a large component of estuarine sediments, it has been suggested that it provides *E. coli* and possibly other faecal bacteria protection against bacteriophage attack in saline conditions (Roper & Marshall, 1974). In addition, Ghoul *et al.*, (1990) suggested that the uptake of osmoprotecting substances such as betaines from the sediment helped alleviate the deleterious effects of saline waters, but also recommended that further *in situ* studies should be undertaken to confirm this.

1.7 Sedimentary processes in an estuarine environment

The weathering and erosion of rocks, soil and riverbanks result in the formation of sediments in the natural environment (Malham *et al.*, 2014). In addition, human activities such as agriculture, forestry operations and road construction can impact on soil and river bank erosion leading to increased levels of sediment in rivers and streams (Wood & Armitage, 1997). Sediment grain size can range from $< 4 \mu\text{m}$ (clay), to $2000 \mu\text{m}$ (very coarse sand) up to large boulders. Estuaries provide a channel for the transportation of sediments from rivers to the sea, as sediments move towards the sea, the continued deposition and resuspension influences grain size distribution (Dyer, 1995). The larger sediment particles can become deposited on the river channels, whilst the finer particles are transported towards the estuary (Dyer, 1995). Most tide-dominated estuaries are often funnel shaped and comprise large areas of fine-grained sediments which results in the constant mixing of suspended particles from both the rivers and the sea (wells, 1995). In addition, extreme weather conditions such as storms can have a significant impact on sediment formation, transport and resuspension (Liu & Huang, 2009).

1.8 Methods for the detection of FIB in recreational water and regulatory standards

1.8.1 Water quality legislation requirements

The Revised Bathing Water directive (2006/7/EC) (E.C. 2006) implemented through the Water Framework Directive (2000/60/EC) (E.C. 2000) states that based on a 90 percentile-evaluation, *E. coli* concentrations in water measured in any one assessment period should not exceed 500 CFU/100 ml to be classified as ‘good’ or ‘sufficient’ quality in transitional and coastal waters. However, for a classification of ‘excellent’ the percentile values should be no more than 250 CFU/100 ml. Concentrations of intestinal enterococci are also measured, with a limit of 100 CFU/100 ml to be classified as ‘excellent’ and a limit of 200 CFU/100 ml for ‘good’ quality. Bacterial abundance is measured in water samples only, and counts are determined by a cultivation-based assay on a selective microbiological medium. However, the directive does not monitor the levels of other potentially harmful groups such as *Salmonella* or *Campylobacter* and does not consider FIB concentrations in sediments.

1.8.2 Culture-based microbiological enumeration methods

Microorganisms exist in significant numbers and as members of complex communities. Consequently, determining the structure and function of these communities is not an easy task

(Fuhrman, 2009). The main difference between monitoring FIB levels in water samples and sediment samples is the need to efficiently dislodge, separate and extract the microorganisms from the sediment particles. Twenty two methods to extract FIB from sand were evaluated by Boehm *et al.*, (2009), the highest FIB recovery obtained by hand shaking the sand in a solution of phosphate-buffered saline or deionized water (1:10 w/v) for 2 minutes, allowing to settle for 30 seconds and included a rinse step. The results, however, were based on sand and it was suggested that for sediments which have a higher silt and clay content a more rigorous form of extraction method may be more effective (Boehm *et al.*, 2009). Craig *et al.*, (2002) found that when compared with hand shaking or using sonication methods, the sonication bath was the most effective form of FIB extraction from sand, when mixed with 0.1% peptone water but when sediments with a higher content of silt and clay were tested, the most appropriate method was to hand shake the sediment and eluent for one minute. However, Epstein & Rossel, (1995) found that the best form for recovering bacteria from sand was to use an ultrasonic probe, but the efficiency of the method was determined by sample size. Boenigk, (2004) suggested that for sediments with a higher clay content a harsher mechanical treatment would be required to separate the bacteria from the sediment, which could often result in cell damage and so proposed a method which involved chemical disintegration of silicates using hydrofluoric acid, this reduced sediment particles and allowed for a gentle sonication procedure to follow. It would seem that the most effective form of CFU extraction depends on the composition and characteristics of the sediment, and it is therefore essential to use the most effective method to give an accurate result of FIB enumeration.

All of the above techniques have been used for enumeration by classic culture-based methods, which were first introduced in the 1800s and are still widely used today. These methods usually involve the cultivation of bacteria either on selective or non-selective media or in broth, followed by enumeration and possible further identification analysis of presumptive colonies. The introduction of chromogenic and fluorescent substrates which detect specific enzymatic activity and create a colour or light reaction allows for increased sensitivity and a faster detection without the need for further biochemical testing (Perry & Freydiere 2007). Membrane filtration is a common method used to detect bacterial cells in water, the membrane pores retain the bacterial cells as water is filtered through, this can then be used in culture-based methods. Culture-based methods remain to be a time consuming and labour intensive exercise due to the long incubation periods required for cell growth. It is also well established that many pathogens and non-pathogenic bacteria can enter into a metabolically dormant state when exposed to adverse environmental conditions, when in this dormant state they are Viable but non-culturable

(VBNC) on routine bacteriological laboratory media (Oliver, 2005). Where bacterial counts are low, an enrichment broth can be used to stimulate growth so detection can be possible, but this step will eliminate any possible quantitative results and only provides information on presence or absence (Ibekwe *et al.*, 2002). The presence of VBNC cells can have public health consequences if they remain undetected by the routine monitoring of bathing and shellfish harvesting waters.

1.8.3 Molecular-based techniques used to detect and quantify FIB in environmental samples

Classical culture-based methods are limited by their inability to detect VNBC cells. However, modern molecular biological techniques allow for a more rapid detection of FIB with more accurate results (Jasson *et al.*, 2010; Hewson & Fuhrman, 2006). Many of these new processes are based on utilising the amplified nucleic acids that result from the Polymerase Chain Reaction (PCR), but often the extraction and purification of DNA from a sample can be the limiting factors in such methods (Jackson *et al.*, 1997). This is especially true for marine sediments, as quite often these sediments may exhibit a high percentage of organic matter, heavy metals and organic pollutants, all of which interfere with the recovery of Deoxyribonucleic Acid (DNA) and when co-extracted with DNA inhibit PCR. Extraction of DNA from soils and sediments often relies upon commercial kits and can be achieved by either direct lysis of the cells within the matrix or by recovering the cells prior to cell lysis. Leff *et al.*, (1995) compared three different DNA extraction methods that incorporated cell lysis within the sediments and cell lysis following removal from the sediments. DNA yield and purity varied significantly between methods but the method shown to yield the most DNA from soils and sediments was direct lysis. However direct lysis is more likely to extract PCR inhibitors along with the DNA and may also include DNA from other sources, for example dead and decaying cells (Steffan *et al.*, 1988). In another study, four direct lysis methods to extract DNA from three different soil types and one marine sediment were compared. The two most successful methods incorporated a bead beating step but only one yielded enough DNA from the sediment sample to allow visualisation on an agarose gel, indicating that the characteristics of the soil or sediment type has a considerable influence on the results (Carrigg *et al.*, 2007). Methods currently used to extract DNA vary extensively with varying results and often include several purification steps.

Once the DNA has been extracted and purified, the PCR method can be used to amplify a target sequence by a thermal cycling process that first denatures the DNA strands, followed by annealing and extension of the added primers, facilitated by a polymerase enzyme. This is

repeated in a thermocycler which results in an increased number of copies of the target sequence (Leonard *et al.*, 2003). The PCR amplification product can then be detected and analysed by gel electrophoresis, nucleic acid probes or by hybridization. PCR has the advantage of being rapid and accurate with high specificity and sensitivity. It can even detect very low numbers of microbes in a given sample and it has been used to detect specific genes in numerous substances such as food (Elizaquivel *et al.*, 2012), seawater and freshwater (Shanks *et al.*, 2011; Haugland *et al.*, 2005), soil (Hoppener-Ogawa *et al.*, 2007) and sediments (Liao *et al.*, 2011). In addition, the PCR method has the added advantage of being able to detect actual pathogens.

Pathogenic bacteria have the ability to cause intestinal and extraintestinal disease by means of a varied combination of virulence factors such as toxins, invasins and adhesins (Hacker *et al.*, 1997). The genome of pathogenic bacteria can contain regions known as pathogenicity-associated islands (PAIs). These regions carry functional virulence genes that can be detected by PCR. In addition, virulence factors are often encoded by genes that are present on mobile genetic components such as bacteriophages and plasmids, thus enabling the transference of virulence between bacteria (Hacker *et al.*, 1997).

Numerous variations of PCR exist, but the main disadvantage to conventional end-point PCR is that it cannot distinguish between dead or live cells, as it utilises DNA. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) aims to overcome this problem by utilising RNA instead of DNA, thus results are more representative of living cells by only detecting live cells (Yaron & Matthews, 2002). First strand cDNA can be synthesised using the RNA as a template and then amplified. Real-Time quantitative PCR (qPCR) which uses fluorescence to monitor the synthesis of the PCR product in real time allows a quantitative result which can be related back to the number of bacterial cells within a sample (Heid *et al.*, 1996) and multiplex PCR can simultaneously detect several different target genes (Jofre *et al.*, 2005; Ibekwe *et al.*, 2002).

1.9 The Conwy catchment

The Conwy Estuary, North Wales, UK, is an extremely important and productive estuary that provides areas for recreation, food and jobs. It supports a commercial shellfish bed that generates a local annual income of approximately £270,000. In addition, the Conwy estuary impacts on blue flag beaches that attract thousands of tourists every year. A decline in the microbial water quality of the Conwy estuary could have significant ecological and economic consequences as well as human health implications. The Conwy catchment covers approximately 300 km² and has a human population of ~112,000, with 89,000 living in close proximity to the

coast (Thorn *et al.*, 2011). The catchment also incorporates large mountainous areas, with a large proportion utilised for extensive agriculture (Oliver *et al.*, 2008). Consequently, the Conwy estuary is susceptible to inputs of microbial pollution, particularly from the discharge of wastewater effluent. In addition the geographical location of the Conwy catchment means the area is prone to flooding which could add to the flow of microbial contaminants from the land to the sea.

Much of the work for this thesis has been carried out using samples taken from the Conwy estuary, as a reduction in microbial water quality of this area could have major ecological and economic consequences. This thesis is part funded by Welsh Water Ltd who has a particular interest in identifying and accounting for any environmental implications associated with their activities to provide clean, safe drinking water and the disposal of wastewater.

1.10 Aims and objectives of this thesis

Microbial contamination of environmental water can pose a significant risk to human health, particularly in areas utilised for bathing, recreation and the harvesting of shellfish. As anthropogenic activities increase, and an increase in extreme weather events contribute to the transfer of faecal contaminants in to environmental waters, there is a necessity to fully understand the complex interactions of faecal bacteria within the environment. Management strategies to safeguard human health have included the implementation of legislative measures to monitor water quality. For example the Revised Bathing Water directive (2006/7/EC) (E.C. 2006) implemented through the Water Framework Directive (2000/60/EC) (E.C. 2000) details measures to be applied for the monitoring of bathing waters. Subsequently, most of the previous research has focused on the survival and persistence of FIB in the water column. It is only recently that sediments have been recognised as a potential and important reservoir of FIB, thus the interactions between FIB and their associated pathogens and sediments is currently under-researched.

By using a suite of multi-disciplinary techniques this thesis focuses on addressing the paucity of information regarding the interactions between FIB and other potentially pathogenic bacteria in sediments, in order to characterise the mechanisms that underpin the transport and persistence of pathogens in the Conwy catchment. In addition, the abundance of aquatic *Vibrio* spp. and culturable heterotrophic bacteria have been assessed in order to compare the interactions of the indigenous bacterial communities with the interactions of FIB communities within sediments.

The key objectives of the thesis are as follows:

1. To investigate the role of sediments as a reservoir for **FIB** and other potentially pathogenic bacteria.
2. To examine the spatial ecology of **FIB** in relation to sediment composition (grain size and organic matter content)
3. To investigate the influence of point and diffuse pollution sources on the abundance of **FIB**, other potential pathogens and the indigenous microbial community in marine and estuarine sediments
4. To investigate the abundance of **FIB** and human pathogenic bacteria in estuarine sediments using both culture-based and molecular methods.

5. To investigate the influence of suspended particulate matter (SPM) on the survival of FIB in both fresh and brackish water.

This thesis is presented as a succession of manuscripts that are prepared for publication in peer reviewed scientific journals. Each chapter in this thesis is linked by the key objectives detailed above

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CHAPTER 2

Spatial and temporal distribution of faecal indicator bacteria in marine sediments surrounding an offshore sewage outfall pipe

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Abstract

The release of municipal waste water into the ocean environment is a cost effective form of waste disposal that has been used for many years in several countries. Many studies have focused on bacterial decay rates in seawater in order to safeguard human health. However, information on the deposition of faecal indicator bacteria (FIB) and their associated pathogens from an offshore outfall to bottom sediments is limited. Here, the abundance of culturable, sediment-associated *Escherichia coli*, total coliforms, enterococci (FIB), *Vibrio* spp. and culturable heterotrophic bacteria were determined from a 15 point sampling array around a sewage outfall pipe for a 12 month period, to evaluate their spatial and temporal variation in relation to sediment grain size and a tidally driven effluent dispersal model. The results revealed significant positive correlations between the abundance of each cultured bacterial group and the abundance of all other groups enumerated. Significant positive correlations were also observed between all cultured bacterial groups and fine sediments comprising silt and clay, whilst larger grain sizes (medium and coarse sands) had significant negative correlations with bacterial abundance. In addition, spatial variation in sediment type suggested that the release of sewage effluent impacts on sediment composition. Furthermore, the observed spatial variation in cultured FIB abundance reflected hydrodynamic model predictions for the sewage effluent plume and thus increases the confidence that the model predictions are accurate. The identification of faeces contaminated sediments surrounding an offshore sewage outfall cannot be ruled out as a contributor to microbial contamination of nearshore waters, which may pose a risk to human health and have socioeconomic consequences, if used for recreation or shellfish harvesting.

KEY WORDS: Sewage effluent, sediment, faecal bacteria, outfall pipe

2.1 Introduction

Sewage outfall pipes that extend into the ocean have been used for many years to dispose of municipal wastewater. In European and many other countries there are conditions of consent to discharge wastewater into the ocean, which include set quantities, dilutions and effluent requirements. There are several advantages to this form of disposal. These include large initial dilution of contaminants, which can be in the order of several hundred-fold within the immediate vicinity of discharge (Koh & Brooks, 1975) and rapid bacterial decay rates within the water column, usually within days of discharge (Loutit & Lewis, 1985). The Directive for England and Wales states that secondary treatment (the biological break down and reduction of organic matter), with a secondary settlement or other process that allows the receiving waters to meet the relevant water quality standards, is required for the discharge of sewage into freshwater and estuaries from wastewater treatment plants that serve agglomerations of between 2,000 and 10,000 p.e. (population equivalent), and are not classed as sensitive areas (areas that sewage release may impact on, i.e. bathing and shellfish waters). However, less stringent processes may be performed, such as primary treatment which involves the removal of suspended solids before discharge, from agglomerations of between 10,000 and 150,000 out to coastal waters and from agglomerations of between 2,000 and 10,000 out to estuaries if research indicates there will be minimal environmental impact as a result. Tertiary treatment requires the additional use of treatment processes to address each pollutant and is required when the area is classed as “sensitive”, whereby the release of sewage may cause eutrophication of environmental waters or impact on bathing and shellfish harvesting waters (91/271/EEC) (E.C. 1991). Consequently, the release of sewage into the open ocean can be cost effective compared with other options for disposal.

The design and length of offshore outfall pipes usually reflect the required conditions of consent and should promote the dispersal of wastewater out to sea and not towards nearshore waters, to minimise the impact on human health (Koh & Brooks, 1975). However, studies tracking the movement of sewage plumes emanating from offshore outfalls have revealed that this may not always be the case. Boehm *et al.*, (2002) discovered that under certain tidal conditions and temperatures, cold water and associated plume effluent can be advected to nearshore waters. If faecal contaminants enter the surf zone, entrainment can result in their transportation over significant distances along the beach before dispersal, causing a health risk for bathers (Grant *et al.*, 2005). To further complicate matters, FIB and enteric pathogens can become attached to particles in the water column, which can provide protection from

environmental stresses such as sunlight inactivation (Fujioka *et al.*, 1981), thus promoting their dispersal and subsequent deposition (Malham *et al.*, 2014). It is well documented that the sediment bed can provide conditions that enhance the survival and persistence of FIB when compared to the water column (Hood & Ness, 1982; Grimes, 1975). Stephenson & Rychert (1982) reported 760 times greater concentrations of *E. coli* in bottom sediments when compared to the overlying water with many investigations attributing the increase to the higher proportions of organic matter present in sediments (Perkins *et al.*, 2014; Craig *et al.*, 2004; Gerba & McLeod, 1976). Consequently, disturbance of faecally contaminated sediments during storms or other forms of hydrodynamic events, such as tidal conditions and bottom trawling, can resuspend the particles and associated bacteria back into water column, with the possibility of their transportation to nearshore waters. Faecal contamination of nearshore waters can therefore pose a risk to human health if used for recreation or the harvesting of shellfish (Shuval, 2003).

Detection of human bacterial and viral pathogens in coastal environments can be problematic because of their usually low concentrations (Straub & Chandler, 2003). In addition, detection can be time consuming and expensive. Therefore microbiological assessment of environmental water quality is usually determined by the enumeration of more abundant bacteriological indicators (Edge & Boehm, 2011). Testing for compliance with EU legislation on bathing water quality currently involves the enumeration of *E. coli* and intestinal enterococci from water samples as a proxy for levels of faecal contamination and associated enteric pathogens. However, despite numerous studies highlighting the often large differences in concentration of FIB in sediment beds when compared to the overlying water column, the microbial contamination of sediments are not considered in routine water quality testing.

It is estimated that globally over 120 million annual instances of gastrointestinal infections and over 50 million instances of severe respiratory infections can be attributed to bathing in faecally contaminated coastal waters (Shuval, 2003). In addition, the consumption of raw or undercooked seafood is thought to be responsible for 40 thousand deaths per year and 40 thousand incidents of long term disability, predominantly associated with liver disease (Shuval, 2003)

Whilst most recorded information details bacterial decay rates in seawater, there are few published data on the fate of FIB and associated pathogens that may become deposited in the sediments around an offshore outfall pipe. To our knowledge there have only been a few studies that determined faecal bacterial abundance in sediments around an ocean sewage outfall (Loutit & Lewis, 1985; Rittenberg *et al.*, 1958; Nusbaum & Garver 1955). Nusbaum & Garver (1955) briefly described finding densities of up to 2.4 billion coliforms in 100 ml of wet mud in areas

surrounding a sewage outfall in San Diego Bay, California, whilst Loutit & Lewis (1985) reported faecal coliforms in sediments up to 8 km from an ocean outfall and stated that the wind and ocean currents can have an impact on the distribution of faecal bacteria (FB). (Rittenberg *et al.*, 1958) determined the abundance of coliform bacteria in deposits surrounding three marine sewage outfalls and found that the number of coliforms was greater in sediments that lay beneath the effluent plume in the water.

Whilst there are economic advantages to the disposal of wastewater into the ocean environment, the discharge of FB and enteric pathogens causes concern for public health. Furthermore, the discharge of FB has socioeconomic consequences, if water quality deteriorates as a result of this discharge in recreational and shellfish harvesting areas. It is therefore important to identify not only the source of faecal pollution but also the fate and ecology of pathogens entering environmental waters to adequately assess and alleviate any risk to human health and to local economies.

Kinmel Bay sewage treatment works releases secondary treated waste-water effluent through an ocean outfall pipe approximately 4 km offshore from the coast of Rhyl, North Wales, UK, and under high flow conditions such as storm flow events, untreated storm water may be discharged. The aim of this research was to determine the abundance of culturable *E. coli*, enterococci and total coliforms (FIB), *Vibrio* spp. and heterotrophic bacteria in sediments taken from a 15 point sampling array around the Kinmel Bay offshore sewage outfall. We compared the spatial and temporal pattern of culturable FIB, *Vibrio* spp. and heterotrophic bacteria in contaminated sediments over a 12-month period, with sediment composition and the predictions of a tidally driven effluent dispersal model for the outfall. In addition, this investigation allows us to gain information on the effect that a relatively isolated point source of sewage release has on surrounding marine sediments and develop our understanding of FIB behaviour upon discharge into the ocean.

2.2 Materials and methods

2.2.1 Study site

The study site was an offshore sewage outfall pipe at Kinnel Bay, North Wales, UK (53.336901N, 3.569200W (WGS84) (Fig. 2.1). The Kinnel Bay wastewater treatment plant serves a population of 77,953, with consent to discharge up to 38,860 m³/d with a dry weather flow that does not exceed 15,941 m³/d. The sewage receives primary and secondary treatment before the effluent is discharged into the ocean; no tertiary treatment is applied. However, untreated effluent is discharged from this outfall under certain high flow conditions such as those caused by a storm event.

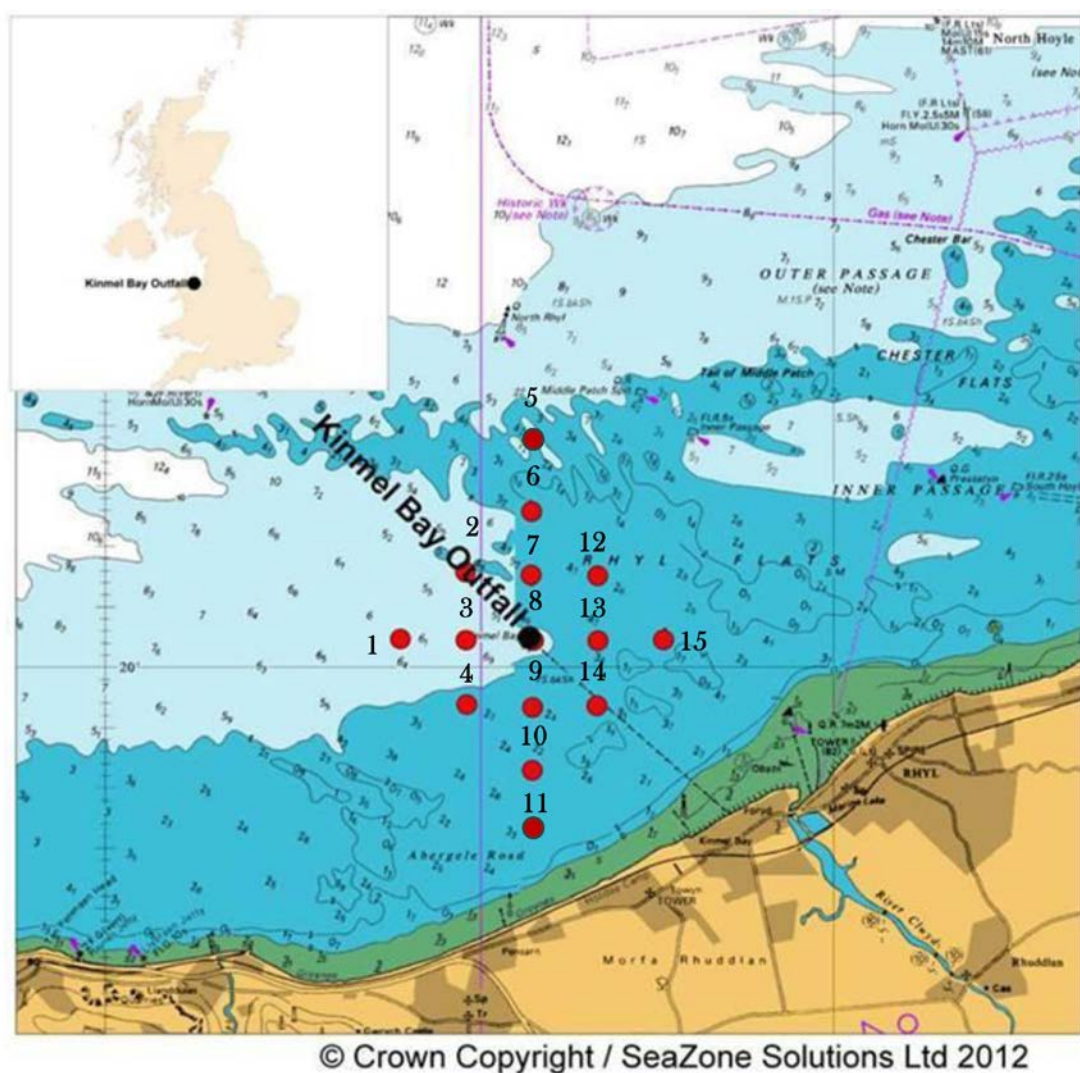


Fig. 2.1 Map of the study site showing the position of the offshore sewage outfall pipe at Kinnel Bay, North Wales, UK, detailing the 15 point grid array.

2.2.2 Sample collection

Sediment samples were collected once a month (due to adverse weather conditions only 12 sampling trips were achieved) from March 2012 to June 2013 inclusive. Sample points were arranged in a 15-point diamond shape grid array around the sewage outfall pipe (Fig. 2.1). The sample points were approximately 1 km apart and starting with site 8 at the point of discharge, the grid array was designed to incorporate the area that the sewage plume occupies according to a tidally driven effluent dispersal model. According to the model, the effluent plume moves in an east and west direction from the discharge point (site 8) occupying sites 3 and 13 before dispersal. The Danish Hydraulic Institute (DHI)'s MIKE21 hydrodynamic and water quality modelling system by Metoc (Intertek Ltd, Liphook, Hampshire) was used to formulate the model for Welsh Water Ltd (Dŵr Cymru Ltd, Treharris, UK) (Fig. 2.2).

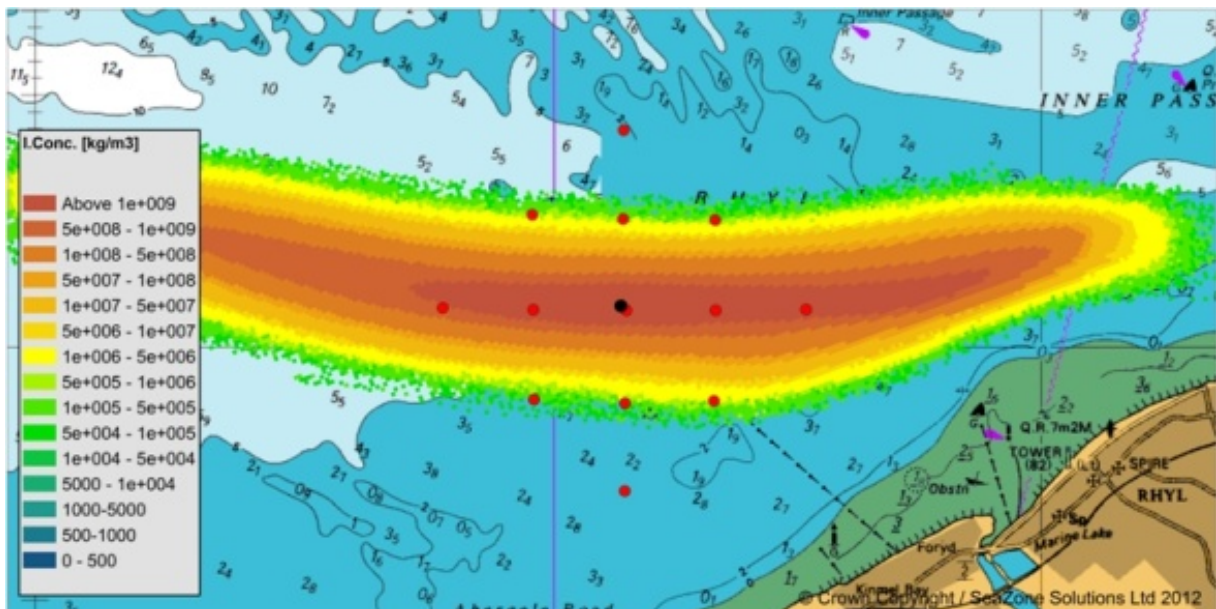


Fig. 2.2 A visual of the plume effluent released from the offshore sewage outfall pipe, Kinmel Bay, North Wales, UK. Formulated by The Danish Hydraulic Institute (DHI)'s MIKE21 hydrodynamic and water quality modelling system by Metoc (Intertek Ltd, Liphook, Hampshire).

A manually operated Van Veen sediment grab was used to collect sediment samples and operated from the side of Bangor University's 7.9 m Cheetah catamaran research boat. Three

replicate sediment samples from 3 separate grabs, were taken from each site. Approximately 50 g of sediment was taken from the centre of each grab and placed into sterile 50 ml polypropylene tubes (VWR International Ltd., Leicestershire, UK).

2.2.3 Isolation and cultivation of target bacterial groups from sediment

Target bacterial groups were isolated as described in Perkins *et al.*, (2014). Briefly, 1 g of sediment was added to 5 ml Ringer’s solution (Oxoid Ltd., Basingstoke, UK) and vortexed for 90 seconds to obtain a 1:5 dilution. Aliquots of the supernatant were transferred aseptically onto agar plates containing a selective medium for culturable *E. coli*, total coliforms, enterococci, *Vibrio* spp. and a medium to culture heterotrophic bacteria. The medium used to culture heterotrophic bacteria was previously tested to confirm the growth of *E. coli* at room temperature after 24 hrs incubation. Results were positive for the growth of *E. coli* and they are therefore included in the heterotrophic bacterial counts. The optimum volume of supernatant used to inoculate each selective medium for countable bacterial colonies was modified after the first two months of sampling due to low bacterial counts (initially 100 µl for all media then modified to 500 µl for *E. coli*, total coliforms and enterococci cultures).

Culture plates were inverted and incubated, according to the manufacturer’s recommendations (Table 2.1). The subsequent colony forming units (CFUs) were counted to determine bacterial abundance. CFU data represent the average of three independent replicate samples (one plate per replicate sample). Sediments from site 5 were not collected for April 2012 due to adverse weather conditions.

Table 2.1 Selective media used to enumerate target bacterial groups.

Bacteria group	Media	Incubation period
----------------	-------	-------------------

<i>E. coli</i> / coliforms	Harlequin <i>E. coli</i> / Coliform Medium (LabM HAL008)	24 h, 37°C
<i>Salmonella</i> spp.	Harlequin <i>Salmonella</i> ABC Medium (LabM HAL001)	24 h, 37°C
<i>Enterococcus</i> spp.	Slanetz & Bartley Medium (LabM LAB166)	48 h, 37°C
<i>Vibrio</i> spp.	Cholera Medium TCBS (Oxoid Ltd, Basingstoke, UK)	24 h, room temperature
<i>Campylobacter</i> spp.	Campylobacter Blood Free Selective Medium (Modified CCDA) (LabM 112) Selective supplements (X112/X212)	37°C for 48 hours in an atmosphere of 5% oxygen, 10% carbon dioxide and 85% nitrogen (Campygen sachets) (Oxoid Ltd, Basingstoke, UK)
Marine heterotrophic bacteria	Marine Agar (Laboratorios Conda, S.A)	24 h, room temperature

2.2.4 Sediment grain size analysis

A Malvern Hydro 2000MU particle size analyser was used in conjunction with the Mastersizer 2000 software to determine the particle size distribution of all samples. Particles were separated by 1 min sonication and grain size determined by laser diffraction. Approximately 1 g of sediment was added to the particle size analyser and 3 independent size measurements were made. All data represent the average of three independent replicate samples. Grain size statistics were then entered into the GRADISTAT software programme for analysis and subsequent classification of sediment type.

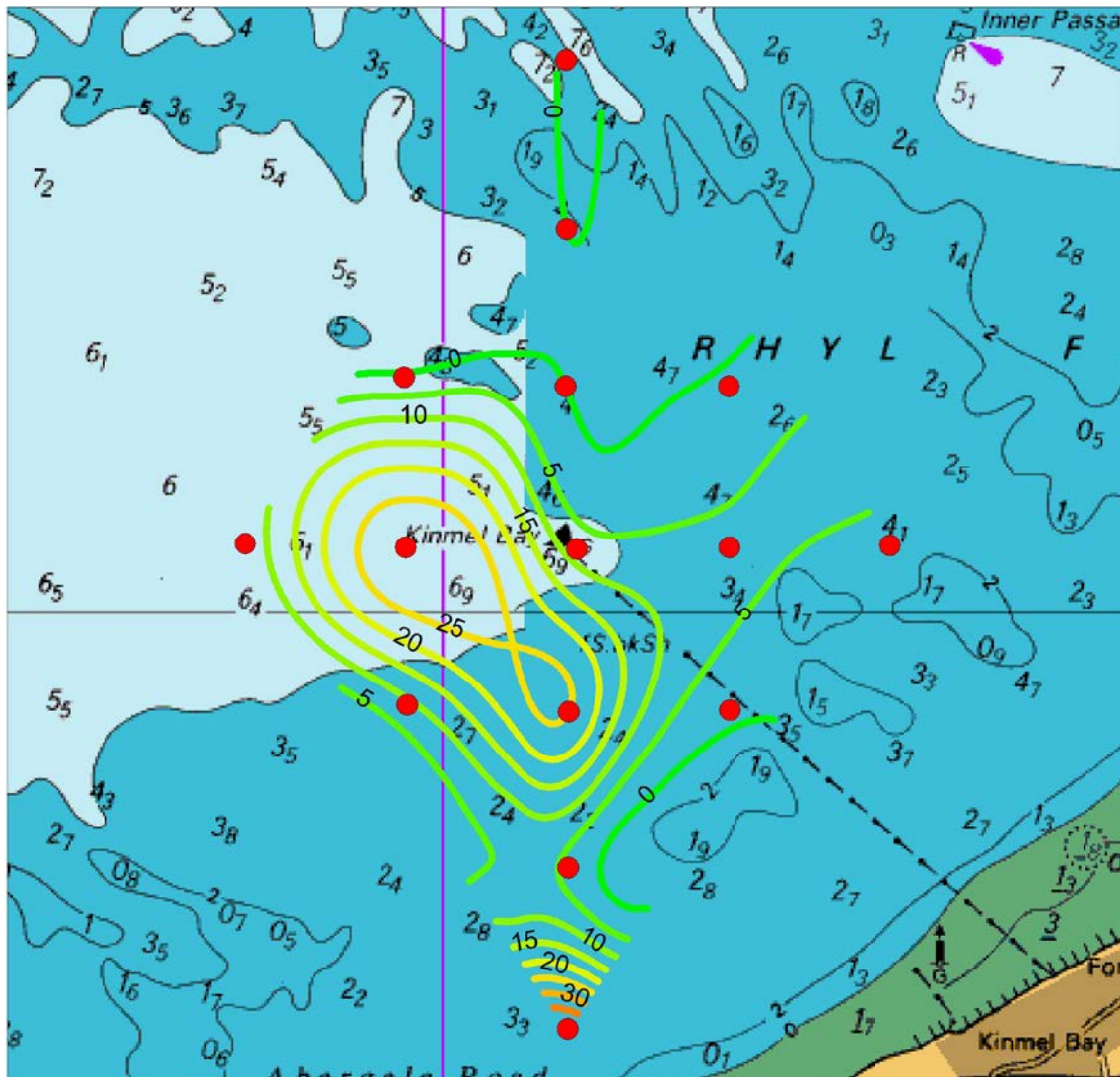
2.2.5 Statistical analysis

The Statistical Package for Social Sciences SPSS v22 (IBM Corp., Armonk, NY) was used to determine basic correlations between bacterial abundance and grain size and between the abundance of each cultured bacterial group. The data were not normally distributed. Therefore, the non-parametric Spearman Rank Correlation Coefficient (r_s) was used.

2.3 Results

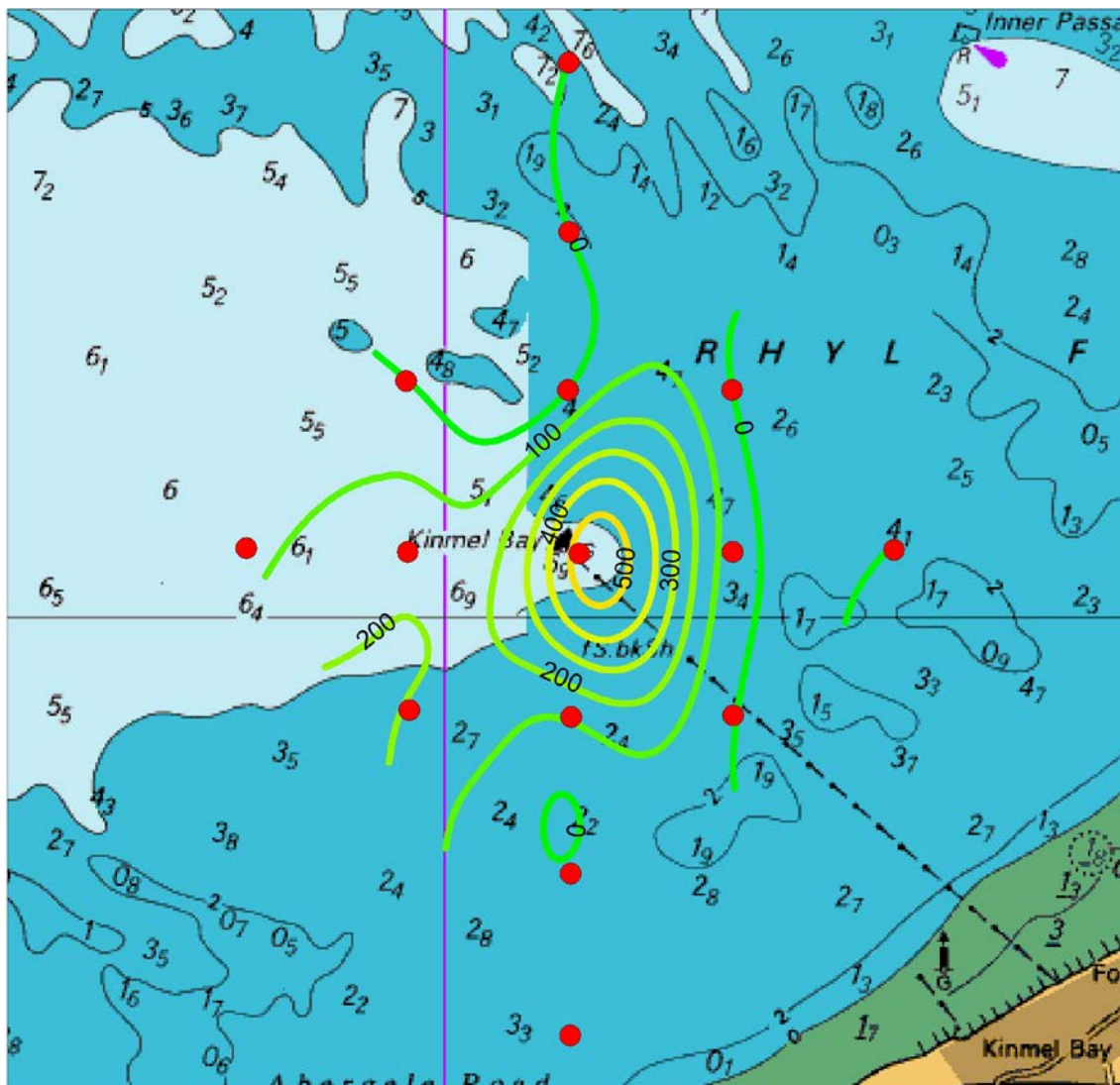
2.3.1 Spatial distribution patterns of sediment-associated culturable FIB, Vibrio spp. and heterotrophic bacteria surrounding an offshore sewage outfall pipe

Culturable *E. coli*, enterococci, total coliforms, *Vibrio* spp. and heterotrophic bacteria were regularly detected in the sediments directly under the outfall pipe, with marked spatial differences in their distribution that extended considerable distances around the outfall, up to 3 km in places (Fig. 2.3-2.7). The highest levels of FIB and *Vibrio* spp. were detected in sediments taken directly under the outfall at sample point 8 and sample point 9 which is 1 km south of the outfall and in a distinct effluent plume pattern running in an east and west direction (sites 3 and 13) from the point of discharge. In addition, high levels of FIB and *Vibrio* spp. were observed at the most southerly sample point (site 11, onshore) 4 km from the outfall. There was unusually high FIB contamination of bed sediments at site 4 in April 2012 (*E. coli* 100 CFU/g, enterococci 83 CFU/g total coliforms 2150 CFU/g) with subsequent low counts for only 3 other months. With the exception of sample site 4 and the near shore sample site (site 11) the calculated average bacterial abundance data revealed a pattern of contamination that ran in an east and west direction of the outfall pipe, which reflected the predicted distribution of the tidally-driven effluent plume. In contrast, FIB abundance was consistently low North of the outfall and towards the open ocean (Fig. 2.3, 2.4 and 2.5).



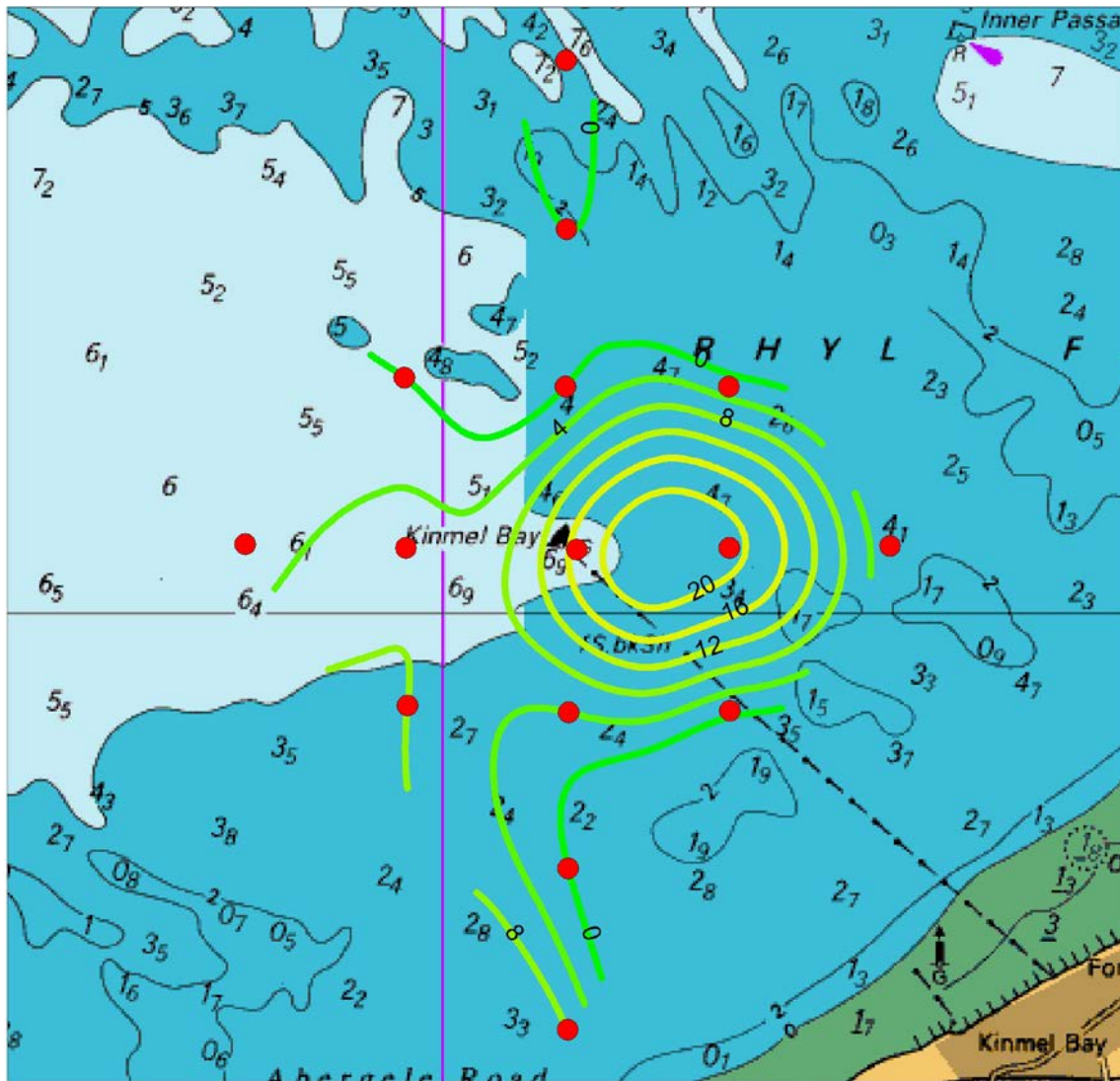
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Fig. 2.3 Contour map showing the average abundance of culturable *E. coli* in sediments (CFU/g wet weight) surrounding on offshore sewage outfall. ($n = 3$ replicate samples collected once a month for 12 months over a 16 month period).



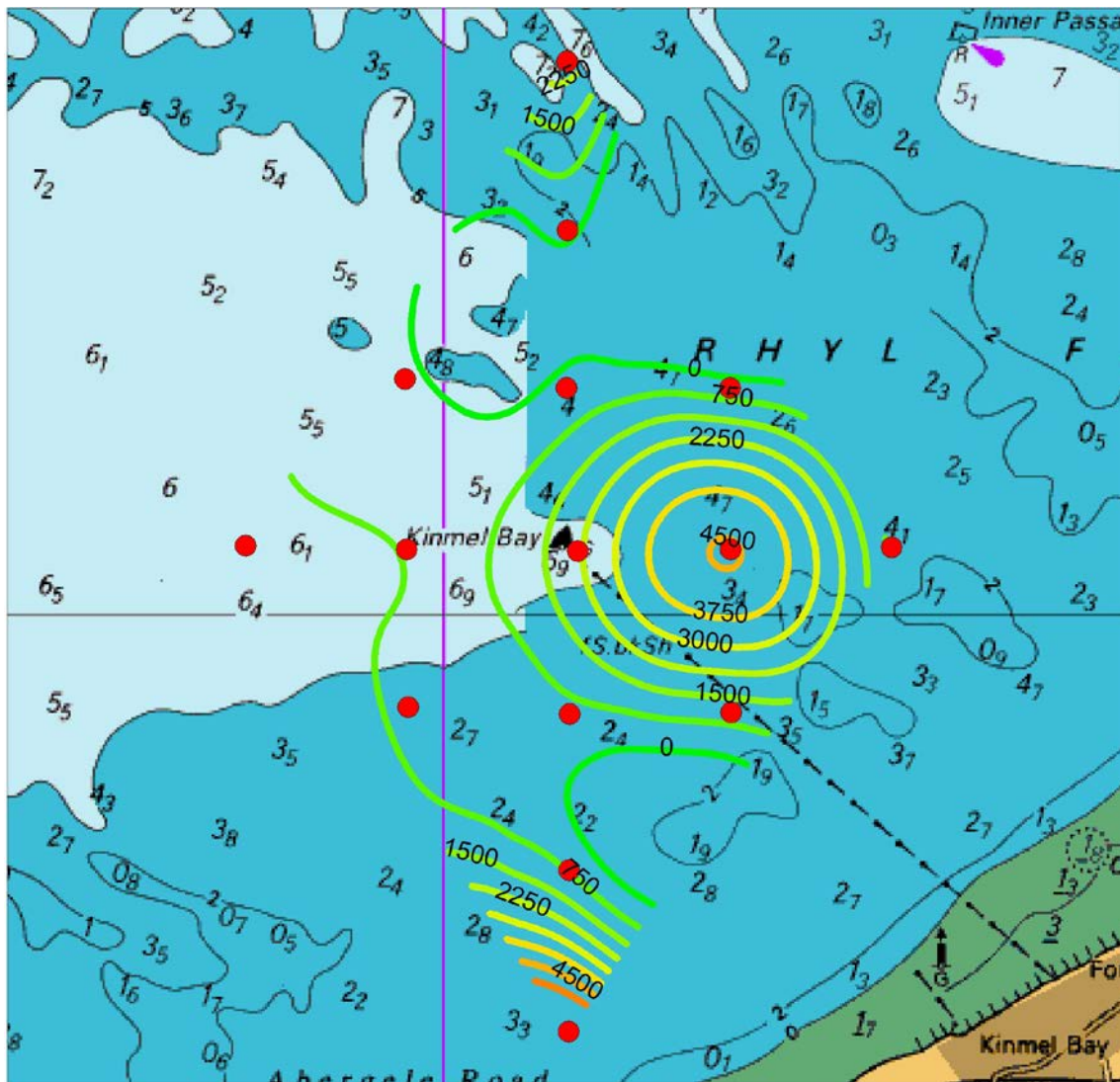
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Fig. 2.4 Contour map showing the average abundance of culturable coliform bacteria in sediments (CFU/g wet weight) surrounding an offshore sewage outfall pipe. ($n = 3$ replicate samples collected once a month for 12 months over a 16 month period).



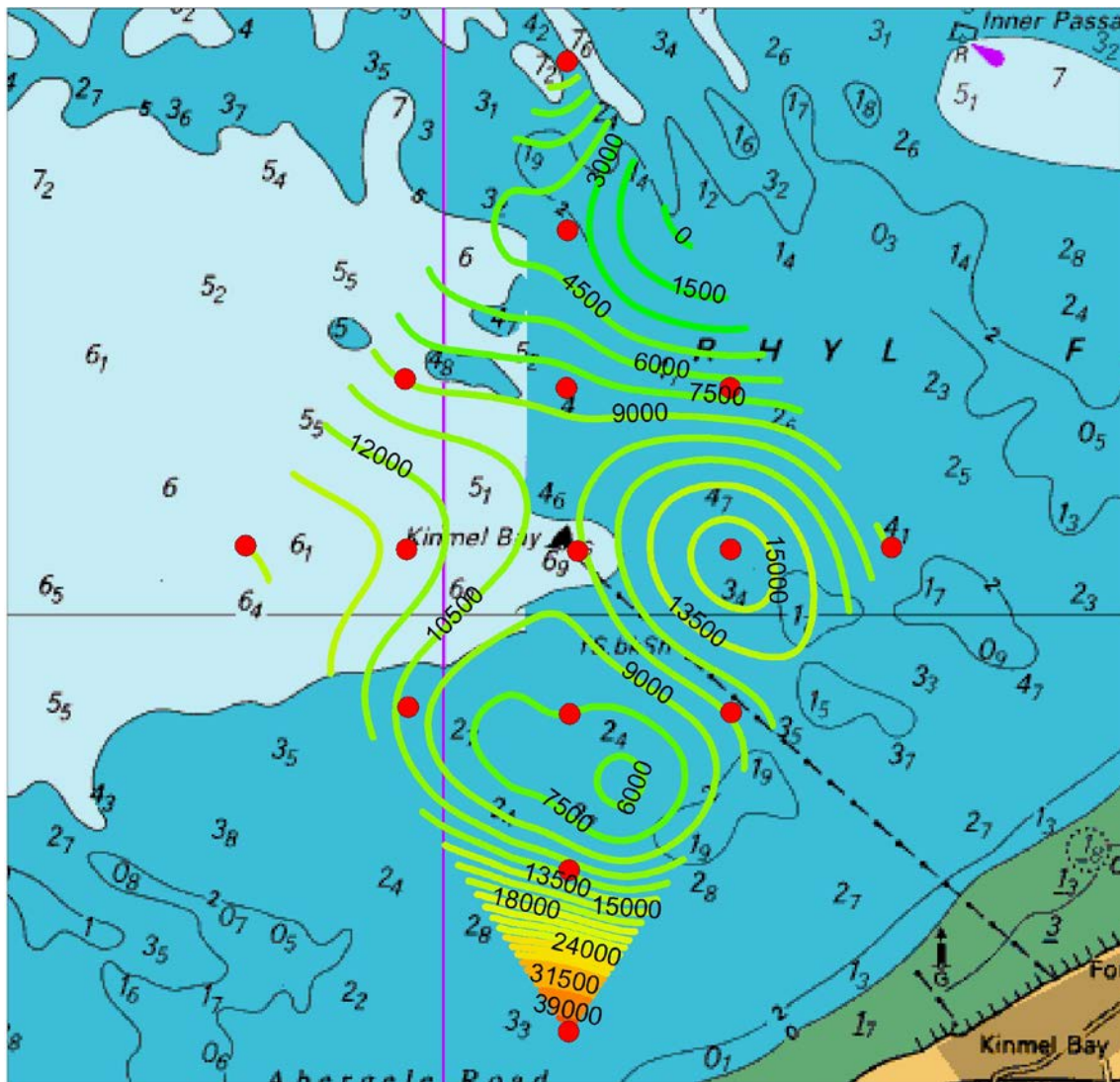
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Fig. 2.5 Contour map showing the average abundance of culturable enterococci in sediments (CFU/g wet weight) surrounding an offshore sewage outfall pipe. ($n = 3$ replicate samples collected once a month for 12 months over a 16 month period).



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Fig. 2.6 Contour map showing the average abundance of culturable *Vibrio* spp. in sediments (CFU/g wet weight) surrounding an offshore sewage outfall pipe. ($n = 3$ replicate samples collected once a month for 12 months over a 16 month period).



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Fig. 2.7 Contour map showing the average abundance of culturable heterotrophic bacteria in sediments (CFU/g wet weight) surrounding an offshore sewage outfall pipe. ($n = 3$ replicate samples collected once a month for 12 months over a 16 month period).

2.3.2 Temporal variation in culturable FIB, Vibrio spp. and heterotrophic bacterial abundance around an offshore sewage outfall pipe over a 13-month period

E. coli abundance ranged from 0 to 3.1×10^2 CFU/g wet weight, with highest counts in February 2013 (Table 2.2). Total coliform abundance ranged from 0 to 4.5×10^3 CFU/g wet weight, with highest counts also in February 2013 (Table 2.3). Culturable enterococci varied from 0 to 1.1×10^2 CFU/g wet weight, with the highest abundance observed in January 2013 (Table 2.4). As an indigenous marine microbe, *Vibrio* spp. abundance was relatively high in comparison to FIB ranging from 0 to 2.4×10^4 CFU/g wet weight (Table 2.5). Heterotrophic bacteria were cultured and enumerated as a proxy for the indigenous microbial community with abundances ranging from 5.8×10^1 to 1.3×10^5 CFU/g wet weight (Table 2.6).

Table 2.2 Heat map detailing the abundance of culturable *E. coli* CFU/g wet weight, within marine sediments collected over a 16 month period (only 12 months sampled due to weather conditions) ($n = 3$).

Sample site	March 2012	April 2012	May 2012	June 2012	August 2012	September 2012	October 2012	November 2012	January 2013	February 2013	April 2013	June 2013
1	0	0	0	57	0	3	0	3	3	0	0	0
2	0	0	0	0	0	0	0	0	3	3	0	0
3	0	17	0	0	0	0	3	0	17	310	0	0
4	0	100	0	0	0	0	3	0	0	7	0	0
5	0	0	0	0	0	0	0	0	3	7	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	3	0	0	0	53	0	0	40	0	0
9	0	0	0	290	0	0	0	0	3	3	0	0
10	0	0	0	43	0	0	0	0	3	0	0	0
11	0	0	0	7	0	290	170	3	7	27	0	0
12	0	0	0	7	0	0	0	0	3	0	0	0
13	0	50	0	7	0	0	3	3	13	0	0	0
14	0	0	0	7	0	0	0	0	0	0	0	0
15	0	0	0	7	0	0	0	23	0	3	0	0

Table 2.3 Heat map detailing the abundance of culturable total coliforms CFU/g wet weight, within marine sediments collected over a 16 month period (only 12 months sampled due to weather conditions) ($n = 3$).

Sample site	March 2012	April 2012	May 2012	June 2012	August 2012	September 2012	October 2012	November 2012	January 2013	February 2013	April 2013	June 2013
1	17	33	0	700	3	13	20	20	43	0	0	3
2	0	0	0	0	7	0	0	7	3	3	0	0
3	0	134	27	30	7	0	17	20	93	1680	0	0
4	0	2250	0	0	0	0	60	0	3	30	0	0
5	0	0	0	0	0	0	0	0	3	17	0	0
6	0	0	3	0	0	0	0	0	0	0	0	0
7	0	0	3	0	0	0	10	3	0	0	0	0
8	0	283	53	7	3	0	1020	123	313	4527	0	0
9	17	0	0	290	7	827	10	3	3	167	0	23
10	0	0	3	46	0	3	0	0	10	23	0	0
11	0	0	0	7	0	330	483	7	10	90	7	0
12	0	0	0	7	3	0	0	0	7	0	0	0
13	0	267	0	220	0	0	13	77	43	0	0	7
14	0	17	0	17	0	3	3	0	7	10	0	0
15	0	0	0	7	0	0	13	27	7	10	0	0

Table 2.4 Heat map detailing the abundance of culturable enterococci CFU/g wet weight, within marine sediments collected over a 16 month period (only 12 months sampled due to weather conditions) ($n = 3$).

Sample site	March 2012	April 2012	May 2012	June 2012	August 2012	September 2012	October 2012	November 2012	January 2013	February 2013	April 2013	June 2013
1	0	0	0	3	0	3	7	0	0	7	0	10
2	0	0	0	0	0	0	0	0	0	0	0	0
3	17	0	0	3	0	0	3	0	13	33	0	0
4	0	83	0	0	0	0	0	0	0	10	0	0
5	0	0	0	0	0	0	0	0	0	23	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	3	0	0	0	0	0	0	0
8	0	33	13	7	0	0	3	0	103	37	3	3
9	0	0	0	43	0	0	0	0	0	7	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	3	3	3	13	3	13	37	23	0
12	0	0	0	0	0	23	0	3	0	0	0	0
13	0	83	0	7	0	3	23	3	110	23	0	0
14	0	0	0	3	3	0	0	0	0	3	0	7
15	0	0	0	3	0	0	0	0	7	3	0	3

Table 2.5 Heat map detailing the abundance of culturable *Vibrio* spp. CFU/g wet weight, within marine sediments collected over a 16 month period (only 12 months sampled due to weather conditions) ($n = 3$).

Sample site	March 2012	April 2012	May 2012	June 2012	August 2012	September 2012	October 2012	November 2012	January 2013	February 2013	April 2013	June 2013
1	133	583	67	717	300	1267	2833	250	0	1500	133	2933
2	17	167	183	67	500	300	150	33	17	17	0	17
3	167	417	717	133	683	1667	1217	183	500	450	17	2583
4	33	4217	67	117	167	583	533	150	0	250	50	267
5	17	0	1	100	183	600	317	100	17	2850	0	24567
6	17	350	67	50	300	100	67	33	0	0	0	233
7	33	150	67	0	433	550	283	100	17	83	8	667
8	283	4300	100	17	433	217	1767	133	933	4483	25	12950
9	200	150	17	267	2900	217	550	383	17	150	17	1667
10	67	333	217	0	1783	1450	400	183	100	467	67	3300
11	1150	5467	1550	50	6733	21683	15367	8717	8717	5033	2083	8367
12	167	83	0	133	200	433	317	100	100	0	8	2600
13	167	9700	300	67	17	2817	9300	267	21667	2467	8	7700
14	433	200	0	1017	8133	1467	300	217	33	33	17	1100
15	17	0	0	0	2417	333	250	300	17	17	17	3950

Table 2.6 Heat map detailing the abundance of culturable heterotrophic bacteria CFU/g wet weight, within marine sediments collected over a 16 month period (only 12 months sampled due to weather conditions) ($n = 3$).

Sample site	March 2012	April 2012	May 2012	June 2012	August 2012	September 2012	October 2012	November 2012	January 2013	February 2013	April 2013	June 2013
1	70766	4750	23200	9833	4550	15900	6633	4183	1150	11967	1692	25317
2	42733	1233	32467	5717	10550	3950	750	1617	617	1967	167	5083
3	10900	12967	67933	3450	10167	9250	2850	3350	4817	3967	1442	24217
4	2850	60233	15533	1967	14150	4733	4200	3600	1050	2467	1150	7183
5	5300	0	547	2283	3817	6617	1417	2050	1200	13633	583	76800
6	2933	26250	2150	2750	5950	783	650	617	100	267	58	2283
7	7967	1625	54633	1567	8050	8950	2633	2567	583	650	442	9067
8	6233	11225	19000	1317	3583	11083	7183	1933	12867	16167	808	32200
9	6417	3733	5100	6800	29800	5150	3700	2017	1017	1667	425	23900
10	4433	17425	20167	700	14767	7200	1900	7067	850	2267	1367	58467
11	13900	39133	136000	2167	45233	85067	33300	38200	32067	27800	14092	53200
12	2917	4267	8700	3350	2700	4417	1700	1883	933	467	492	48200
13	3800	20333	23533	5467	1050	11650	25417	2817	28017	18817	667	46000
14	6067	6583	10050	11333	51933	13033	2033	2433	1367	1133	467	22000
15	22617	2050	13800	1817	15617	2483	967	2783	717	700	417	40933

2.3.3 The relationship between culturable FIB, Vibrio spp. and heterotrophic bacterial abundance, sediment grain size and organic matter content

Significant positive correlations were revealed between the abundance of each bacterial group and all other groups cultured (Table 2.7). In addition, significant positive correlations were determined between the abundance of all cultured bacterial groups and finer sediments, clay (grain size < 4 μm) (*E. coli*, enterococci, total coliforms, *Vibrio* spp. and total heterotrophic bacteria, $r_s = 0.151$ ($p < 0.001$) $r_s = 0.293$ ($p < 0.001$), $r_s = 0.240$ ($p < 0.001$), $r_s = 0.305$ ($p < 0.001$) $r_s = 0.245$ ($p < 0.001$) and silt (grain size 4 μm -63 μm) (*E. coli*, enterococci, total coliforms, *Vibrio* spp. and total heterotrophic bacteria, $r_s = 0.160$ ($p < 0.001$) $r_s = 0.302$ ($p < 0.001$), $r_s = 0.245$ ($p < 0.001$), $r_s = 0.316$ ($p < .001$) $r_s = 0.254$ ($p < 0.001$) respectively) (Table 2.7).

Table 2.7 Correlation coefficient (r) matrix demonstrating the relationship between the abundance of each cultured bacterial group and between each bacterial group and sediment grain size ($n = 540$).

	<i>E. coli</i>	Total coliforms	Enterococci	Heterotrophs	<i>Vibrio</i> spp.	clay	silt	Very fine sand	Fine sand	Medium sand	Coarse sand	Very coarse sand
<i>E. coli</i>	1.000											
Total coliforms	0.613**	1.000										
Enterococci	0.388**	0.427**	1.000									
Heterotrophs	0.86*	0.159**	0.240**	1.000								
<i>Vibrio</i> spp.	0.182**	0.289**	0.355**	0.618**	1.000							
Clay	0.151**	0.240**	0.293**	0.245**	0.305**	1.000						
Silt	0.160**	0.245**	0.302**	0.254**	0.316**	0.995**	1.000					
Very fine sand	0.117**	0.180**	0.236**	0.264**	0.336**	0.460**	0.478**	1.000				
Fine sand	-0.085*	-0.021	-0.161**	-0.032	-0.038	-0.326**	-0.310**	0.279**	1.000			
Medium sand	-0.190**	-0.251**	-0.278**	-0.291**	-0.328**	-0.485**	-0.504**	-0.557**	0.278**	1.000		
Coarse sand	-0.170**	-0.264**	-0.200**	-0.281**	-0.303**	-0.257**	-0.279**	-0.415**	0.028	0.818**	1.000	
Very coarse sand	-0.051	-0.088*	0.005	-0.046	-0.073	-0.048	-0.048	-0.070	-0.174**	-0.035**	0.256**	1.000

** . Correlation is significant at the 0.01 level (2-tailed).

* . Correlation is significant at the 0.05 level (2-tailed).

2.3.4 Distribution of sediment type in relation to a 15 sample point array

The sediment grain size data were entered into the software GRADISTAT (Blott & Pye, 2001) to classify each sediment sample as mud, muddy sand or sand. Site 4 samples consisted of particles over 2 mm and were therefore classified as gravel and did not undergo laser diffraction. Only two sites were classified as mud: site 8 (point of discharge) and site 11 (nearshore). Muddy sand ran in an east and west direction from site 8, once again reflecting the plume distribution, as described by the MIKE21 model. All other sites were described as sand, with the exception of site 5, which was also muddy sand.

2.4 Discussion

This study revealed that sewage discharge into offshore environmental waters resulted in microbial contamination of the underlying bed sediments. These results not only showed FIB contamination of marine sediments surrounding an offshore sewage outfall pipe, but also confirmed that spatial variation of FIB abundance was concordant with sediment composition, and that the spatial variation of FIB abundance was associated with hydrodynamic model predictions for sewage effluent dispersal. Although treatment of sewage is in many cases applied before discharge into the ocean, the release of faecal bacteria into the ocean environment may still occur, thus many studies have investigate their decay rates in the water column.

A bacterial decay model was previously tested on an outfall plume in the Ipanema ocean, which proved predictions to be efficient with die off rates for plume associated coliforms varying from 0.48 to 60.30 hours using a parameter of T90 (Time to reduce bacterial populations by 90%) (Carvalho *et al.*, 2006). However, prediction rates may differ depending on many aspects related to the geometric characteristics of the plume. For example, die off rates reduce with increasing depth of submergence and decreasing light intensity (Carvalho *et al.*, 2004). In addition, suspended particles in the water column may provide FIB with protection from sunlight (Fujioka *et al.*, 1981), provide a platform for bacterial attachment which may result in their transportation throughout the environment and also promote their deposition (Malham *et al.*, 2014). Rittenberg *et al.*, (1958) suggested that the settling of the sludge contained in the sewage discharge can result in the sedimentation of coliforms.

The present study revealed FIB contamination of sediments surrounding an offshore sewage outfall for at least 8 out of the 12 months. The winter months of January and February

yielded the highest counts. These data support previous studies that attribute the extended persistence of enteric bacteria in marine waters to low ambient temperatures (McFeters *et al.*, 1993; Halton & Nehlsen, 1968). Rittenberg *et al.*, (1958) described how the flow of plume effluent discharged from an offshore outfall was reflected in the spatial contamination of coliforms in surrounding sediments. Here, the average abundance data calculated for total coliforms in sediments across all 15 sample sites revealed highest overall contamination at site 8 (positioned directly under the outfall) and in a distinct pattern running east to west from the discharge point, which reflects the movement of the effluent plume (Fig. 2.4). Although the average abundance of coliform bacteria was high at site 4, this was caused by an unusual high concentration enumerated from sediments in April 2012, and although anecdotal, it is reasonable to suggest that storm conditions that occurred a few days prior to sample collection on this date could have increased wave action and resulted in the transportation of FIB discharged from the outfall. The average abundance data for enterococci also showed a distinct pattern of sediment contamination extending east and west of the discharge point. Overall, the highest counts of enterococci were found at site 13 (1 km east of discharge) and site 8 (directly beneath the point of discharge) also reflecting the movement of the effluent plume (Fig. 2.5). These data support a previous investigation which showed that a constant release of FIB into freshwater from wastewater pipes can cause an accumulation of FIB in the surrounding sediments (Thevenon *et al.*, 2012). Although the presence of *Vibrio* spp. in marine waters is unrelated to faecal pollution, their abundance in bed sediments was determined because *Vibrio* spp. are an example of an autochthonous pathogen present in marine ecosystems that have the ability to cause infection, in particular from the consumption of raw or undercooked seafood (Malham *et al.*, 2014). Similar distribution patterns and the same correlations were observed for *Vibrio* spp. (Fig. 2.6) and heterotrophic bacteria (Fig. 2.7) in relation to FIB, suggesting that the detection of FIB in sediments indicates the presence of other potentially pathogenic bacteria. Furthermore, the discharge of sewage into the marine environment has an impact on *Vibrio* spp. and heterotrophic bacterial abundance in bed sediments.

The Sediment particle size analysis revealed a dominance of sand and muddy sand. However, site 8 and site 11 contained mud and yielded continually higher concentrations of FIB than the other sampled sites, which reflects the significant positive correlations found between the abundance of all target bacterial groups and silt and clay. Similar correlations have been previously reported using the same bacterial groups (Perkins *et al.*, 2014). It is however, important to note that site 11 is approximately 3 km from the sewage discharge point and lies closest to the shore line. Therefore, it is probable that the elevated results of FIB at this site

result from other point and diffuse sources of microbial pollution. All other sites, with the exception of site 5, which contained muddy sand and site 4 which was predominantly gravel, were predominantly sand. Site 4 consisted of gravel, FIB were enumerated in 4 out of the 12 months sampled with 1 month showing high contamination which skewed the average data. A distribution of muddy sand extended an east and west direction from site 8 (the discharge point) which corresponded to the visual path of the effluent plume, thus supports a previous study which suggested that the constant deposition of sludge released in the sewage effluent can effect sediment composition by the deposition of organic rich particles (Thevenon *et al.*, 2011). It is well documented that the presence of organic matter in bed sediments can provide a nutrient rich environment that can enhance the survival and persistence of FIB and other potentially pathogenic bacteria when compared to the water column (Anderson *et al.*, 2005).

Conclusion

Although many studies have established faecal bacterial decay rates in seawater (Davies & Evison, 1991; Fujioka *et al.*, 1981) the information on the fate of offshore sewage outfall derived faecal bacteria in ocean sediments is limited. We have shown that the accumulation of faecal bacteria can occur in sediments in close proximity to a sewage discharge point and extend to considerable distances of up to 3 km. The variation in the spatial distribution of sediment grain size around the sewage outfall demonstrates that the release of sewage effluent can impact sediment composition which in turn influences bacterial abundance. In addition, the release of wastewater into the ocean can have an impact on the abundance of the potentially pathogenic *Vibrio* spp. in marine sediments. The spatial distribution of faecally contaminated sediments reflected the movement of the effluent plume as predicted by the hydrodynamic model, thus revealing the accuracy of the model but highlighting the implications of resuspension and transportation of contaminated sediments to nearshore waters. Microbial contamination of nearshore waters can have both human health implications and economic repercussions for areas that rely on income from tourism and the harvesting of shellfish. To eliminate any socioeconomic consequences as a result of poor water quality and to safeguard human health, further research is needed to understand the interactions of FIB and their associated pathogens with sediments. In addition to identifying the mechanisms that promote microbial transport throughout the environment.

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CHAPTER 3

Sediment composition influences spatial variation in the abundance of human pathogen indicator bacteria within an estuarine environment

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Abstract

Faecal contamination of estuarine and coastal waters can pose a risk to human health, particularly in areas used for shellfish production or recreation. Routine microbiological water quality testing highlights areas of faecal indicator bacteria (FIB) contamination within the water column, but fails to consider the abundance of FIB in sediments, which under certain hydrodynamic conditions can become resuspended. Sediments can enhance the survival of FIB in estuarine environments, but the influence of sediment composition on the ecology and abundance of FIB is poorly understood. To determine the relationship between sediment composition (grain size and organic matter) and the abundance of pathogen indicator bacteria (PIB), sediments were collected from four transverse transects of the Conwy estuary, UK. The abundance of culturable *Escherichia coli*, total coliforms, enterococci, *Campylobacter*, *Salmonella* and *Vibrio* spp. in sediments was determined in relation to sediment grain size, organic matter content, salinity, depth and temperature. Sediments that contained higher proportions of silt and/or clay and associated organic matter content showed significant positive correlations with the abundance of PIB. Furthermore, the abundance of each bacterial group was positively correlated with the presence of all other groups enumerated. *Campylobacter* spp. were not isolated from estuarine sediments. Comparisons of the number of culturable *E. coli*, total coliforms and *Vibrio* spp. in sediments and the water column revealed that their abundance was 281, 433 and 58-fold greater in sediments (colony forming units (CFU)/100 g) when compared with the water column (CFU/100 ml), respectively. These data provide important insights into sediment compositions that promote the abundance of PIB in estuarine environments, with important implications for the modelling and prediction of public health risk based on sediment resuspension and transport.

KEY WORDS: Sediment, estuary, faecal indicator bacteria, *E. coli*, coliforms, pathogens, pathogen indicator bacteria.

3.1 Introduction

Estuarine environments represent some of the most biologically productive systems in the biosphere and consequently provide a wealth of economic, social and natural ecosystem services that include food, employment, recreation and habitat (Costanza *et al.*, 1998). However, the sustainability of such systems can be severely compromised along developed and urbanised coastlines, and this is predominantly due to anthropogenic influences (Vitousek *et al.*, 1997). Almost half of the world's population are thought to live within a few hundred kilometers of the coast (Shuval, 2003) and consequently, anthropogenic activities have a significant influence upon the health of estuarine and ocean ecosystems. Furthermore, as the global climate changes, meteorological events such as storms and floods present further impacts upon estuarine environments, and increased rainfall will significantly impact on the flow and transportation of microbial pollution from the terrestrial environment into the coastal zone (Wetz & Yoskowitz *et al.*, 2013)). Human pathogenic microorganisms are introduced into estuarine ecosystems via the release of effluent from wastewater treatment plants, ineffective septic tank systems and storm water runoff (Hong *et al.*, 2010). Agricultural runoff from livestock farming can also represent a major source of microbial pollution, particularly when excreted waste from poultry and livestock is re-applied to land (Topp *et al.*, 2009). Wildlife, especially migratory wildfowl and other birds also represent an important source of zoonotic bacterial pathogens in natural environments (Obiri-Danso & Jones, 2000).

Human pathogenic bacteria often occur at low levels in the environment (Straub & Chandler, 2003) and their specific detection is a laborious and costly process (Edge & Boehm, 2011) Consequently, the detection and enumeration of faecal indicator bacteria that are present in the gastrointestinal tract of warm-blooded animals in much higher quantities represent a more effective 'indicator' of risk (Carrero-Colon *et al.*, 2011). Current legislation on water quality testing relies on the enumeration of faecal coliforms (including the well-characterised *E. coli*) and enterococci to assess and classify water quality (E.C. 2006). However, several other groups of potentially pathogenic bacteria have been detected in estuarine and marine environments. These include *Salmonella* spp. (Berthe *et al.*, 2008), *Campylobacter* spp. (Alonso & Alonso, 1993) and *Vibrio* spp. (Cox & Gomez-Chiarri, 2012). *Salmonella* spp. are well-known foodborne pathogens and recognised as one of the main causes of gastroenteritis in humans (Polo *et al.*, 1998), resulting in around 1.3 billion annual cases of infection worldwide (Coburn *et al.*, 2007) but their presence in bathing waters is not routinely monitored (Figueras *et al.*, 1997). Pathogenic strains of *Campylobacter* spp. are also thought to be responsible for a large proportion of enteric illnesses

in humans living in developed and industrialized countries (Levin, 2007). Contamination of surface and coastal waters by *Salmonella* and *Campylobacter* spp. is thought to occur predominantly via faecal contamination from wildfowl and other birds, although other animals including domestic livestock are also reservoirs (Thorns, 2000). *Vibrio* spp. are ubiquitous in aquatic environments and some strains are pathogens of humans; *V. cholera* has been responsible for several previous pandemics, resulting in high human mortality rates (Dziejman *et al.*, 2002), some of which originated from the consumption of contaminated seafood (Cabral, 2010). Consequently, filter-feeding shellfish such as mussels, scallops and oysters are especially susceptible to contamination with bacterial pathogens if grown in contaminated waters (Bakr *et al.*, 2011).

Spatial and temporal variations in bacterial abundance in an environment are controlled by the interactions of complex physical, chemical and biological parameters, such as available nutrients (Pommepuy *et al.*, 1992), organic matter (Pote *et al.*, 2009), sediment grain size (Garzio-Hadzick *et al.*, 2010), clay content (Burton *et al.*, 1987), heavy metal content (Jones, 1964), predation by protozoa (Davies *et al.*, 1995), competition (Marino & Gannon, 1991), temperature (Singleton *et al.*, 1982), salinity (Bordalo *et al.*, 2002), sunlight intensity (Davies & Evison, 1991) and seasonal variations (Faust *et al.*, 1975). Consequently, association with particulate material and sediments offers several advantages in terms of the survival and persistence of bacterial pathogens, such as protection from UV light (Davies-Colley *et al.*, 1999; Sinton *et al.*, 1994), protection from phage attack in saline conditions (Roper & Marshall, 1974), shelter from predation (Davies & Bavor, 2000) and a greater organic matter content compared to the water column (Gerba & McLeod, 1976). However, hydrodynamic processes (e.g. tides and storms), recreational activities and mechanical disturbances such as commercial dredging all have the potential to re-suspend sediment particles and their associated pathogens back into the water column, resulting in periodic elevated levels of pollution (An *et al.*, 2002; Grimes, 1975). Currently, the classification of bathing water quality relies solely upon the enumeration of FIB in water samples, despite the well-established paradigm that allochthonous bacterial pathogens in the water column become preferentially attached to particulate material, which promotes their downward flux to the bottom sediments where they are typically found in greater abundance (Danovaro *et al.*, 2009; Droppo *et al.*, 2009).

The Conwy catchment, North Wales, UK, has a population of approximately 112,000, 80% of which live in coastal resorts that represent the main economic and tourist areas, with over 5.4 million visitors per annum. The Conwy estuary directly impacts on commercially important shellfish beds, bathing waters and beaches; hence deterioration of the water quality could have

major socioeconomic consequences. The catchment covers an area of ~ 300 km² which consists of large areas of land utilised for agriculture (Thorn *et al.*, 2011), some of the highest mountains in the UK (Oliver *et al.*, 2008), in addition to residential and commercial areas. Consequently, the Conwy river and its tributaries (such as the river Gyffin and river Ganol) receive wastewater effluent from several waste water treatment plants in addition to septic tank discharges. Microbiological inputs from wildlife, agriculture, wastewater effluent, septic tank discharges and run-off from storm events therefore represent potential point and diffuse sources of FIB in the Conwy estuary.

Previous analysis of FIB concentrations in the water column across four transverse transects of the Conwy estuary revealed significant spatial variations in waterborne *E. coli* numbers on contrasting sides of the estuary (Quilliam *et al.*, 2011). These data suggested that significantly different levels of microbial pollution were present in the east and west sides of the river (Quilliam *et al.*, 2011) and consequently, the choice of sampling points for routine water quality monitoring could have a significant impact upon the classification of microbiological water quality in this area. The Conwy estuary has a dynamic tidal cycle, with resuspension and deposition of finer sediment particles observed on the shallower banks of the river, resulting in the formation of mud flats. Consequently, the localised resuspension of sediment associated FIB into the water column was one proposed explanation for such contrasting FIB counts in the water column of the Conwy estuary (Quilliam *et al.*, 2011), yet the spatial relationships between sediments composition (grain size and organic matter content) and FIB abundance in the Conwy and beyond have received little attention.

Whilst most studies focus on enumeration of *E. coli* and enterococci in water samples for the assessment of public health risk, the aim of our study was to address the paucity of information regarding the ecology of FIB along with other potentially human-pathogenic bacteria in estuarine sediments, and to identify sediment characteristics that promote pathogen abundance in estuarine environments. A second aim was to determine if the enumeration of *E. coli*, coliforms and enterococci, that represent the current standard ‘indicator’ organisms for faecal contamination and water quality monitoring, are a suitable indicator for the co-occurrence of other potentially pathogenic bacterial groups. This was achieved by determining spatial variations in the abundance of culturable *E. coli*, total coliforms, enterococci, *Salmonella*, *Campylobacter* and *Vibrio* spp. (to be used as a proxy for potential human bacterial pathogens in the estuary and referred to as ‘pathogen indicator bacteria’ (PIB) herein) in estuarine sediments of the Conwy Estuary, UK, in relation to sediment grain size, organic content and other physico-chemical factors.

3.2 Materials and methods

3.2.1 Study site

Sample locations were selected to correspond to those tested in a previous study that observed spatial variations of waterborne *E. coli* within the study area (Quilliam *et al.*, 2011), in addition to possible point and diffuse sources of pollution that include mudflats used by wading birds and agricultural land (transect 1), input from the river Ganol (transect 2), the river Gyffin (transect 3) and Conwy marina (transect 4), (Fig. 3.1).

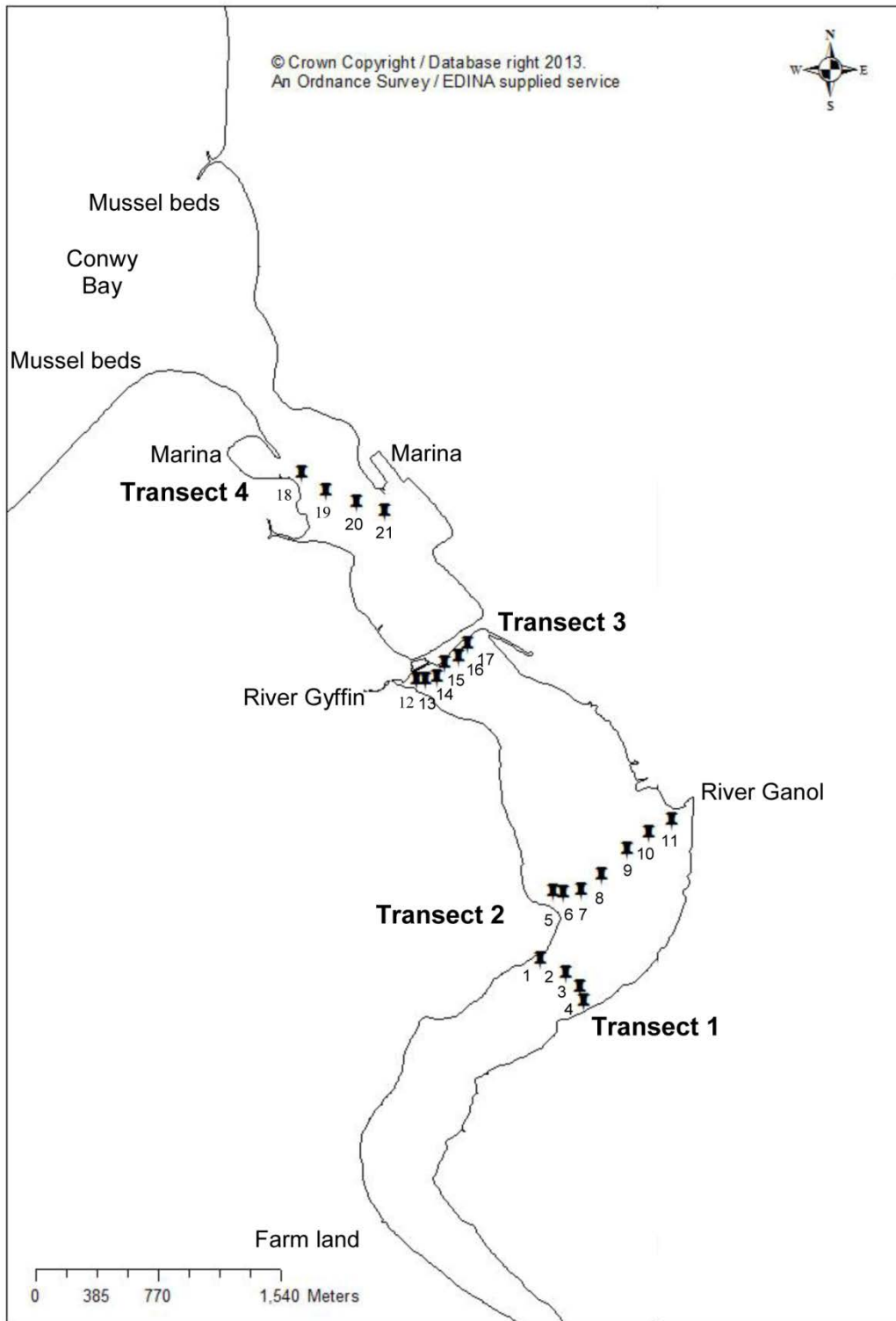


Fig. 3.1. A map of the study site; the Conwy Estuary, North Wales, UK. Water and sediment samples were collected in triplicate from four transverse transects of the Conwy estuary (twenty one sampling sites).

3.2.2 Estuarine sampling

Estuarine sediment and water samples were collected from four transverse transects of the estuary over two consecutive sampling days (transects 1 and 2 collected on the 14/05/12, transects 3 and 4 on the 15/05/12). The Bangor University research vessel “Macoma”, a 7.9 m Cheetah catamaran survey boat was utilised for sample collection, which was initiated at slack water. For each sampling point, three replicate sediment samples were collected using a manually operated Van Veen sediment grab, approximately 50 g of bottom sediment from the centre of each grab was transferred into sterile 50 ml polypropylene tubes (VWR International Ltd., Leicestershire, UK), water samples were collected in triplicate from 0.2 m below the water surface using a sterile 1 L polypropylene container, then transported to the laboratory where water samples were processed within 4 h and sediment samples within 6 h.

3.2.3 Isolation and cultivation of target bacterial groups from sediment

One gram of each sediment sample was transferred to a 7 ml sterile bijoux tube (Starlab UK Ltd., Milton Keynes, UK) and suspended in 5 ml Ringer’s solution (Oxoid Ltd., Basingstoke, UK) to obtain a 1:5 (w/v) dilution. Each sample was vortexed for 90 s to disassociate and resuspend bacteria from the sediment. There are numerous methods described to disassociate and resuspend bacteria from sediments in to a solution, these include hand shaking and sonication (Craig *et al.*, 2002). However, the recovery of bacteria may vary depending on the sediment sample size (Epstein and Rossel, 1995) and the composition (grain size) (Boenigk, 2004). Therefore, an investigation was undertaken to assess the recovery of bacteria from sediment samples of varying composition by vortexing (data not shown). Briefly, various sediment samples were suspended in 5 ml Ringer’s solution (Oxoid Ltd., Basingstoke, UK) to obtain a 1:5 (w/v) dilution and spiked with a known number of bacterial cells. Samples were vortexed for 90 and 150 seconds. The enumeration of the recovered bacterial cells by culture based methods revealed that vortexing the sample at a maximum speed for 90 s yielded the greatest recovery and thus was used throughout the studies detailed in thesis. Aliquots of the resulting supernatant for each sample were transferred aseptically onto agar plates containing a selective medium for *E. coli*, total coliforms, enterococci, *Vibrio* spp., *Campylobacter* spp., *Salmonella* spp. and total heterotrophs. The optimum volume of supernatant used to inoculate each selective medium was determined in a previous study (data not shown). All selective medium plates were inverted and incubated according to manufacturer’s recommendations

(Table 2.1). Resulting colony forming units (CFUs) provided enumeration of bacterial groups. The CFU data for sample point 13 represents the average of only two sediment samples due to one of the replicates being lost as a result of the presence of gravel clasts. All other CFU data represent the average of three independent replicate samples

3.2.4 Isolation and cultivation of target bacterial groups from water

Water samples were processed within 4 h in accordance with the Revised Bathing Water Directive (2006/7/EC). Enumeration of bacteria in water samples was achieved by using vacuum-filtration as described in (Quilliam *et al.*, 2011). Briefly, water samples were homogenised by shaking and 50 ml of water filtered under vacuum through a 0.2 µm cellulose acetate membrane (Sartorius Stedim Biotech., Gottingen, Germany). Subsequently, the membranes were aseptically transferred onto sterile agar plates containing selective medium for the enumeration of *E. coli*, total coliforms, *Vibrio* spp. and heterotrophic bacteria. Agar plates were inverted and incubated according to manufacturer's recommendations (Table 2.1). Resulting Colony Forming Units (CFUs) were enumerated 24 h post incubation.

2.2.5 Sediment particle size analysis

Sediment grain size was determined by laser diffraction after 1 min sonication to separate particles, using a Malvern Hydro 2000MU particle size analyser in conjunction with the Mastersizer 2000 software. Three replicate sediment samples from each site were pooled and homogenised. Approximately 1 g of sediment was added to the particle size analyser and 3 independent size determinations were made. This was repeated 3 times using the same pooled sample to determine an overall average.

2.2.6 Determination of sediment organic matter content

The loss on ignition method (LOI) was used to determine organic matter content of sediment samples. Three replicate sediment samples from each site were pooled and homogenised. Approximately 20 g of fresh sediment from each sample was placed in a pre-weighed crucible and dried at 95°C for 24 h. Approximately 4 g of the resultant dried sediment was pre-weighed, transferred to another crucible and placed into a muffle furnace at 550°C for 4 h. Organic matter content was calculated as the difference between the weight of the dry sediment

and weight of the residue post-combustion. This was repeated 3 times using the same pooled sample to determine an overall average. Moisture content per g of fresh sediment was determined by calculating the percentage difference between wet weight and dry weight after 24 h at 95°C.

2.2.7 In situ physico-chemical measurements of estuarine transect sample sites

Conductivity (calculated to practical salinity units (PSU)), temperature and depth measurements were recorded *in situ* using a YSI 600LS CTD scanner attached to a YSI 650 MDS data logger. This was deployed at each sample site in parallel with collection of water and sediment samples. Salinity and temperature measurements were recorded from the water immediately above the sediment bed and 0.2 m below the water surface. (Conductivity readings were calculated to determine salinity).

2.2.8 DNA extraction from bacterial isolates, PCR and sequencing of the 16S rRNA gene.

To validate the identity of bacterial colonies on selective microbiological medium, DNA was extracted from a total of 30 isolates that matched or were similar to the expected colony morphology/phenotype for *E. coli*, enterococci, *Campylobacter* and *Vibrio spp.* for 16S rRNA gene PCR amplification and sequencing. For *E. coli* and enterococci, little variation in colony morphology and phenotype was observed across all of the colonies counted, and so only a small number of isolates were sequenced for confirmation.

DNA was extracted from 30 isolated bacterial colonies using the ISOLATE genomic DNA mini kit (Bioline Reagents Ltd., London, UK) following the manufacturer's protocol. Agarose gel electrophoresis (1%) was used to visualise the DNA extracted from each bacterial isolate. Subsequently, the 16S rRNA gene of each isolate was amplified via PCR using the primer pair pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3'), which are conserved regions and enable amplification of nearly the full length of the 1.5 kb 16s rRNA gene (Edwards *et al.*, 1989). The PCR reactions consisted of 10 pmol of each primer (pA and pH), 1 x MyTaq red mix (Bioline), approximately 100 ng of template DNA and ddH₂O to a total reaction volume of 50 µl. The PCR conditions were as follows: an initial denaturation step at 95°C for 1 min, followed by 35 cycles of 95°C for 15 s, 55°C for 15s, 72°C for 10 s and a final hold at 4°C. PCR amplicons were visualised using 1% agarose gel. The expected 16S rRNA gene amplicon size was approximately 1500 bp and amplicons of the expected size were subsequently excised from the agarose gel and purified using

the Isolate PCR and Gel Kit (Bioline Reagents Ltd., London, UK) following the manufacturer's instructions. Purified 16S rRNA gene amplification products for each bacterial isolate were sequenced in both the forward and reverse orientation by Macrogen Europe (Netherlands). The Geneious Pro software package, Geneious 6.1 version created by Biomatters, (Available from <http://www.geneious.com/>) was used to quality clip each sequence and assemble the forward and reverse reads of each strain into a contiguous sequence. The sequence identity of each contig was determined using NCBI BLASTn.

3.3 Results

3.3.1 The relationship between PIB abundance, sediment grain size and organic content.

There were marked spatial differences in the abundance of sediment-associated PIB across all of the 21 sample sites within the estuary (Fig. 3.2), as determined by culture counts from 1 g of sediment wet weight on selective medium (for dry weight see Table 3.1).

Table 3.1 Sediment dry weight determined from 1 g wet weight.

Sample site	Sediment dry weight (g)
1	0.950127
2	0.949253
3	0.912751
4	0.844045
5	0.920252
6	0.94133
7	0.945941
8	0.940672
9	0.919224
10	0.914467
11	0.891909
12	0.900297
13	0.91571
14	0.934392
15	0.946164
16	0.941073
17	0.935374
18	0.909405
19	0.943176
20	0.947442
21	0.947442

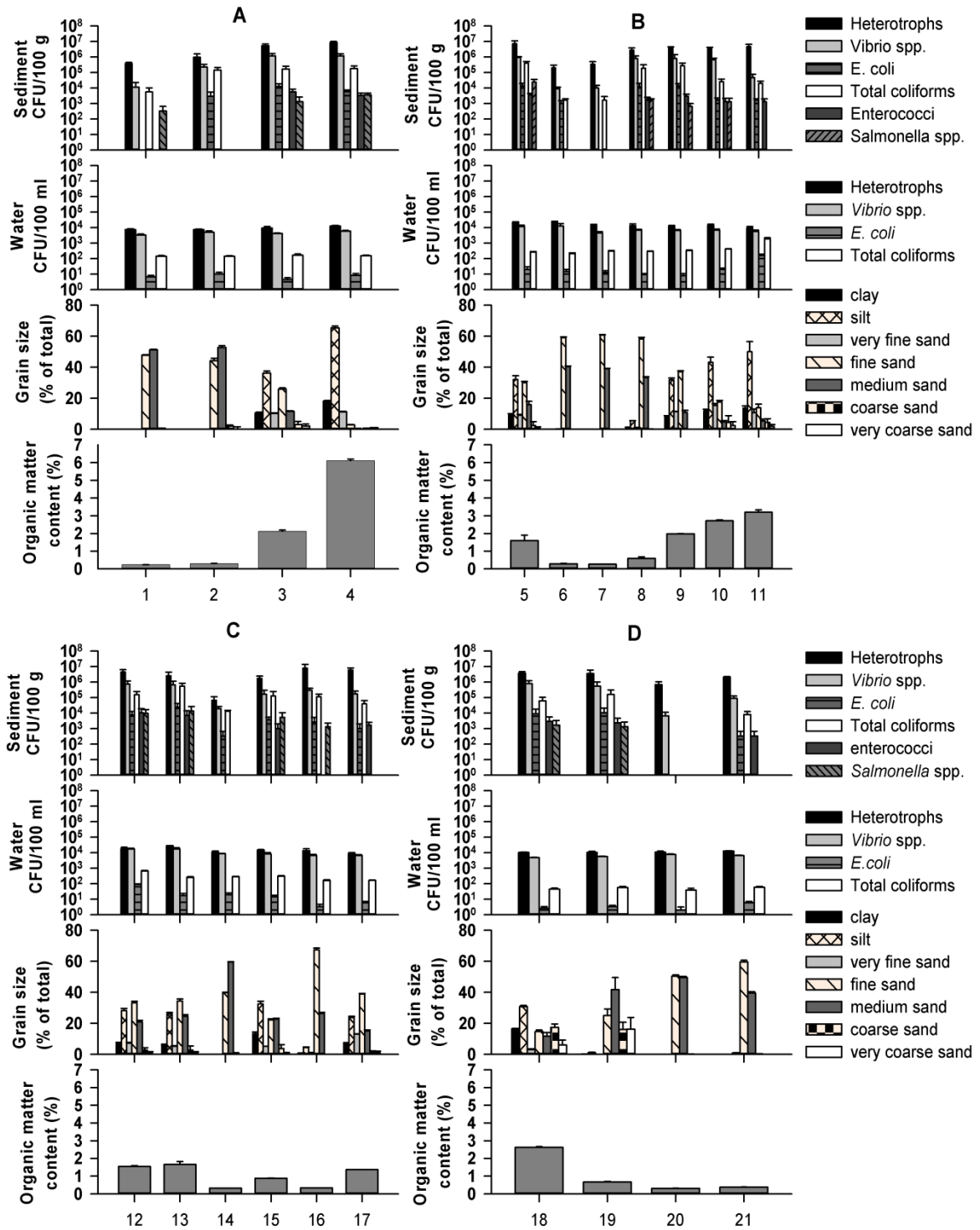


Fig. 3.2 Bacterial abundance in sediments (CFU/100 g wet weight) compared to sediment grain size, organic matter content and bacterial abundance in the water column (CFU/100 ml), across four transverse transects. (A) Transect 1, (B) Transect 2, (C) Transect 3, (D) Transect 4. The X-axis represents sample points ($n = 3$ replicate samples for A, B, C and D except for sediment samples site 13, $n = 2$), mean values are plotted and error bars represent the SEM).

Mean densities of *E. coli* and total coliforms in sediments ranged from 0 to 2.4×10^4 CFU/100 g and 0 to 5.4×10^5 CFU/100 g wet weight, respectively (Table 3.2). *Salmonella* abundance varied from 0 to 1×10^4 CFU/100 g and 0 to 2.5×10^4 CFU/100 g wet weight, respectively. *Vibrio* spp. were detected at all 21 sample sites (6.7×10^3 to 1.2×10^6 CFU/100 g wet weight) and this reflects their status as indigenous members of marine and aquatic environments (Table 3.2). In addition, direct colony counts on *Campylobacter* selective media indicated the presence of 0 to 3.5×10^4 CFU/100 g wet weight. However, 16S rRNA gene sequencing of putative *Campylobacter* spp. isolated from a subsequent sampling survey on the same selective medium revealed that none of the sequenced isolates were *Campylobacter* spp. (Table 3.3). Culturable heterotrophic bacteria were enumerated as a proxy for the abundance of the indigenous estuarine microbial community and their abundance ranged from 6.6×10^4 to 8.7×10^6 CFU/100 g wet weight. The enumeration of culturable heterotrophic bacteria alongside the target PIB groups enabled analysis of the relationship between total culturable heterotrophic bacteria counts and PIB counts in sediments, these data demonstrate that the abundance of heterotrophic bacteria within the sediments showed significant positive correlations with the abundance of all PIB groups measured (Table 3.4).

Table 3.2 Bacteria counts, sediment (CFU/100 g) versus water (CFU/100 ml). Data shown as mean ($n = 3$, sample point 13 $n = 2$ for sediment).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
<i>E. coli</i> CFU/100 g	0	3000	12667	6000	13667	1000	0	12000	13333	1667	1667	7667	24000	333	3667	2667	1000	9000	10667	0	333
<i>E. coli</i> CFU/100 ml	7	10	5	9	20	15	13	9	8	21	159	88	17	19	14	3	6	3	3	2	6
Coliforms CFU/100 g	5667	145667	173667	185667	399333	1667	1667	191000	281667	26000	20667	153667	546000	13000	126000	114667	39333	60333	156000	0	8000
Coliforms CFU/100 ml	147	147	171	157	265	215	311	297	333	423	1865	664	255	281	302	161	163	45	57	39	59
<i>Vibrio</i> spp. CFU/100 g	11667	224167	1208333	1204167	934167	9167	10000	801667	817500	676667	48333	774167	671250	19167	164167	300833	173333	795833	546667	6667	87500
<i>Vibrio</i> spp. CFU/100 ml	3467	5067	4200	6067	12200	12933	4667	7000	6733	7000	5667	17267	17200	8600	8133	6733	6733	4867	5733	7600	6467

Table 3.3 Identification of sequenced isolates.

Presumed	Closest blast search identity match	Querycov/ Max ident %	Sequence length
<i>Campylobacter</i> spp.	<i>Acinetobacter calcoaceticus</i>	99 / 100	1425
<i>Campylobacter</i> spp.	<i>Acinetobacter calcoaceticus</i>	100 / 100	1409
<i>Campylobacter</i> spp.	<i>Acrobacter butzleri</i>	100 / 99	1250
<i>Campylobacter</i> spp.	<i>Ochrobactrum intermedium</i>	99 / 100	1382
<i>Campylobacter</i> spp.	<i>Escherichia coli</i>	100 / 99	1440
<i>Campylobacter</i> spp.	<i>Agrobacterium tumefaciens</i>	100 / 100	1372
<i>Campylobacter</i> spp.	<i>Pseudomonas fluorescens</i>	100 / 99	1416
<i>Escherichia coli</i>	<i>Escherichia coli</i>	100 / 100	1425
<i>Escherichia coli</i>	<i>Escherichia coli</i>	100 / 99	1420
<i>Escherichia coli</i>	<i>Escherichia coli</i>	100 / 99	1430
<i>Escherichia coli</i>	<i>Escherichia coli</i>	100 / 99	1428
<i>Escherichia coli</i>	<i>Escherichia coli</i>	100 / 99	1428
<i>Escherichia coli</i>	<i>Escherichia coli</i>	100 / 99	1428
<i>Escherichia coli</i>	<i>Escherichia coli</i>	100 / 99	1437
<i>Escherichia coli</i>	<i>Escherichia coli</i>	100 / 99	1441
<i>Escherichia coli</i>	<i>Escherichia coli</i>	100 / 99	1438
<i>Escherichia coli</i>	<i>Escherichia coli</i>	100 / 99	1428
<i>Escherichia coli</i>	<i>Acinetobacter</i> spp.	100 / 99	1434
<i>Vibrio</i> spp.	<i>Shewanella</i> spp.	100 / 99	1430
<i>Vibrio</i> spp.	<i>Vibrio splendidus</i>	99 / 99	1448
<i>Vibrio</i> spp.	<i>Vibrio splendidus</i>	100 / 99	1450
<i>Vibrio</i> spp.	<i>Vibrio splendidus</i>	100 / 99	1440
<i>Vibrio</i> spp.	<i>Vibrio splendidus</i>	100 / 99	1451
<i>Vibrio</i> spp.	<i>Shewanella piezotolerans</i>	100 / 99	1439
<i>Vibrio</i> spp.	<i>Vibrio artabrorum</i>	100 / 97	1449
<i>Vibrio</i> spp.	<i>Photobacterium frigidiphilum</i>	100 / 98	1442
<i>Vibrio</i> spp.	<i>Vibrio</i> spp.	100 / 96	1445
<i>Enterococcus</i> spp.	<i>Enterococcus faecalis</i>	100 / 99	1358
<i>Enterococcus</i> spp.	<i>Enterococcus faecalis</i>	100 / 99	1447
<i>Enterococcus</i> spp.	<i>Enterococcus hirae</i>	100 / 99	1335

Sediment grain size composition within sediment samples ranged from clay (0 to 18%), silt (0 to 65%), very fine sand (0 to 15%), fine sand (3 to 67%) medium sand (0 to 59%), coarse sand (0 to 17%) and very coarse sand (0 to 16%) (Fig. 3.2). The organic matter content of the sediment samples varied from 0.3 to 6 % across the sample sites (Fig. 3.2). The abundance of each cultured bacterial group within the sediments showed significant positive correlations with the abundance of all other measured bacterial groups (Table 3.4). *Vibrio* spp. were also more abundant in sediments that had higher densities of FIB.

Table 3.4 Correlation coefficient (r) matrix demonstrating the relationship between the abundance of each cultured bacterial group within estuarine sediments (n = 21).

	<i>E. coli</i>	Total coliforms	<i>Salmonella</i> spp.	Enterococci	Heterotrophs	<i>Vibrio</i> spp.
<i>E. coli</i>	1.000					
Total coliforms	0.945**	1.000				
<i>Salmonella</i> spp.	0.763**	0.759**	1.000			
Enterococci	0.817**	0.780**	0.729**	1.000		
Heterotrophs	0.536*	0.574**	0.476*	0.645**	1.000	
<i>Vibrio</i> spp.	0.847**	0.859**	0.709**	0.817**	0.720**	1.000

** . Correlation is significant at the 0.01 level (2-tailed).

* . Correlation is significant at the 0.05 level (2-tailed).

The abundance of all isolated PIB groups showed a significant positive correlation with both sediment clay content (grain size < 4 µm) (*E. coli*, enterococci, total coliforms, and *Vibrio* spp. $r_s = 0.543$ ($p < 0.011$), $r_s = 0.664$ ($p < 0.001$), $r_s = 0.495$ ($p < 0.023$), $r_s = 0.663$ ($p < 0.001$) respectively) and silt content (grain size 4 µm-63 µm) (*E. coli*, enterococci, total coliforms and *Vibrio* spp., $r_s = 0.570$ ($p < 0.007$), $r_s = 0.687$ ($p < 0.001$), $r_s = 0.547$ ($p < 0.010$), $r_s = 0.688$ ($p < 0.001$) respectively). Significant negative correlations were observed between the abundance of all PIB groups with fine sand (125 µm-250 µm) and medium sand (250 µm-500 µm) (Table 3.5). Sediments with high amounts of clay and silt along with very fine sand contained the greatest proportion of organic material (significant positive correlation; organic matter content and clay, $r_s = 0.917$ ($p < 0.001$), organic matter content and silt, $r_s = 0.926$ ($p < 0.001$), organic matter content and very fine sand, $r_s = 0.810$ ($p < 0.001$). Significant negative correlations were evident between organic matter content and fine sand and also organic matter content and medium sand (Table 3.5). Sediments with high organic matter therefore also showed significant positive correlations with the abundance of all isolated PIB (Table 3.5).

Table 3.5 Correlation coefficient (r_s) matrix demonstrating the relationship between the abundance of each cultured bacterial group within estuarine sediments and sediment grain size and organic matter content ($n = 21$).

	<i>E. coli</i>	Total coliforms	<i>Salmonella</i>	Enterococci	<i>Vibrio</i> spp.	organic matter	Clay	Silt	Very fine sand	Fine sand	Medium sand	Coarse sand	Very coarse sand
<i>E. coli</i>	1.000												
Total coliforms	0.945**	1.000											
Enterococci	0.763**	0.759**	1.000										
<i>Salmonella</i>	0.817**	0.780**	0.729**	1.000									
<i>Vibrio</i> spp.	0.847**	0.859**	0.709**	0.817**	1.000								
Organic matter	0.594**	0.545*	0.497*	0.765**	0.712**	1.000							
Clay	0.543*	0.495*	0.568**	0.664**		0.917**	1.000						
Silt	0.570**	0.547*	0.578**	0.687**	0.663**	0.926**	0.958**	1.000					
Very fine sand	0.432	0.446*	0.368	0.625**	0.688**	0.810**	0.804**	0.828**	1.000				
Fine sand	-0.457*	-0.403	-0.462*	-0.618**	0.605**	-0.504*	-0.834**	-0.795**	-0.840**	-0.539*	1.000		
Medium sand	-0.450*	-0.416	-0.419	-0.650**	0.648**	-	-0.903**	-0.899**	-0.923**	-0.935**	0.668**	1.000	
Coarse sand	0.334	0.223	0.361	0.448*	0.266	0.527*	0.486*	0.517*	0.214	-0.774**	-0.292	1.000	
Very coarse sand	0.443*	0.353	0.398	0.634**	0.504*	0.736**	0.625**	0.696**	0.461*	-0.851**	-0.578**	0.864**	1.000

** . Correlation is significant at the 0.01 level (2-tailed).

* . Correlation is significant at the 0.05 level (2-tailed).

3.3.2 Comparison of PIB abundance between sediment and water samples

The average abundance data for culturable PIB (*E. coli*, total coliforms and *Vibrio* spp.) in sediment and water samples across all 21 sample sites revealed that *E. coli*, coliforms and *Vibrio* spp. were 281, 433 and 58-fold more abundant in the sediment (CFU/100 g) than the water column (CFU/100 ml), respectively (Table 3.6).

Table 3.6 Average abundance of culturable pathogen indicator bacteria in sediment (CFU/100g wet weight) and water (CFU/100 ml) across 21 estuarine sample sites.

	Sediment	Water	Fold - difference
<i>E. coli</i>	5.9x10 ³	2.1x10 ¹	281
Total coliforms	1.3x10 ⁵	3.0x10 ²	433
<i>Vibrio</i> spp.	4.5x10 ⁵	7.8x10 ³	58

3.3.3 The effect of depth, salinity and temperature on PIB abundance

Collection of water samples was initiated at slack water, with a range of water column depths from 0.5 to 12.3 m across all sites (Table 3.7). Salinity and temperature measurements taken from above the bottom sediment varied between 9.3 to 30.0 psu and 10.8 to 11.2°C, respectively. At 0.2 m below the water surface the temperature varied from 10.8 to 11.2°C and salinity ranged from 10.1 to 27.8 psu (Table 3.7).

Table 3.7 Details the depth at which the sediment samples were collected, salinity measurements taken for both water samples and above the sediment samples, calculated as Practical Salinity Units (PSU), temperature taken from the water and above the sediment.

	Depth (m)	Salinity (water) PSU	Salinity (sediment) PSU	Temperature (water) (°C)	Temperature (sediment) (°C)
1	5.0	18.0	25.9	11.2	11.0
2	3.6	17.9	25.8	11.2	11.0
3	5.2	20.1	25.8	11.2	11.0
4	1.2	19.8	23.2	11.2	11.2
5	4.9	23.7	25.1	11.1	11.1
6	4.0	23.7	26.4	11.1	11.0
7	2.1	19.6	26.8	11.2	11.0
8	4.0	21.4	27.7	11.1	11.0
9	4.1	22.5	27.4	11.1	11.0
10	0.5	22.4	23.4	11.1	11.1
11	0.5	10.11	9.3	10.9	11.0
12	4.3	25.3	27.1	10.8	11.0
13	12.3	26.1	27.8	10.8	11.0
14	6.3	23.7	28.0	11.0	11.0
15	4.0	24.2	26.3	11.0	11.0
16	5.0	24.8	26.8	11.0	11.0
17	1.4	23.5	26.2	11.1	11.0
18	10.2	24.3	30.0	11.1	11.0
19	4.4	26.0	29.6	11.0	11.0
20	4.7	27.8	29.7	11.0	11.0
21	4.6	26.3	29.3	11.1	11.0

Bacterial abundance within the sediments revealed no significant correlations with any of the physico-chemical parameters measured (Table 3.8). *Vibrio* spp. enumerated from the water samples were the only cultured species to show significant correlations with depth, temperature or salinity. Temperature had a significant negative correlation with the abundance of *Vibrio* spp. ($r_s = 0.614$ $p < 0.003$), and salinity had a significant positive correlation with their abundance ($r_s = 0.544$ $p < 0.011$) (Table 3.8).

Table 3.8 Correlation coefficient (r_s) matrix demonstrating the relationship between the abundance of each cultured bacterial group within estuarine sediments and physico-chemical parameters measured directly above the bottom sediments ($n = 21$).

	<i>E. coli</i>	Total coliforms	<i>Salmonella</i> spp.	Enterococci	<i>Vibrio</i> spp.
<i>E. coli</i>	1.000				
Total coliforms	0.945**	1.000			
<i>Salmonella</i> spp.	0.763**	0.759**	1.000		
Enterococci	0.817**	0.780**	0.729**	1.000	
<i>Vibrio</i> spp.	0.847**	0.859**	0.709**	0.817**	1.000
Salinity	-0.002	-0.106	-0.042	-0.049	-0.175
Temperature	-0.129	-0.014	0.077	-0.048	0.084
Depth	0.253	0.170	0.288	0.165	0.098

** . Correlation is significant at the 0.01 level (2-tailed).

* . Correlation is significant at the 0.05 level (2-tailed).

3.3.4 Taxonomic identification of bacterial isolates using 16S rRNA gene sequencing

None of the isolates derived from the selective medium for *Campylobacter* ($n = 7$) were positively identified as the target group. However, 91% of *E. coli* ($n = 11$), 100% of enterococci ($n = 3$) and 67% of *Vibrio* ($n = 9$) isolates were positively identified as the target group (Table 3.2). No sequence data were obtained for *Salmonella* isolates and they should therefore be considered as presumptive *Salmonella* spp.

3.4 Discussion

Results from this study demonstrate that sediments represent a significant reservoir for PIB and that sediment characteristics (grain size and organic matter content) influence spatial variations in PIB abundance in estuarine environments. Here, clay and silt fractions comprised a higher organic matter content in comparison to medium and coarse sand and harboured significantly higher densities of PIB. Previous studies have also concluded that the presence of finer sediment particles can have a positive impact on FIB abundance (Garzio-Hadzick *et al.*, 2010; Howell *et al.*, 1996) and that organic material is a contributing factor to the prolonged persistence and survival of FIB in sediments (Haller *et al.*, 2009; Pote *et al.*, 2009; Craig *et al.*, 2004). However, almost all of the previous studies focus specifically on ‘classic’ faecal indicators such as *E. coli*, coliforms and enterococci, and here we have expanded knowledge on comparative PIB-sediment interactions with sediments for a greater repertoire of bacterial groups.

Results revealed there was not only a large spatial variation in PIB abundance that correlated to sediment grain size and organic matter content, but PIB abundance was also found to be significantly higher in the sediments when compared to the overlying water column, with levels of *E. coli* reaching over 3 orders of magnitude higher in the sediment. It is well documented that bacterial association with sediments circumvents the negative effects of environmental stresses, such as providing protection from UV light (Davies-Colley *et al.*, 1999), which in turn can augment the survival and even growth of FIB. For example, DNA fingerprinting analyses performed on *E. coli* populations in beach sand and sediment revealed the possibility that some strains may have become naturalized to this environment (Ishii *et al.*, 2007) and growing evidence also suggests that there may be free-living strains of FIB surviving and multiplying within the water column of environmental waters, independently of a host (Power *et al.*, 2005).

The abundance of *E. coli* and enterococci in sediments had a significant positive correlation with the abundance of other PIB. Consequently, the enumeration of FIB as the ‘classic’ bacterial indicators currently conducted as part of routine water quality monitoring represent suitable indicators for the presence of other PIB groups. *E. coli* and enterococci are the predominant indicator species for monitoring faecal pollution of aquatic environments within the European Union (EU) despite numerous studies highlighting the differential environmental survival of different FIB groups (Haller *et al.*, 2009; Korhonen & Martikainen, 1991), in addition to different strains of the same group (Anderson *et al.*, 2005). In this study, the presence and abundance of enterococci had a stronger significant positive correlation with silt and clay when compared with other FIB, which may suggest greater survival times of enterococci under favourable conditions that certain sediment types may provide. However, it is well established that the survival times of different species, and even strains of the same species, varies considerably in aquatic environments, both between and within species, and this must be taken into consideration. For example, in comparison to other FIB, enterococci survive for longer periods in sediments (Haller *et al.*, 2009) and within estuarine environments (Bordalo *et al.*, 2002), and it has been proposed that the increase in survival under harsh conditions may be due to their membrane composition (Okpookwasili & Akujobi, 1996; Evison, 1989). Such evidence suggests that enterococci may be a more robust indicator of the persistence of faecal contamination rather than more recent contamination within coastal and estuarine environments.

The enumeration of total heterotrophs revealed significant positive correlations with all other cultured PIB groups and therefore the same trends were seen within the indigenous microbial community (Table S6). It should be noted that despite obtaining high colony counts on selective microbiological media for *Campylobacter* spp., 16S rRNA gene sequence analysis of some of these isolates suggested that none were *Campylobacter* spp. These data highlight the potential pitfalls of culture-based analyses of microbial taxa using a selective microbiological medium, and care must be taken when interpreting microbial culture counts. Despite this, the sequenced isolates of enterococci, *Vibrio* spp. and *E. coli* indicate that these media were selective for the desired bacterial target groups (Table 3.3). Little variation in temperature and salinity could be explained by the small geographical variation along each transect. There was no significant correlation between temperature and salinity and the abundance of PIB, with the exception of *Vibrio* spp. enumerated from the water column. Here, *Vibrio* spp. had a significant positive correlation with salinity and a significant negative correlation with temperature, supporting previous proposals that temperature and salinity have important implications for

Vibrio spp. population dynamics (Singleton *et al.*, 1982). The depth at which sediment samples were taken had no impact on the abundance of PIB enumerated from the sediments.

This study highlights the risk of periodic elevated FIB and other PIB in the water column due to the resuspension of microbial contaminated sediments. Furthermore, the risk of microbial pollution from the resuspension of sediments is not taken into consideration when assessing microbial pollution of recreational and shellfish harvesting waters. The time and place of sampling in addition to tidal and hydrodynamic conditions may impact upon FIB concentrations in the water column. Despite several reports on the importance of sediments as a reservoir for FIB (Davies *et al.*, 1995; Marino & Gannon, 1991), the enumeration of waterborne FIB has received much more attention. Quilliam *et al.*, (2011) revealed significant spatial variations in waterborne *E. coli* numbers on contrasting sides of the same four transverse transects of the Conwy estuary studied here, indicating that significantly different levels of microbial pollution were present in the east and west sides of the river, and the localised re-suspension of sediment associated FIB into the water column was one proposed explanation for such contrasting FIB counts in the water column (Quilliam *et al.*, 2011). Despite this, the dynamics of sediment transport and re-suspension in relation to PIB concentrations is poorly understood. The attachment of PIB to particulate matter in the water column provides a platform for transportation and downward flux to the bed sediment, cyclical changes in tidal flow and the salinity of the water will also impact the downward flow of particle-associated PIB to the bed sediments. Conversely, under certain hydrodynamic conditions (e.g. tidal cycles and storm flow events), bed stress and turbulence can also impart the re-suspension of sediments and subsequent transportation and deposition of particle associated FIB to other areas of the estuary. Due to the hydrodynamics of an estuarine system, fine sediments are usually deposited around the banks of the basin (Malham *et al.*, 2014) and our data support this trend, with finer particles detected in greater abundance in sediments on the east and west sides of the estuary and coarser sand deposited in the central channel. FIB levels in water can be affected by other factors such as temperature (Faust *et al.*, 1975), exposure to sunlight and salinity (Bordalo *et al.*, 2002), nutrient concentrations (Hong *et al.*, 2010), predation by protozoa (Barcina *et al.*, 1997) and competition (Korhonen & Martikainen, 1991). However, here we demonstrate that sediments also contribute to the distribution of FIB and other PIB in an estuarine environment.

Experimental results confirm that estuarine sediments harbour PIB, which may potentiate their prolonged persistence and survival in this environment. This study also identifies areas of high microbial contamination within the Conwy estuary, which highlights the risk of sediment and PIB resuspension back into the water column under turbulent hydrodynamic

conditions that result in sediment bed stress and promote the erosion of sediments. To our knowledge, this is the first comprehensive study of the co-occurrence of *E. coli*, coliforms, enterococci, *Salmonella* and *Vibrio* spp. (PIB) in relation to sediment composition. These data show that all PIB groups studied are strongly correlated with the presence of clay, silt and organic matter content in sediments. In addition, the presence of *E. coli* and total coliforms strongly correlates with the abundance of the other PIB tested, both allochthonous and autochthonous, suggesting that culture-based determinations of *E. coli* and coliform abundance in sediments represent a useful surrogate for the presence of other PIB groups.

Conclusion

Faecal contamination in aquatic environments is currently assessed by measuring culturable *E. coli* and enterococci counts in water samples only. Here, we demonstrate that sediment composition, specifically clay, silt and organic matter content, are linked with greater PIB abundance in sediments. The enhanced abundance of viable PIB in sediments therefore has implications for water quality and public health when considering the potential for resuspension of bed sediments, particularly finer particles such as clay and silt that we have demonstrated to contain higher concentrations of PIB. It may therefore be necessary to incorporate PIB loadings in bottom sediments into routine monitoring protocols and hydrodynamic models to adequately assess their risk to human health. The detection of spatial variations of PIB within sediments also highlights the necessity for further research on the interactions of pathogens with sediments and their role in the survival, persistence and transportation of PIB within environmental waters.

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CHAPTER 4

**Detection and quantification of FIB and human pathogenic bacteria in estuarine sediments
using culture-based, PCR and qPCR methods**

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Abstract

Estuarine environments deliver valuable ecosystem services such as food and recreation. The presence of microbial pathogens derived from diffuse and point pollutant sources in

estuarine sediments represents a threat to water quality and public health, yet the role of sediments as a reservoir for pathogenic bacteria has received little attention. To address this, sediments were collected from a longitudinal transect of the Conwy estuary, UK, to determine the abundance of culturable Faecal Indicator Bacteria (FIB) (*E. coli*, total coliforms, enterococci and *Salmonella* spp.) and *Vibrio* spp. In addition, PCR and qPCR were used to detect and quantify 14 species-specific and/or virulence genes of known microbial pathogens in estuarine sediment samples. Sediment composition (grain size and organic matter content) were determined to ascertain any relationship with gene presence and abundance and the abundance of cultured bacteria. Results revealed that estuarine sediments are a reservoir for FIB, *Vibrio* spp. and human pathogenic bacteria. Spatial variation of FIB, *Vibrio* spp. and pathogen abundance in estuarine sediments is influenced by sediment type (grain size) and subsequent organic matter content. In general, significant positive correlations were determined between the presence of silt, clay and very fine sand and the abundance of cultured bacteria in addition to the abundance of species specific virulence genes. The use of qPCR to enumerate FIB and other potential pathogens suggested greater proportions of FIB than culture-based methods. In particular, detection of *E. coli* (*uidA* gene) via qPCR suggested that *E. coli* were 3 orders of magnitude more abundant than culture counts, indicating the possible presence of viable but non-culturable (VBNC) FIB in estuarine sediments. In addition, the detection of *stx* virulence genes in the absence of other virulence factors suggests the presence of free bacteriophage that have the potential to transmit virulence factors to non-pathogenic bacteria. Furthermore, this study highlights the various limitations using a single method has on determining FIB abundance and that of human pathogens in estuarine sediments. The resuspension of human pathogen contaminated sediments in an estuarine environment can pose a risk to human health particularly in a productive estuary that impacts on both bathing waters and shellfish harvesting waters. This study highlights the need for further investigations to understand the interactions of human pathogens in an estuarine environment to minimize any human health risk.

KEY WORDS: Estuary, sediment, faecal indicator bacteria, public health, microbial water quality.

4.1 Introduction

Estuarine environments are extremely valuable coastal systems and provide several ecosystem services that include recreation, areas for tourism, employment and food resources (Wetz & Yoskowitz, 2013; Costanza *et al.*, 1998). However, the proportion of human

populations residing in coastal areas is increasing, with approximately one in every three people now living no less than 100 km from the ocean (GESAMP, 2001). In addition, growing tourism adds to the pressures on the coastal zone (Davenport & Davenport, 2006). Consequently, an increase in anthropogenic activities can have a negative impact on the health and productivity of estuarine and coastal systems (Lotze *et al.*, 2006).

Human pathogenic microorganisms excreted in the faeces of animals and humans can reach environmental waters via several point and diffuse sources, including sewage discharge, agricultural runoff, and malfunctioning septic tanks (Hong *et al.*, 2010; Topp *et al.*, 2009a). In addition, recent climate observations indicate that in certain areas throughout the world, storm and flood events have increased in frequency (Wetz & Yoskowitz, 2013), thus increasing the flow of microbial pollutants from land to sea. In some cases, the inundation of wastewater arising from storm flow events result in the need for treatment plants to discharge untreated sewage via combined sewer overflows (CSOs) (Rose *et al.*, 2001). Consequently, a reduction in water quality in estuarine and coastal zones can have significant socioeconomic consequences, particularly for areas where tourism and the harvesting of shellfish provide a major source of income (Malham *et al.*, 2014; Geary & Davies, 2003).

It is estimated that globally, each year, bathing in coastal waters contaminated with wastewater is the cause of over 170 million cases of disease in humans (Shuval, 2003). Commercially grown shellfish such as mussels and oysters are often found in the shallow waters of estuaries where nutrient levels are high (Lees, 2000). However, these near shore waters can be frequently exposed to sewage contamination. Consequently, these filter-feeding organisms can accumulate high concentrations of faecal bacteria and viruses, thus posing a risk to human health if eaten raw or undercooked (Malham *et al.*, 2014; Lees, 2000). It is estimated that approximately 40 thousand deaths a year can be attributed to the consumption of contaminated shellfish/molluscs that are harvested from sewage contaminated waters, in addition to ca. 40 thousand instances of long-term disability and 4 million cases of hepatitis A and E (Shuval, 2003).

It is impractical and not economically viable for regulatory authorities to monitor the often low levels of diverse pathogens that can be found in environmental waters (Wade *et al.*, 2010). Consequently, enumeration of indicator organisms that are present in faecal matter in much higher quantities and represent a proxy for pathogen content has become the paradigm for water quality determinations (Wade *et al.*, 2010). Whilst testing for the presence of coliform bacteria is an accepted method for the assessment of microbial water quality, *E. coli* is considered to be a more precise indicator of faecal pollution (WHO, 2006). EU legislation requires the use of traditional culture-based methods to determine concentrations of *E. coli* and enterococci in

recreational waters to assess the microbial water quality. However, the proficiency of these methods may be under question as it is evident that indicator species such as *E. coli* can enter a viable but non-culturable (VBNC) state, meaning that their lack of detection by microbiological laboratory media can result in a false negative outcome (Oliver, 2005). In addition, naturalized free living strains of *E. coli* that are able to propagate outside of a host have been detected in the environment. Subsequently, their veracity as an effective indicator for anthropogenic pollution has been challenged (Power *et al.*, 2005). Several other potentially pathogenic bacterial groups have been isolated from the environment and are not routinely monitored for water quality purposes; these include *Campylobacter* (Alonso & Alonso, 1993), *Salmonella* (Berthe *et al.*, 2008) and *Vibrio* spp. (Gutierrez West *et al.*, 2013).

Campylobacter infection arises predominantly from the consumption of contaminated meat and is considered one of the most common causes of acute diarrhoea, globally (Levin, 2007), with *C. coli* and *C. jejuni* most commonly associated with foodborne infections (Humphrey *et al.*, 2007). Numerous studies have isolated *Campylobacter* from secondary environments such as rivers (Bolton *et al.*, 1987) and coastal waters (Alonso & Alonso, 1993). *Salmonella* have also been isolated from natural environments, including estuarine water and sediments (Sayler *et al.*, 1976). The primary reservoir for *Salmonella* spp. are the intestines of animals, insects and humans, but intensive farming practices have seen the colonisation of *Salmonella* and *Campylobacter* spp. particularly in poultry (Humphrey *et al.*, 2007; Humphrey 2006; Bryan & Doyle, 1995). Consequently, faecal pollution from livestock can enter environmental waters from agricultural runoff (Topp *et al.*, 2009). Furthermore, *Salmonella* and *Campylobacter* have been isolated from shellfish harvested for human consumption posing a risk to human health (Bakr *et al.*, 2011; Wilson & Moore, 1996). Indigenous members of marine and estuarine environments such as *Vibrio* spp. have also been implicated in gastrointestinal illness in humans that can often lead to death if left untreated (Dziejman *et al.*, 2002). *V. cholera* has been responsible for several pandemics throughout the 19th and 20th Centuries, resulting in high mortality rates (Dziejman *et al.*, 2002). Most cases of infection arise from the consumption of contaminated sea foods potentially arising from *V. cholera*, *V. parahaemolyticus* and *V. vulnificus* (Cabral, 2010).

The survival and distribution of Faecal Indicator Bacteria (FIB) and microbial pathogens in the environment is determined by several environmental factors such as available nutrients (Pommepeuy *et al.*, 1992), temperature and salinity (Singleton *et al.*, 1982), seasonal variation (Faust *et al.*, 1975) and competition (Marino & Gannon, 1991). Much attention has been given to the survival and persistence of FIB in environmental waters (Bonjoch *et al.*, 2011; Bordalo *et*

al., 2002; Alkan *et al.*, 1995) whilst investigations on the interactions between FIB and sediments have arguably been less comprehensively studied until relatively recently (Perkins *et al.*, 2014; Thevenon *et al.*, 2012). The attachment of FIB to suspended particles in the water column can promote their deposition, leading to an accumulation of FIB in bottom sediments (Droppo *et al.*, 2009). Numerous studies have shown that when compared to the water column, FIB abundance is much greater in the underlying sediments (Stephenson & Rychert, 1982), with sediment-associated concentrations of FIB as much as 433-fold greater than the overlying water (Perkins *et al.*, 2014). Sediments are not only a reservoir for FIB, but they can also provide a nutrient rich environment in which FIB can proliferate (Haller *et al.*, 2009; Pote *et al.*, 2009). In addition, sediment type can impact spatial variation in the abundance of FIB and *Vibrio* spp., with clay and silt sediment fractions harbouring higher concentrations of FIB than coarser, sandy sediments (Perkins *et al.*, 2014). Consequently, disturbance of benthic sediments due to storm events, natural turbulence and recreation can result in the resuspension of sediments, potentially releasing high concentrations of FIB in to the overlying water column. Despite this, sediments are not considered when assessing the microbial quality of environmental water. Furthermore, there is a paucity of information on the potential risk linked with sediment-associated FIB. This is due to a lack of quantitative data regarding the presence and abundance of actual bacterial pathogens associated with the high levels of FIB detected in sediments, and their potential impact on water quality and public health following their resuspension and transport through the coastal zone.

The Conwy catchment, North Wales, UK, has a human population of ~112,000, with over 75% residing in coastal areas. Consequently, potential point and diffuse sources of microbial pollution in the Conwy estuary include waste effluents released by waste-water treatment plants (WWTPs), discharges from septic tank systems and run-off from large areas of land used for agriculture (Perkins *et al.*, 2014). In addition, the mudflats of the Conwy estuary are also utilised by wildfowl, including migratory birds. Water quality in the Conwy estuary has a direct impact on commercial shellfish beds towards the mouth of the estuary, in addition to bathing water and beaches (Quilliam *et al.*, 2011) that represent popular recreational areas for local residents and over 5 million tourists every year (Thorn *et al.*, 2011). A reduction in water quality could therefore have significant socioeconomic implications, affecting tourism, blue flag beach status and shellfish bed classification.

Our previous investigation of culturable FIB and *Vibrio* spp. abundance in sediments of the Conwy estuary revealed that spatial variation in FIB abundance could be attributed to sediment composition (Perkins *et al.*, 2014). In addition, there was significant positive

correlations between the abundance of all bacterial groups cultured (*E. coli*, total coliforms, enterococci, *Salmonella* and *Vibrio* spp.), demonstrating that enumeration of culturable *E. coli* and enterococci in estuarine sediments represent a useful surrogate for the presence of other potentially pathogenic bacterial groups (Perkins *et al.*, 2014). However, the use of traditional microbiological culture-based techniques does not distinguish between harmless ‘indicator’ bacteria and potentially pathogenic types that reside in estuarine systems. Pathogenic microorganisms possess genes encoding virulence factors (Hacker *et al.*, 1997) that can be detected by molecular methods such as PCR and quantitative PCR (qPCR) to detect and quantify bacterial pathogens in environmental matrices (Oster *et al.*, 2014) .

In this chapter, the paucity of information on the proportions of pathogenic bacteria in estuarine sediments, their relationship with the presence of FIB, and their spatial distribution in relation to sediment composition are investigated. Here the presence and abundance of pathogenic *E. coli*, *Enterococcus*, *Campylobacter* and *Salmonella* spp. in the sediments of the Conwy estuary were determined by the detection of virulence genes via PCR and qPCR and compared with the abundance of culturable *E. coli*, total coliforms, *enterococci*, *Vibrio* and *Salmonella* spp. from the same sediment sample. Spatial variation of both culturable FIB abundance and the frequency of specific virulence genes in estuarine sediments was also determined in relation to sediment grain size and organic matter content. These data enabled comparisons between FIB and bacterial pathogen abundance in estuarine sediments derived from both culture and molecular-based approaches.

4.2 Materials and methods

4.2.1 Study Site

Sediment samples were collected in triplicate from 11 sites along a longitudinal transect of the Conwy estuary, North Wales, UK. Sampling locations were designed to incorporate a contrasting range of sediment types within the estuary and representative areas of sediment

associated with faecal contamination that we previously identified (Perkins *et al.*, 2014). Sediment samples were collected at slack water on 08/05/2013 (Fig. 4.1).

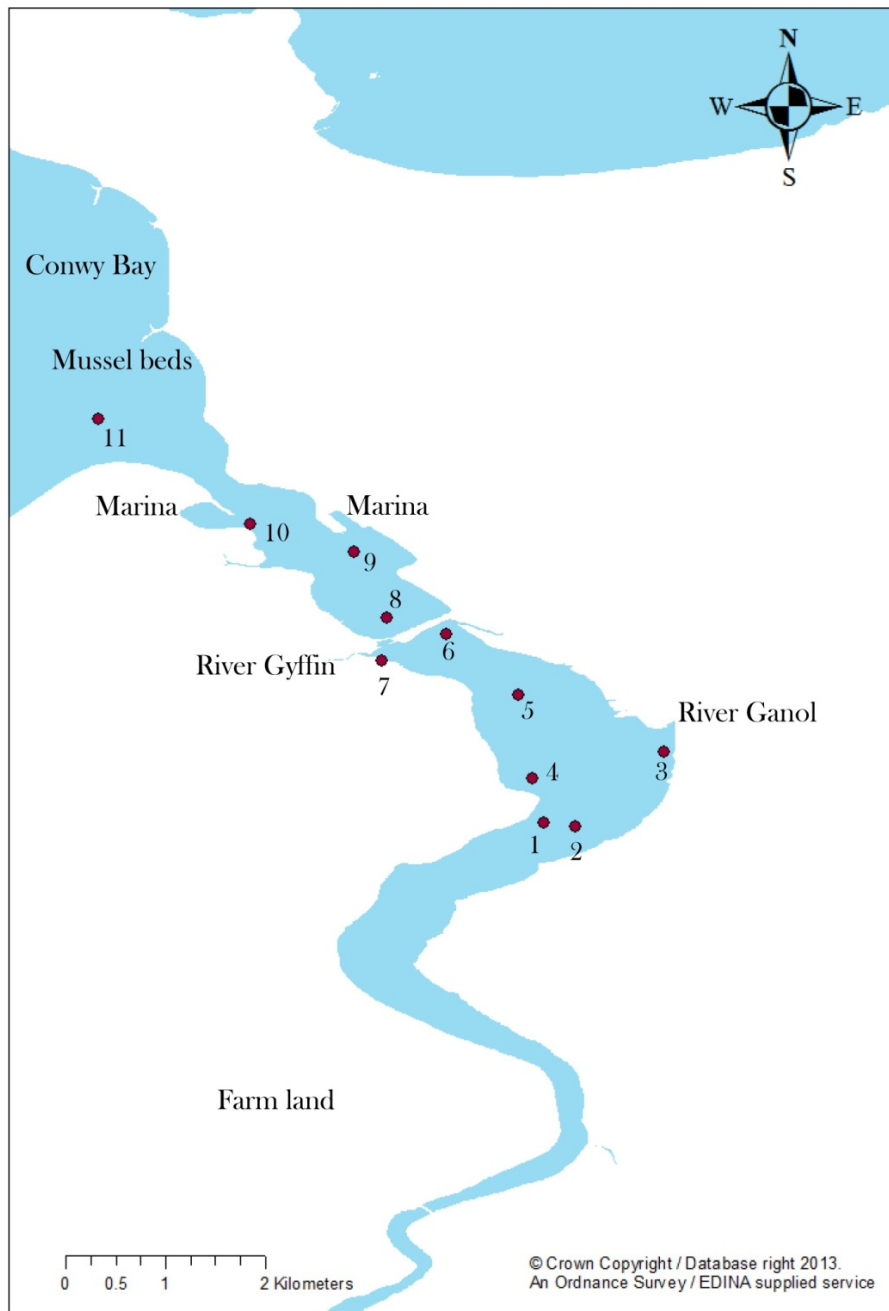


Fig. 4.1 A map of the study site; the Conwy Estuary, North Wales, UK. Sediment samples were collected from 11 sites.

4.2.2 Estuarine sampling

Estuarine sediments were collected in triplicate at each site, using a 1.5 l Van Veen sediment grab, manually operated from the side of the Bangor University 7.9 m catamaran research boat. Approximately 50 g of sediment was transferred from the centre of the grab in to

a sterile 50 ml polypropylene tube (VWR International Ltd., Leicestershire, UK). Sediment samples were transported to the laboratory and immediately processed, for bacterial culture counts (within 6 hours of collection). Samples were also divided into three aliquots and stored at 4°C for sediment grain size and organic content analysis, and at -80°C for subsequent molecular analysis.

4.2.3 Isolation and enumeration of target bacterial groups from sediments

Determination of *E. coli*, total coliforms, enterococci, *Salmonella* and *Vibrio* spp. concentrations in sediment was based on the procedure described in (Perkins *et al.*, 2014). Briefly, 1 g of sediment sample was transferred to a sterile 7 ml bijoux tube (Starlab UK Ltd., Milton Keynes, UK), a 1:5 (w/v) dilution was obtained by adding 5 ml of Ringer's solution (Oxoid Ltd., Basingstoke, UK). Each sample was processed using a vortex mixer for 90 s and 500 µl of the sediment supernatant was directly transferred on to agar plates containing selective medium for *E. coli*, total coliforms, enterococci and *Salmonella* spp. under aseptic conditions. Due to the typically high counts of *Vibrio* spp. previously found in these sediments (Perkins *et al.*, 2014), only 200 µl of sediment suspension was transferred on to *Vibrio* spp. selective medium. Agar plates were inverted and incubated according to the manufacturer's recommendations (Table 2.1). Subsequent colony forming units (CFU) were counted to obtain viable bacterial cell counts.

4.2.4 Sediment particle grain size analysis

Sediment grain sizes were determined by laser diffraction using a Malvern Hydro 2000 MU particle size analyser and the Mastersizer 2000 software. Approximately 1 g of each sediment sample was added to the analyser, followed by 1 min sonication to separate particles. Three independent sediment grain size determinations (technical replicates) were produced for each of the three replicate sediment samples from each site (biological replicates) and an overall average was calculated.

4.2.5 Determining sediment organic matter content

The organic matter content of each sample was determined by the loss on ignition method (LOI). A pre weighed crucible containing approximately 20 g of fresh sediment was dried at 95°C for 24 h then re-weighed. The percentage difference between wet weight and dry weight per g of sediment could then be calculated. Approximately 4 g of the resulting dried

sediment was transferred into another crucible, weighed and placed into a muffle furnace for 4 h at 550°C and then re-weighed. Organic matter content was calculated as the difference between the weight before and after combustion. One independent calculation was made for each of the three replicate sediment samples from each site and an overall average was calculated.

4.2.6 DNA extraction of positive control pure cultures using the Isolate II Genomic DNA Kit (Bioline)

Pure cultures of *Enterococcus faecalis*, *Enterococcus faecium* and *Salmonella* spp. were obtained for use as positive control DNA in PCR reactions. DNA was extracted from pure cell cultures using the Isolate II Genomic DNA Kit (Bioline), modified by the elution of DNA into 50 µl of nuclease-free water instead of 100 µl as described in the protocol.

4.2.7 DNA quantification

DNA extracts from cell cultures were quantified using a Nanodrop 1000 spectrophotometer. DNA yields were extremely low from both *E. faecalis* and *E. faecium*, so another method was adopted (Griffiths *et al.*, 2000). *Salmonella* DNA was diluted using nuclease-free water to give a final concentration of 20 ng/µl and stored at -20°C until further use.

4.2.8 DNA extraction from cell cultures (Griffiths *et al.*, 2000).

Using a sterile loop, approximately 3 cultured bacterial colonies were aseptically transferred from agar plates into a pre sterilised 2 ml graduated skirted tube (Starlab UK Ltd, Milton Keynes, UK) containing 0.5 g acid washed sterile glass beads (Sigma-Aldrich Company Ltd, Dorset, UK) and 500 µl of 5% CTAB buffer/phosphate buffer (120 mM pH 8.0) [equal volumes of 10% CTAB in 0.7 M NaCl and 240 mM potassium phosphate buffer pH 8.0] to resuspend colonies. 500 µl of phenol/chloroform/isoamylalcohol (25:24:1) (Sigma) was added. For cell disruption, tubes were placed in a Thermo Electron Corporation FastPrep® FP120 Savant Homogenizer, for 30 s at a setting of 5.5 m/s. Tubes were cooled on ice then centrifuged at 4°C for 5 min at 14,000 rpm. The top aqueous layer was transferred to a new sterile 1.5 ml tube and an equal volume of chloroform/isoamylalcohol (24:1) was added then mixed to form an emulsion. Tubes were again centrifuged at 4°C for 5 min at 14,000 rpm and the aqueous phase was transferred to a new sterile 1.5 ml tube. To eliminate RNA an RNase treatment step

was performed. RNase A (Sigma) was added at a final concentration of 100mg/ml and incubated for 30 min at 37°C. Each sample was made up to 500 µl with sterile distilled water where necessary. Equal volumes of chloroform/isoamylalcohol (24:1) were added, mixed well to form an emulsion and centrifuged at 4°C for 5 min at 14,000 rpm. The top aqueous phase was transferred to a new sterile 1.5 ml tube, 2 volumes of 30% PEG 6000/1.6 M NaCl solution was added, mixed by shaking and stored at 4°C overnight to precipitate DNA. DNA was then pelleted by centrifugation at 4°C for 10 min at 15,000 rpm, the supernatant was removed and the pellet washed with 200 µl of ice cold 70% ethanol. Pellets were air dried and resuspended in 30 µl of nuclease-free water. DNA concentration was determined using a Nanodrop 1000 spectrophotometer, samples were diluted where possible to give a final volume of 20 ng/µl and stored at -20°C until further use.

Genomic DNA of *E. coli* strain EDL933 was obtained to use as a positive control from the University of Liverpool, UK.

4.2.9 DNA extraction from estuarine sediment using the PowerLyzer™ PowerSoil® DNA Isolation Kit (MOBIO Laboratories, Inc. Carlsbad, CA)

DNA was extracted from 0.25 g of each sediment sample from all 11 sites using a PowerLyzer™ PowerSoil® DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, CA) with the PowerLyzer® 24 bench top bead-based homogenizer (MOBIO Laboratories, Inc., Carlsbad, CA). The procedure was modified by eluting DNA into 30 µl of nuclease-free water. DNA concentration was determined using a Qubit® 2.0 Fluorometer and purity assessed using a Nanodrop 1000 spectrophotometer, DNA was then stored at -20°C until further use.

4.2.10 End point PCR using MyTaq™ HS DNA polymerase (Bioline)

One single and three multiplex PCR assays were used to analyse DNA extracts from estuarine sediments for the presence of bacterial virulence and species-specific genes. Primer pairs were described in previously published studies (Table 4.1). The primers utilised in this study are non-degenerate (Table 4.1). Therefore, there is no flexibility in the sequence which could result in missed targets. To overcome this limitation and reduce the risk of missing the required target, degenerate primers that have flexibility in targeting the desired gene could be

used. However, it would be extremely costly and time consuming to design non-degenerate primers for the amount of gene targets required in this study. Subsequently, the chosen primers have been used and specificity confirmed in previous studies (Table 4.1).

Table 4.1 Primers used in this study.

Gene	function	specificity	Primer name	Oligonucleotide sequence (5' to 3')	Optimized annealing temp.	Published annealing Temp.	(bp)	Reference
<i>Enterococcus</i> spp. multiplex								
<i>asaI</i>	Aggregation substance	<i>Enterococcus faecalis</i>	ASA11 ASA12	GCACGCTATTACGAACTATGA TAAGAAAAGAACATCACCACGA	56°C	56°C	375	Vankerckhoven <i>et al.</i> , 2004, Ahmed <i>et al.</i> , 2012
<i>gelE</i>	Gelatinase	<i>Enterococcus faecalis</i>	GEL11 GEL12	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	56°C	56°C	213	Vankerckhoven <i>et al.</i> , 2004, Ahmed <i>et al.</i> , 2012
<i>cylA</i>	Cytolysin	<i>Enterococcus faecalis</i>	CYTI CYTIIb	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	56°C	56°C	688	Vankerckhoven <i>et al.</i> , 2004, Ahmed <i>et al.</i> , 2012
<i>esp</i>	Enerococcal surface protein	<i>Enterococcus faecalis</i> & <i>Enterococcus faecium</i>	ESP14F ESP12R	AGATTTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	56°C	56°C	510	Vankerckhoven <i>et al.</i> , 2004, Ahmed <i>et al.</i> , 2012
<i>hyl</i>	Hyaluronidase	<i>Enterococcus faecium</i>	HYLn1 HYLn2	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	56°C	56°C	276	Vankerckhoven <i>et al.</i> , 2004, Ahmed <i>et al.</i> , 2012
<i>Campylobacter</i> spp. multiplex								
<i>Cj-cdtB</i>	Cytolethal distending toxin	<i>Campylobacter jejuni</i>	Cj- CdtBU5 Cj- CdtBR6	ATCTTTTAACCTTGCTTTTGC GCAAGCATTAAAATCGCAGC	56°C	56°C	714	Asakura <i>et al.</i> , 2008, Shiramaru <i>et al.</i> , 2012
<i>Cc-cdtB</i>	Cytolethal distending toxin	<i>Campylobacter coli</i>	Cc- CdtBU5 Cc- CdtBR5	TTTAATGTATTATTTGCCGC TCATTGCCTATGCGTATG	56°C	56°C	413	Asakura <i>et al.</i> , 2008, Shiramaru <i>et al.</i> , 2012
<i>cadF</i>	outer membrane protein associated with adhesion	<i>Campylobacter jejuni</i> & <i>Campylobacter coli</i>	cadF cadR	TTGAAGGTAATTTAGATATG CTAATACCTAAAGTTGAAAC	56°C	52°C 49°C	400	Nayak <i>et al.</i> , 2005 Amri <i>et al.</i> , 2007. (Added to multiplex described n Shiramaru <i>et al.</i> , 2012).

Table 4.1 Primers used in this study.

<i>E. coli</i> multiplex								
<i>stx₁</i>	Shiga toxin	<i>Escherichia coli</i>	stx1F stx1R	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	60°C	65°C decreasing to 60°C	180	Paton & Paton, 1998
<i>stx_{2b}</i>	Shiga toxin	<i>Escherichia coli</i>	stx2F stx2R	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	60°C	65°C decreasing to 60°C	255	Paton & Paton, 1998
<i>eae</i>	intimin	<i>Escherichia coli</i>	eaeAF eaeAR	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	60°C	65°C decreasing to 60 °c	384	Paton & Paton ,1998
<i>hlyA</i>	enterohemolysin	<i>Escherichia coli</i>	hlyAF hlyAR	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	60°C	65 °c decreasing to 60°C	534	Paton & Paton, 1998
<i>uspA</i>	Universal stress protein A	<i>Escherichia coli</i>	Ec1 Ec2	CCGATACGCTGCCAATCAGT ACGCAGACCGTAGGCCAGAT	60°C	72°C	884	Chen & Griffiths, 1998. (Added to multiplex described in Paton & Paton, 1998)
<i>Salmonella</i> spp.								
<i>invA</i>	Invasion gene	<i>Salmonella</i> spp	invAF invAR	ACAGTGCTCGTTTACGACCTGAAT AGACGACTGGTACTGATCGATAAT	60°C	60°C	244	Hur <i>et al.</i> , 2011 Bhatta <i>et al.</i> ,2007

All PCR reactions were performed on a Techne TC-5000 PCR Thermal Cycler (Scientific Laboratory Supplies Ltd., Nottingham, UK). Assays contained 5 µl of 5x MyTaq Reaction Buffer (5mM dNTPs, 15mM MgCl₂ stabilizers and enhancers), 10 pmol (each) of forward and reverse primer, 0.25 µl MyTaq™ HS RED DNA polymerase (Bioline Reagents Ltd., London, UK) and 20 ng of DNA template and nuclease-free water to a total volume of 25 µl. Positive (target DNA) and negative (nuclease-free water) controls were performed for each PCR except for *C. jejuni* and *C. coli* as no control DNA was available. Cycling conditions were as follows: 95°C 1 min initial denaturation, followed by 35 cycles of 95°C for 15 s, 15 s at a specific annealing temperature (Table 4.1) and 72°C for 90 s, followed by a final hold at 4°C. (For singleplex PCR extension time was altered from 90 s to 10 s). PCR products were separated by gel electrophoresis on 2% agarose.

Visualisation of PCR amplicons were extremely difficult, as amplicons were faint and a positive result was difficult to determine. Therefore, 1 µl of amplicon was used in a second identical reaction to re-amplify any products (described as a nested PCR). PCR cycling conditions were performed as previous. Re-amplified PCR products were visualised on a 2% agarose gel. Although previous tests had been carried out to verify specificity of the primer pairs used in this study a selection of the PCR products of expected size bands were excised from the gel and purified using QIAquick Gel Extraction Kit (Qiagen, Manchester, UK) following the manufacturer's instructions, and sent for nucleotide sequencing (Macrogen, Amsterdam, The Netherlands). Amplicon sequences were checked against the NCBI BLASTn programme (<http://www.ncbi.nlm.nih.gov/>). Results validated the specificity of primer pairs for *stx₁*, *stx_{2b}*, *esp*, *gelE* and *asaI* in environmental samples.

4.2.11 qPCR analysis of bacterial virulence gene copies and species-specific gene copies in estuarine sediments

A TaqMan-based qPCR approach was taken to quantify species-specific and virulence gene copy numbers for *E. coli* (*stx₁*, *stx_{2b}*, *eae* and *tir₀₁₅₇*), *E. faecalis* (*groES*), *E. faecium* (*groES*), *C. jejuni* (*cadF*), and *Salmonella* (*invA*). TaqMan primers and probes were designed, verified and supplied by Primerdesign Ltd, Southampton, UK, (Table 4.2). Assays were performed according to the manufacturer's guidelines.

Table 4.2 Details of the qPCR kits (Pimerdesign, Southampton, UK) used for the detection and simultaneous quantification of numerous bacteria.

Bacteria	gene	function	kit	Catalogue reference
<i>E. faecalis</i>	<i>groES</i>	Heat shock protein (enterococcal cytoplasmic protein)	Genesig® standard kit	E.faecalis-EASY
<i>E. faecium</i>	<i>groES</i>	Heat shock protein (enterococcal cytoplasmic protein)	Genesig® standard kit	E.faecalis-EASY
<i>E. coli</i>	<i>uidA</i>	Glucuronidase	Genesig® standard kit	Path-E.coli generic-EASY
<i>E. coli</i>	<i>stx₁</i>	Shiga toxin	Genesig® standard kit	Path-E.coli_stx1-EASY
<i>E. coli</i>	<i>stx_{2b}</i>	Shiga toxin	Genesig® standard kit	E.coli_stx2b-EASY
<i>E. coli</i>	<i>eae</i>	Intimin	Genesig® standard kit	EPEC-EASY
<i>E. coli</i> O157	<i>tir</i>	Translocated intimin receptor	Genesig® standard kit	Path-E.coli_0157-EASY
<i>C. jejuni</i>	<i>cadF</i>	Outer membrane protein associated with adhesion	Genesig® standard kit	C.jejuni-EASY
<i>Salmonella</i> spp.	<i>invA</i>	Invasion gene	Genesig® standard kit	S.enterica-EASY

Each reaction mix consisted of 5 µl 2x qPCR MasterMix (PrimerDesign, Southampton, UK), 0.5 µl primer/probe mix, between 1 and 12.5 ng of DNA template, 0.05 µl of ROX was added as the passive reference and nuclease-free water in a total volume of 10 µl. Cycling conditions were as follows: Enzyme activation 95°C 15 mins, 40 cycles of denaturation at 95°C for 10 s and annealing/extension/data collection at 60°C for 60 s. Enzyme activation was changed to 10 min for detection of the *uidA* gene.

A 5 point standard curve comprising a ten-fold serial dilution range from 2 to 200,000 gene copies, was included for each qPCR assay. All samples and negative controls were performed in triplicate. All standards were performed in either duplicate or triplicate. The number of gene copies in each reaction was ascertained from the standard curve based on the Cycle threshold (Ct) for each standard. A separate set of calibrator reactions were produced for each assay and so the level of detection may differ. The number of target genes per g of sediment

were determined by the following equation: total DNA (ng) extracted (from 0.25 g sediment (wet weight)) / ng of DNA per reaction x average gene copy number x 4.

4.2.12 Statistical Analysis

Due to the data not having a normal distribution, basic correlations were determined by using the Statistical Package for Social Sciences SPSS v22, (IBM Corp., Armonk, NY) and the non-parametric Spearman Rank Correlation Coefficient (r_s). In addition, the Mann-Whitney U test was performed to establish significant differences.

4.3 Results

4.3.1 Mean densities of culturable pathogen indicator bacteria and specific gene copy number from estuarine sediments across all 11 sample sites.

Enumeration of culturable sediment-associated bacterial groups and the quantification of species specific and virulence genes revealed marked spatial differences across all 11 sites. *Salmonella* were not detected at any site using culture-based techniques. *E. coli* were cultured from estuarine sediments taken from sites 6, 7 and 9, with mean densities based on 3 independent replicates ranging from 0 to 5.0×10^1 CFU/g wet weight. Total coliforms were cultured from 7 out of the 11 sites and mean densities ranged from 0 to 2.5×10^2 CFU/g wet weight. Enterococci were cultured from sites 1, 3, 6, 7, 9 and 11, and their abundance ranged from 0 to 4.8×10^2 CFU/g wet weight. In comparison, the indigenous *Vibrio* spp. were detected at all sites and ranged from 2.5×10^1 x 1.8×10^4 CFU/g wet weight. Sites 6 and 7 produced the highest densities of culturable *E. coli*, total coliforms and enterococci. In addition, site 7 also produced the highest densities of culturable *Vibrio* spp. The *E. coli* species-specific *uidA* gene was detected by qPCR at all sites; mean densities determined by qPCR and based on 9 assays (3 replicate assays for each of three biological replicates) ranged from 1.4×10^3 to 2.4×10^4 gene copies/g wet weight. The *E. faecalis groES* gene and the *E. faecium groES* gene were detected at sites 3, 6, 7 and 9. In addition *E. faecalis* was detected at site 1. Mean densities of the *E. faecalis groES* and the *E. faecium groES* gene ranged from 0 to 1.5×10^4 gene copies/g and 0 to 2.7×10^4 gene copies/g respectively. The *C. jejuni cadF* gene was only detected at site 6 at a density of 1.8×10^2 gene copies/g, whilst the *invA* gene of *Samonella* spp. was detected at sites 2, 4, 5, 8 and 11 with densities ranging from 0 to 2.8×10^3 gene copies/g (Fig. 4.2).

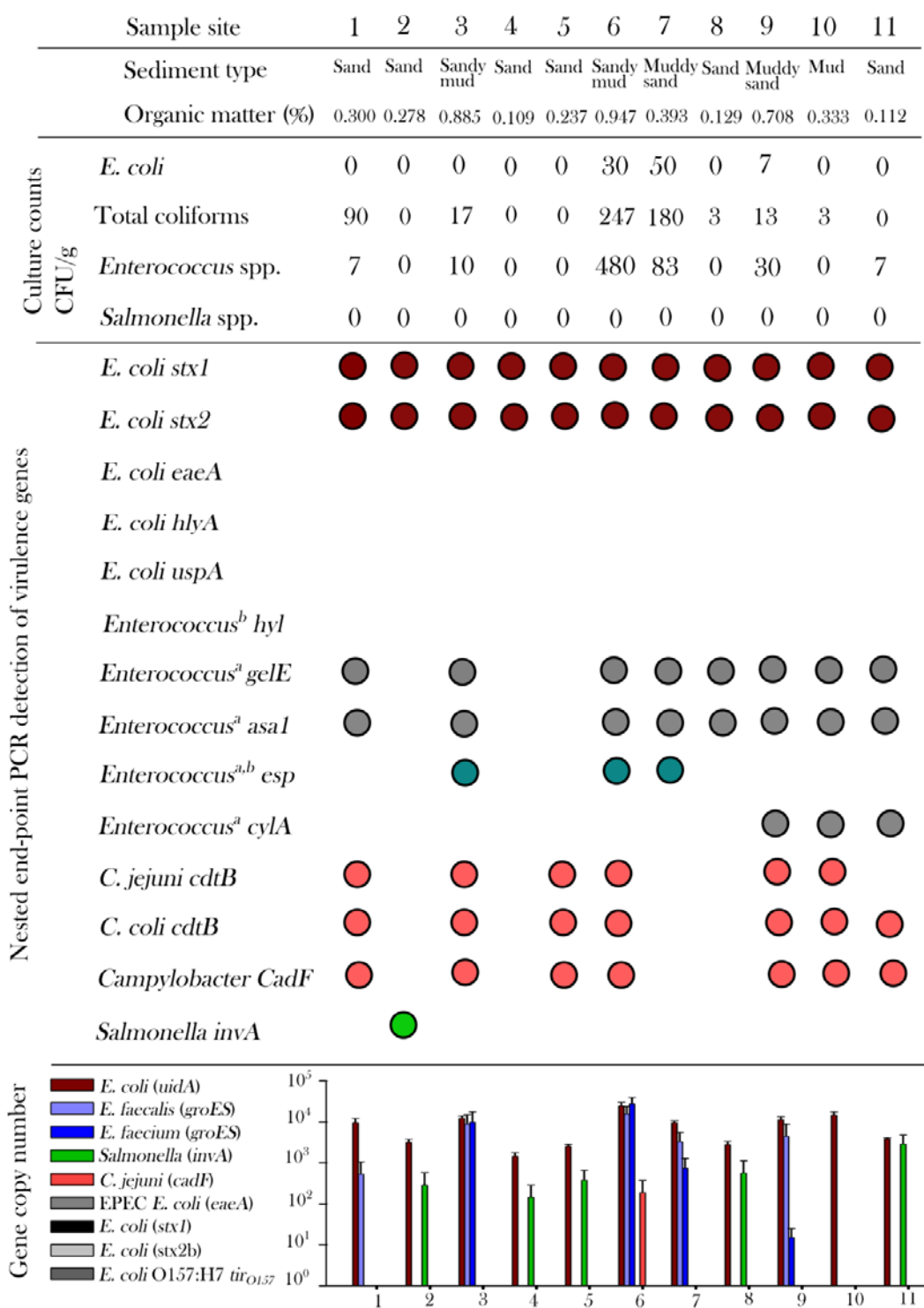


Fig. 4.2 Bacterial abundance (CFU/g wet weight) in sediments compared to sediment grain size, organic matter content, the presence (PCR) and quantification (qPCR) of species-specific and/or virulence genes in sediments.

4.3.2 Comparison of cultured bacterial abundance data, specific gene detection and quantification data using PCR and qPCR

Sites 6, 7 and 9 produced culturable *E. coli*, abundances ranging from 7 CFU/g to 5.0×10^1 CFU/g. However, PCR produced positive results for the detection of the *E. coli*-associated virulence genes *stx₁* and *stx_{2b}* and all sites sampled. In contrast, PCR did not detect any of the other *E. coli* virulence genes targeted (*eae*, *hlyA* and *uspA*). The use of qPCR determined concentrations of *E. coli* by detection of the species specific *uidA* gene at all sites ranging from 1.4×10^3 to 2.4×10^4 gene copies/g but did not detect any of the species specific virulence genes tested (*eae*, and *tir_{O157}*) (Fig. 4.2). Enterococci were cultured from sediments from 6 out of the 11 sites (sites 1, 3, 6, 7, 9 and 11), their abundance ranged from 7 CFU/g to 4.8×10^2 CFU/g. PCR amplification revealed *E. faecalis* virulence genes *gelE* and *asaI* at sites 3, 6, 7, 8, 9 and 10. In addition, sites 3, 6 and 7 were positive for the *esp* gene and samples 9, 10 and 11 were positive for the *cytA* gene via end-point PCR. The qPCR results showed concentrations of enterococci at sites 1, 3, 6, 7 and 9 ranging from 6.8×10^1 to 6.1×10^4 gene copies/g. With the exception of site 10, detection of the *groES* gene by qPCR corresponded with sites that produced culturable enterococci and positive PCR results. Due to previously identified problems with the specificity of *Campylobacter* spp. selective medium (data not shown) no attempt was made to culture *Campylobacter* spp. However PCR amplification revealed PCR products for the *C. jejuni* specific *cdtB* gene at sites 1, 3, 5, 6, 9 and 10. Amplification of the *C. coli* *cdtB* gene and the *Campylobacter* spp. specific *cadF* gene using multiplex PCR generated similar size products, therefore a positive result is described as detection of *Campylobacter* spp., with detection at all sites excluding sites 2, 4 and 8. In contrast, qPCR only detected and quantified the *cadF* gene at site 6, thus it can be assumed that all the other sites positive for either the *cc-cdtB* or the *cadF* gene are positive for the *cc-cdtB* gene. Culturable *Salmonella* spp. were not detected from sediments at any of the 11 sites sampled. However, PCR amplification revealed the presence of the *Salmonella* specific *invA* gene at site 2, whilst qPCR detected *invA* at sites 2, 4, 5, 8 and 11, with concentrations ranging from 1.2×10^3 to 1.3×10^4 gene copies/g. Culturable total coliforms were enumerated from 7 out of the 11 sites. *Vibrio* spp. were cultured from all sites (Fig. 4.2).

4.3.3 Spatial relationship between culturable FIB and *Vibrio* spp. abundance, sediment particle size and organic matter content of estuarine sediments

Sediment grain size composition ranged from clay (0 to 15%), silt (0 to 71%), very fine sand (0 to 11%), fine sand (2 to 40%), medium sand (0.5 to 75%), coarse sand (0 to 12%) and very coarse sand (0 to 0.6%) (Fig. 4.3). The organic matter content of sediment samples ranged from 0.1 to 0.9% across all 11 sites sampled (Fig. 4.4). Significant positive correlations were revealed between the abundance of all cultured bacterial groups and sediments that consisted of higher proportions of very fine sand (grain size 63 μm - 125 μm) (*E. coli*, total coliforms, enterococci and *Vibrio* spp. $r_s = 0.436$ ($p = 0.011$), $r_s = 0.560$ ($p < 0.001$), $r_s = 0.698$ ($p < 0.001$), $r_s = 0.792$ ($p < 0.0001$) respectively) (Table 4.3). With the exception of *E. coli*, significant positive correlations were also revealed between the abundance of cultured groups and sediments that comprise higher proportions of silt (total coliforms, enterococci and *Vibrio* spp. $r_s = 0.426$ ($p < 0.013$), $r_s = 0.627$ ($p < 0.001$), $r_s = 0.698$ ($p < 0.001$)) (Table 4.3). In addition, significant negative correlations were observed between culturable enterococci and *Vibrio* spp. abundance and the coarser sediment fractions of fine sand $r_s = -0.379$ ($p < 0.030$), $r_s = -0.558$ ($p < 0.001$), medium sand $r_s = -0.588$ ($p < 0.0001$), $r_s = -0.616$ ($p < 0.001$) and coarse sand $r_s = -0.447$ ($p < 0.009$), $r_s = -0.544$ ($p < 0.001$), respectively. Furthermore sediments that comprise higher clay, silt and very fine sand were shown to have a significant positive correlation with organic matter content (Table 4.3). Results also showed significant positive correlations between each cultured bacterial group (Table 4.3).

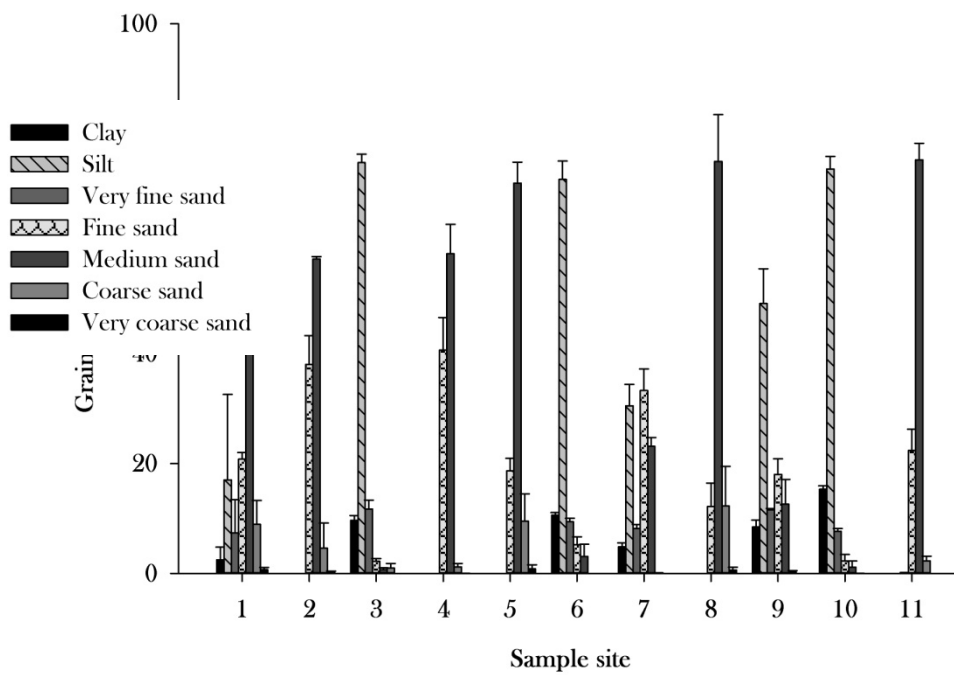


Fig. 4.3 Grain size composition of sediment samples. Three independent

sediment grain size determinations (technical replicates) were produced for each of the three replicate sediment samples from each site (biological replicate) and an overall average was calculated. Error bars represent the SEM.

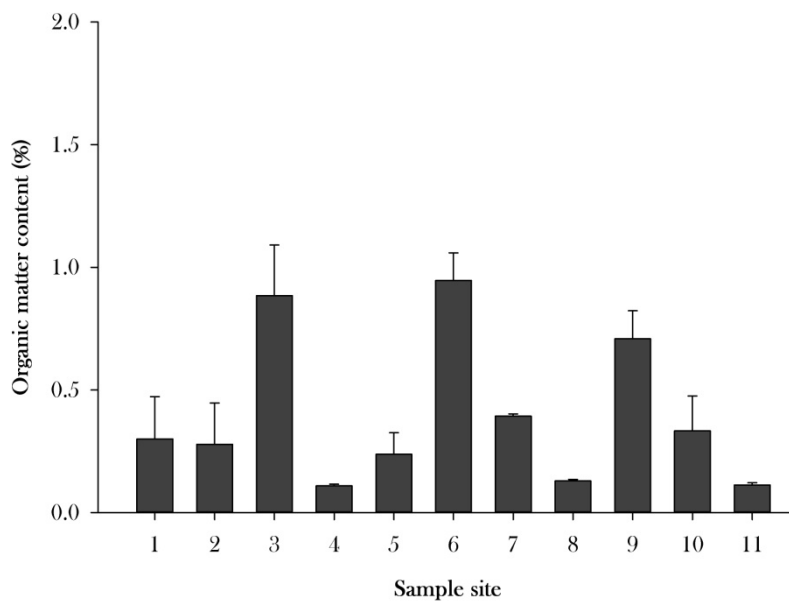


Fig.4.4 Percentage organic matter content of sediment samples. Error bars represent SEM (n = 3).

Table 4.3 Correlation coefficient (r_s) matrix demonstrating the relationship between the abundance of each cultured bacterial group within estuarine sediments and sediment grain size and organic matter content ($n = 33$).

	<i>E. coli</i>	Enterococci	<i>Vibrio</i> spp.	Total coliforms	Organic matter content	Clay	Silt	Very fine sand	Fine sand	Medium sand	Coarse sand	Very coarse sand
<i>E. coli</i>	1.000											
Enterococci	0.673**	1.000										
<i>Vibrio</i> spp.	0.423*	0.685**	1.000									
Total coliforms	0.658**	0.677**	0.481**	1.000								
Organic matter content	0.234	0.631**	0.611**	0.301	1.000							
Clay	0.220	0.594**	0.757**	0.342	0.759**	1.000						
Silt	0.226	0.627**	0.698**	0.426*	0.789**	0.956**	1.000					
Very fine sand	0.436*	0.698**	0.792**	0.560**	0.695**	0.753**	0.776**	1.000				
Fine sand	0.029	-0.379*	-0.558**	-0.208	-0.644**	-0.811**	-0.812**	-0.474**	1.000			
Medium sand	-0.199	-0.588**	-0.616**	-0.339	-0.708**	-0.895**	-0.898**	-0.748**	0.596**	1.000		
Coarse sand	-0.344	-0.447**	-0.544**	-0.237	-0.185	-0.474**	-0.360*	-0.383*	0.215	0.462**	1.000	
Very coarse sand	-0.177	-0.308	-0.385*	-0.103	-0.376*	-0.329	-0.270	-0.306	0.127	0.416*	0.620**	1.000

** . Correlation is significant at the 0.01 level (2-tailed).

* . Correlation is significant at the 0.05 level (2-tailed).

4.3.4 Spatial relationship between species specific and virulence gene abundance via qPCR, sediment particle size and organic matter in estuarine sediments

The abundance of the *E. coli* (*uidA*), *E. faecalis* and *E. faecium* (*groES*) genes determined by qPCR revealed significant positive correlations with sediments that consisted of higher proportions of clay (grain size <4 µm) $r_s = 0.653$ ($p < 0.001$), $r_s = 0.404$ ($p = 0.020$) $r_s = 0.500$ ($p = 0.003$) respectively, silt (4 µm-63 µm) $r_s = 0.638$ ($p < 0.001$) $r_s = 0.453$ ($p < 0.008$) $r_s = 0.541$ ($p < 0.001$), respectively, and very fine sand (63 µm-125 µm) $r_s = 0.578$ ($p < 0.001$) $r_s = 0.493$ ($p = 0.004$) $r_s = 0.495$ ($p < 0.003$), respectively (Table 4.4). In addition, significant negative correlations were revealed between the abundance of the *E. coli* (*uidA*), *E. faecalis* and *E. faecium* (*groES*) genes and sediments that consisted of the coarser fractions of medium sand (Table 4.4). Furthermore, the abundance of the *Salmonella* spp. *invA* virulence gene had a significant positive correlation with medium sand (250 µm-500 µm) $r_s = 0.402$ ($p < 0.021$) and a significant negative correlation with the finer fractions of clay $r_s = -0.444$ ($p = 0.10$), silt $r_s = 0.465$ ($p < 0.006$) and very fine sand $r_s = -0.464$ ($p < 0.006$) (Table 4.4).

Table 4.4 Correlation coefficient (r) matrix demonstrating the relationship between the abundance of each species-specific and virulence genes (using qPCR) and sediment grain size and organic matter content.

	Organic matter	Clay	Silt	Very fine sand	Fine sand	Medium sand	Coarse sand	Very coarse sand	<i>E. coli (uidA)</i>	<i>E. faecalis (groES)</i>	<i>E. faecium (groES)</i>	<i>Salmonella (invA)</i>	<i>C. jejuni (cadF)</i>
Organic matter	1.000												
Clay	0.759**	1.000											
Silt	0.789**	0.956**	1.000										
Very fine sand	0.695**	0.753**	0.776**	1.000									
Fine sand	-0.644**	-0.811**	-0.812**	-0.474**	1.000								
Medium sand	-0.708**	-0.895**	-0.898**	-0.748**	0.596**	1.000							
Coarse sand	-0.185	-0.474**	-0.360	-0.383	0.215	0.462**	1.000						
Very coarse sand	-0.376	-0.329	-0.270	-0.306	0.127	0.416	0.620**	1.000					
<i>E. coli (uidA)</i>	0.437	0.653**	0.638**	0.578**	-0.473**	-0.456**	-0.387	-0.112	1.000				
<i>E. faecalis (groES)</i>	0.486**	0.404	0.453**	0.493**	-0.282	-0.444**	-0.282	-0.197	0.241	1.000			
<i>E. faecium (groES)</i>	0.524**	0.500**	0.541**	0.495**	-0.352	-0.485**	-0.369	-0.235	0.438	0.680**	1.000		
<i>Salmonella (invA)</i>	-0.290	-0.444**	-0.465**	-0.464**	0.315	0.402	0.229	-0.002	-0.428	-0.219	-0.262	1.000	
<i>C. jejuni (cadF)</i>	0.186	0.217	0.156	0.095	-0.149	-0.111	-0.144	-0.074	0.260	-0.083	0.370	-0.083	1.000

** . Correlation is significant at the 0.01 level (2-tailed).

* . Correlation is significant at the 0.05 level (2-tailed).

4.3.5 Spatial relationship between the presence of virulence genes via PCR, sediment particle size and organic matter in estuarine sediments

Significant positive correlations were observed between the presence of the *E. faecalis gelE* and *asaI* genes, the *Campylobacter cdtB* and *cadF* gene and sediments that contained higher proportions of clay $r_s = 0.581$ ($p < 0.001$) $r_s = 0.581$ ($p < 0.001$) $r_s = 0.652$ ($p < 0.001$) $r_s = 0.581$ ($p < 0.001$), silt $r_s = 0.608$ ($p < 0.001$) $r_s = 0.608$ ($p < 0.001$) $r_s = 0.685$ ($p < 0.001$) $r_s = 0.608$ ($p < 0.001$), and very fine sand $r_s = 0.527$ ($p < 0.001$) $r_s = 0.527$ ($p < 0.001$) $r_s = 0.481$ ($p = 0.005$) $r_s = 0.527$ ($p = 0.002$), respectively (Table 4.5). In addition, significant negative correlations were observed between the presence of the same 4 genes and sediments that consisted of higher proportions of fine sand (Table 4.5).

Table 4.5 Correlation coefficient (r.) matrix demonstrating the relationship between the presence of specific virulence genes (using PCR) and sediment grain size and organic matter content.

	<i>gelE</i>	<i>asaI</i>	<i>esp</i>	<i>cylA</i>	<i>Cj-cdtB</i>	<i>cadF/Cc-cdtB</i>	<i>invA</i>	Organic matter	Clay	Silt	Very fine sand	Fine sand	Medium sand	Coarse sand	Very coarse sand
<i>gelE</i>	1.000														
<i>asaI</i>	1.000	1.000													
<i>esp</i>	-0.542	-0.542	1.000												
<i>cylA</i>	0.375	0.375	-0.375	1.000											
<i>Cj-cdtB</i>	0.261	0.261	0.149	0.149	1.000										
<i>cadF/Cc-cdtB</i>	0.542	0.542	-0.083	0.375	0.671	1.000									
<i>invA</i>	-0.516	-0.516	0.516	-0.194	-0.346	-0.516	1.000								
Organic matter	0.422	0.422	0.121	0.014	0.639	0.529	-0.199	1.000							
clay	0.581	0.581	-0.137	0.300	0.652	0.581	-0.300	0.759	1.000						
Silt	0.608	0.608	-0.165	0.165	0.685	0.608	-0.314	0.789	0.956	1.000					
Very fine sand	0.527	0.527	-0.275	0.143	0.481	0.527	-0.272	0.695	0.753	0.776	1.000				
Fine sand	-0.686	-0.686	0.186	-0.407	-0.633	-0.622	0.432	-0.644	-0.811	-0.812	-0.474	1.000			
Medium sand	-0.322	-0.322	0.043	-0.079	-0.537	-0.350	0.078	-0.708	-0.895	-0.898	-0.748	0.596	1.000		
Coarse sand	-0.246	-0.246	0.056	-0.365	0.021	-0.175	-0.012	-0.185	-0.474	-0.360	-0.383	0.215	0.462	1.000	
Very coarse sand	0.057	0.057	-0.057	-0.046	0.036	0.080	-0.133	-0.376	-0.329	-0.270	-0.306	0.127	0.416	0.620	1.000

4.3.6 The Relationship between culture-based, PCR and qPCR methods

Significant positive correlations were determined between culture based, PCR and qPCR methods for the enumeration and detection of enterococci from estuarine sediments. A significant positive correlation was observed between the abundance of culturable *E. coli* and *uidA* gene copy number determined by qPCR.

4.4 Discussion

Results from this study show that there were marked spatial differences in the number of culturable FIB and *Vibrio* spp. in estuarine sediments, in addition to marked spatial differences in the abundance and detection frequency of FIB virulence and species-specific genes in estuarine sediments. The abundance of culturable bacteria, FIB and *Vibrio* spp. in estuarine sediments is influenced by sediment composition. In addition, for the most part, the presence and abundance of *E. coli*, enterococci and *C. jejuni* as determined by PCR and qPCR was also influenced by sediment composition, showing positive detection and higher densities in sediments consisting of higher proportions of clay, silt and very fine sand. Furthermore, the finer fractions of clay silt and very fine sand contain higher densities of culturable FIB, *Vibrio* spp. and *E. coli* species specific gene copies and enterococci virulence gene copies as determined by qPCR. In contrast, higher densities of *Salmonella* (as determined by qPCR), were consistent with the coarser fractions of estuarine sediment. The abundance of *Campylobacter* as determined by qPCR showed no correlation with sediment type. Sediments with higher proportions of clay, silt and very fine sand have consistently higher proportions of organic matter, which some studies have attributed to greater survival of FIB in sediments when compared to the water column (Pote *et al.*, 2009).

Culture based methods revealed low concentrations of *E. coli* in estuarine sediment at 3 out of the 11 sites sampled, but in contrast, the use of qPCR revealed that the *E. coli* species specific *uidA* gene was present at all 11 sites sampled, with quantities reaching up to 3 orders of magnitude higher than culture counts. When exposed to adverse environmental conditions such as saline water (Darcan *et al.*, 2009) and changes in temperature (Patrone *et al.*, 2013) numerous bacteria including those in the genera *Escherichia* (Iijima *et al.*, 1998), *Enterococcus* (Leo *et al.*, 2005), *Vibrio* (Xu *et al.*, 1983), *Campylobacter* (Patrone *et al.*, 2013) and *Salmonella* (Roszak *et al.*, 1984) have demonstrated the ability to enter into a VBNC state. This is a dormant state thought to be a survival strategy induced by environmental stresses (Iijima *et al.*, 1998). The

adaptive response to unfavourable conditions allows the cells to conserve their viability but cannot be cultured using routine bacteriological media (Oliver, 2005). It is also known that cells in a VBNC state can retain their virulence and can be resuscitated when conditions become more favourable (Oliver, 2005). The significant large increase in the abundance of the *E. coli* species specific gene by qPCR methods when compared to the abundance of cultured *E. coli*, suggests the possibility of VBNC *E. coli* cells present in estuarine sediments. A previous study determined the abundance of *E. coli* in coastal marine sediments using culture-based and molecular methods, results revealed enumeration of *E. coli* via molecular methods produced significantly greater quantities of up to 4 logs higher compared to culture-based methods (Luna *et al.*, 2010). However, it should be noted that exogenous DNA or DNA derived from dead *E. coli* cells within the sediments may also have contributed to the increased *E. coli* signal detected via qPCR. Quantitative PCR also consistently yielded greater values for the enumeration of enterococci and *Salmonella* spp. when compared to culture based methods, further supporting the idea that potentially pathogenic *Enterococcus* and *Salmonella* spp. may be abundant in a VBNC state in estuarine sediments.

To address the paucity of information on the presence and abundance of virulent human pathogenic bacteria in estuarine sediments, a qPCR approach was also undertaken for the detection and quantification of *E. coli* specific virulence genes (*stx₁*, *stx_{2b}*, *eae*, *hlyA*, *uspA* and *tir₀₁₅₇*) in estuarine sediments. However, no gene copies of these target genes were detected using qPCR in the sediment samples studied here. One possible explanation for this is that genetically adapted enteric bacteria may have eliminated non-functioning virulence genes (Ochman *et al.*, 2000). Consequently, the absence of virulence genes may indicate the presence of naturalized strains of *E. coli* in estuarine sediments. In contrast, a nested PCR revealed the presence of *E. coli* virulence genes *stx₁* and *stx_{2b}* at all sites but showed negative results for all other targeted *E. coli* virulence genes. These data are concordant with several other studies that have detected the presence of *stx* genes via PCR methods whilst the use of culture-based methods produced negative results (Meng *et al.*, 2014; Fremaux *et al.*, 2006). Bacteriophages carrying *stx* genes have been detected free of a bacterial host in various environments such as waste water (Muniesa & Jofre, 1998) and river water (Dumke *et al.*, 2006) and may explain the detection of *stx* genes in the absence of culturable *E. coli* here. Bacteriophages are often involved in the mobility and transfer of virulence factors between bacteria (Martinez-Castillo & Muniesa, 2014). In particular, bacteriophage that carry *stx* genes are able to transform non-pathogenic enteric bacteria such as *E. coli*, in to Shiga toxin-producing *E. coli* (STEC) (Martinez-Castillo *et al.*, 2013). Furthermore, the persistence of bacteriophages in the environment is determined by their high resistance to

disinfection processes (Allue-Guardia *et al.*, 2014) and their resilience to natural inactivation such as sunlight (Duran *et al.*, 2002). Consequently, the detection of *stx* genes in the absence of other virulence factors appears to indicate the presence of free bacteriophage in estuarine sediments, and would be an interesting focus of further research. The persistence of free infectious bacteriophage in estuarine sediments could result in the conveyance of *stx* genes throughout the environment, converting non-pathogenic bacteria into pathogenic bacteria that are able to produce shiga toxins. Furthermore, Stx bacteriophages in the environment could be implicated in the rise of new pathogenic bacterial strains (Martinez-Castillo & Muniesa, 2014).

The most frequent detection of FIB from a combination of all 3 methods was at sites 6 and 9, positioned on the east side of the estuary. Sediment particle analysis and subsequent sample identification using the software programme GRADISTAT (Blott & Pye, 2001), described site 6 as sandy mud and site 9 as muddy sand. These data corroborate previous studies that confirmed up to five times more *E. coli* in the water on the east side of the estuary compared with the west (Quilliam *et al.*, 2011) and higher concentrations of pathogen indicator bacteria (PIB), (*E. coli*, total coliforms, enterococci, *Salmonella* and *Vibrio* spp.) in sediments that have a higher proportion of clay, silt and fine sand and organic matter content (Perkins *et al.*, 2014). Sites 6, 7 and 9 yielded the highest cultured counts for *E. coli* and enterococci. Site 9 is positioned in close proximity to a marina which could be a potential source of pollution. Site 6 produced the highest cultured counts for total coliforms and also yielded the greatest quantities of all virulence genes detected, with the exception of the *invA* gene which was found in greater quantities at site 11. Site 6 is immediately opposite the outlet of the river Ganol, which receives discharge from septic tanks and wastewater from numerous waste water treatment plants, which could be a source of microbial contaminants. Site 7 is positioned opposite the outlet of the river Gyffin which runs through areas of high agricultural activity and may also be a source of faecal pollution. Sediment composition at site 11 was described as sand; the site lies at the mouth of the estuary amongst commercially important shellfish beds and in close proximity to bathing waters. Culture-based and qPCR methods did not detect *E. coli* or enterococci at this site, however qPCR revealed concentrations of the *Salmonella invA* gene at concentrations of 2892 gene copies/g. Resuspension of *Salmonella* contaminated sediments may potentially pollute the water column. The absence of *E. coli* and enterococci may result in *Salmonella* concentrations remaining undetected if current sampling strategies were used to monitor microbial pollution, representing a human health risk. Polo *et al.*, (1998) also reported the presence of *Salmonella* in beach water when the densities of indicator organisms were low, suggesting that the use of indicator organisms may not identify relatively high densities of *Salmonella* in seawater and

therefore may be a potential health hazard for bathers. Sediments at site 2, 4 and 5 are described as sand, these sites showed the least contamination. However, *Salmonella* were detected at all three of these sites. This study revealed a significant positive correlation between sand and the abundance of the *invA* gene found in *Salmonella* spp., which suggests the ecology of *Salmonella* spp. in estuarine sediment may differ from that of *E. coli* and enterococci. Consequently, the enumeration of *E. coli* and enterococci spp. as an indicator of *Salmonella* presence may result in a significant underestimation. These results are concurrent with a previous study that isolated *Salmonella* from molluscs collected from category A shellfish beds (category A shellfish beds produce molluscs that are considered suitable for direct human consumption, based on the level of indicator organisms such as *E. coli*, present in the mollusc flesh), concluding that the use of indicator organisms does not guarantee the absence of other bacterial pathogens. It was suggested that tests for other pathogens such as *Salmonella* and *Campylobacter* should possibly be considered (Wilson & Moore, 1996). In addition, Alonso & Alonso, (1993) tested eight marine recreational waters for the abundance of *Campylobacter* and found no relationship with the abundance of faecal indicators.

A previous study identified that estuarine sediments are a reservoir for FIB and *Vibrio* spp. (Perkins *et al.*, 2014). Here, we identify that estuarine sediments act as a reservoir for natural and pathogenic FIB, *Vibrio* spp. and other human pathogenic bacteria. The spatial variation of pathogen abundance in estuarine sediments, for the most part can be attributed to sediment composition. Furthermore the ecology of FIB in estuarine sediments may not reflect the ecology of pathogenic species. Hydrodynamic processes such as storm events, recreation, the harvesting of shellfish and natural turbulence may result in the resuspension of pathogen contaminated sediments, resulting in microbial contamination of the water column thus posing a risk to human health.

To our knowledge there is no published literature on the comparative analysis between culture based, PCR and qPCR methods for the detection and quantification of FIB and their associated pathogens in estuarine sediments. This study reveals there are ambiguities associated with each method used in this investigation. Quantification of specific genes by qPCR consistently suggested higher densities of FIB than culture-based methods, but these data should be treated with caution as the use of DNA does not differentiate between live, dead or VBNC cells. Culture-based methods used to determine microbial water quality cannot distinguish between pathogenic and non-pathogenic bacteria. In addition, the disassociation of bacteria from sediments may have resulted in underestimations of CFUs. PCR did confirm the presence of bacterial pathogenicity genes in estuarine sediments, but lacked the ability to quantify their presence. Although some

significant positive correlations did exist between these methods here, a previous study that compared the relationship between qPCR and culture-based methods to determine enterococci abundance from three beaches over various temporal scales, also found a positive correlation between culture based and qPCR methods, but over the course of the sampling campaign (one day) correlations between these methods weakened (Converse *et al.*, 2012).

These results highlight the limitations of using a single method to determine FIB and their associated pathogens in estuarine sediments. Furthermore, the use of a combination of methods may yield better results in order to fully understand the interactions of pathogens with the environment.

A reduction in the water quality of estuarine systems can result in significant socioeconomic consequences, and it is therefore important that we understand not only the route of pathogen transmission to this environment, but how pathogens interact with their environment. This study has determined that estuarine sediments act as a reservoir for human pathogens. In addition, the composition of estuarine sediment influences pathogen abundance. Furthermore, the use of culture-based and molecular methods to enumerate FIB in estuarine sediments has revealed the presence of possible populations of FIB in a VBNC state. Whilst the plate counting technique is still widely used to enumerate FIB from environmental samples it should be noted that in the absence of other methods to confirm cell numbers, the interpretation of the results may vary. The discrepancies transpire with the recovery of cells from the substrate and the dispersal of microbes on to the solid medium to ensure the growth of individual CFUs from individual cells (Skinner *et al.*, 1952).

The results from this study also highlight the possible presence of Stx bacteriophages in estuarine sediments, all of which may have significant consequences for pathogen contamination of environmental waters. To adequately assess the microbial quality of environmental waters, sediments should be considered as a reservoir for pathogenic microorganisms that can potentially re-contaminate the water column. This study emphasizes the necessity for further research in order to fully understand the interactions of pathogenic bacteria with sediments.

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CHAPTER 5

Decay rates of Faecal Indicator Bacteria (FIB) from sewage and sheep faeces in brackish and freshwater microcosms with contrasting suspended particulate matter (SPM) concentrations

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Abstract

To safeguard human health, legislative measures require the monitoring of FIB concentrations in recreational waters. Many studies have therefore focused on the survival of faecal indicator bacteria (FIB) in the water column and more recently in bottom sediments. However, the influence of suspended particulate matter (SPM) on the survival of FIB in the water

column of an estuarine environment to our knowledge has never been addressed. Microcosms of freshwater containing sediment concentrations of 16 mg/l for low particulate matter (LPM) treatment, 160 mg/l for high particulate matter (HPM) treatment, and 1600 mg/l for extreme particulate matter (EPM) and microcosms of brackish water containing 16.5 mg/l for LPM, 165 mg/l for HPM and 1654 mg/l for EPM were inoculated with crude sewage and sheep faeces. The decay rates of *E. coli*, total coliforms and enterococci were determined by enumeration over 5 consecutive days. *E. coli* from sheep faeces proliferated and persisted at high levels in both water matrices. Enterococci from sheep faeces persisted at higher levels in freshwater compared to brackish water. In general, FIB from sheep faeces increased with a decrease in SPM, subsequent decay rates increased with an increase in SPM in estuarine water. Sewage derived FIB showed the same trend in survival rates as sheep faeces derived FIB in relation to SPM concentrations in brackish water. Although this trend in survival was observed for both sources of FIB it was more evident in FIB from sheep faeces. In addition, FIB from sheep faeces proliferated in the water column and persisted at much higher levels than their sewage counterparts irrespective of water type. With the exception of coliform bacteria similar trends in survival were observed for FIB from sewage in freshwater in relation to SPM concentrations over the first two days of the experiment. However, over the duration of the experiment there were no differences overall. In comparison, *E. coli* from sheep faeces and coliform bacteria showed the opposite trend in freshwater compared with brackish water, where concentrations increased with an increase in SPM. Subsequent decay rates of coliform bacteria were not influenced by SPM concentrations. However *E. coli* decay rates decreased with a decrease in SPM. Enterococci from sheep faeces did not show any difference in survival rates in relation to SPM concentrations in freshwater. These results determine that the survival rates of FIB in aquatic environments are system specific, species and source dependant and influenced by SPM concentrations. This study has important implications for future management strategies for catchment-based risk assessments of FIB pollution in aquatic environments.

KEY WORDS: Suspended particulate matter, decay rate, FIB, brackish water, freshwater

5.1 Introduction

Estuarine environments are extremely important ecosystems that sustain many socioeconomic activities and provide vital resources such as food, habitat and areas for recreation (Costanza *et al.*, 1998). However, estuarine and coastal ecosystems are increasingly exposed to environmental pressures, particularly from anthropogenic activities (GESAMP, 2001). Human populations are concentrated in coastal areas and consequently, the close proximity of industrial

sites, extensive agriculture, sewage discharge and recreation can result in an increase in the flow of microbial pollutants to the sea (GESAMP, 2001).

A decline in the microbiological water quality of highly productive coastal systems can have significant social and economic consequences, and this is particularly true for catchment areas that incorporate blue flag beaches and shellfish harvesting waters (GESAMP, 2001). The current EU standard to assess the microbial pollution of bathing waters involves the enumeration of *E. coli* and enterococci as indicators of pathogen content in coastal waters (E.C. 2006), and consequently, the main focus of research has historically been on the survival and persistence of faecal indicator bacteria (FIB) in the water column (Kay *et al.*, 2005; Jin *et al.*, 2004). However, in recent years the importance of sediment-associated FIB has been recognised and it is now well established that sediments act as a significant reservoir for faecal bacteria and their associated pathogens (Perkins *et al.*, 2014). Numerous studies have revealed that FIB concentrations are much higher in sediment compared with the overlying water column (Perkins *et al.*, 2014), and the prolonged survival of sediment-attached FIB can be attributed to an increase in nutrients and organic matter content (Lee *et al.*, 2006). It has also been suggested that the attachment of FIB to suspended solids in the water column can provide protection, particularly from sunlight (Kay *et al.*, 2005), and result in the downward flux of particle-bound microbial pathogens to benthic sediments, where they accumulate. However, there is currently a lack of information regarding the influence that suspended particulate matter (SPM) concentrations in the water column have on FIB survival in aquatic systems, and this is particularly true for extreme levels of SPM that may arise due to future climate scenarios.

Estuaries are the interface between rivers and the sea, resulting in a complex and dynamic ecosystem that regularly processes a large exchange of fresh and seawater, causing the resuspension of bottom sediments and the constant mixing of particulate matter (Malham *et al.*, 2014). Sediment transport and resuspension within an estuarine environment can be influenced by many environmental and human factors (Liu & Huang, 2009). For example, the water column of a tide-dominated estuary is usually high in particulate matter concentrations, as fine sediments are persistently resuspended (Wells, 1995). In addition, recent climate observations have revealed that episodes of extreme weather events such as storms and hurricanes have increased in many parts of the world (Wetz & Yoskowitz, 2013), which can exacerbate the remobilization of sediments in estuarine and other aquatic environments (Liu & Huang, 2009; Williams *et al.*, 2008). Liu & Huang (2009) recorded levels of SPM reaching up to 4 g/l in Apalachicola Bay located in Florida, USA, under severe storm conditions, whilst a reduction in wind-induced currents resulted in levels of SPM as low as 0.2 g/l. Furthermore, human activities such as the

felling of forests and the building of roads can lead to soil erosion, and subsequently, soil running into river systems can be transported downstream and deposited into estuarine and coastal environments (GESAMP, 2001). To ensure that management strategies used to assess microbial pollution of environmental waters are adequate to safeguard human health, it is extremely important particularly in a changing climate to identify how varying levels of SPM concentrations could impact on FIB survival in the coastal zone. Furthermore, due to the heterogenous nature of sources of microbial pollution in estuarine environments, it is important to determine if the survival and decay rates of FIB in environmental waters of varying SPM concentrations are source dependant.

The Conwy catchment, North Wales, UK, is home to ~112,000 people with over 75% living near the coast. Approximately 5.4 million tourists visit the Conwy catchment every year, with many partaking in water sports and swimming (Thorn *et al.*, 2011). The Conwy estuary is an extremely productive system that incorporates blue flag beaches and directly impacts on a commercial shellfishery. Subsequently, a decline in microbial water quality could have significant consequences for both the tourist and shellfish industries and for human health. The catchment covers ~300 km² and includes large areas of land utilised for agribusiness. In addition, land is utilised for homes and commercial properties. Subsequently, the Conwy estuary is subject to inputs of waste water effluent both directly and indirectly from its tributaries (such as the river Gyffin and the river Ganol). Additional microbiological inputs can be attributed to wildfowl such as migratory birds that utilise the Conwy estuary as a feeding ground, ineffective septic tank systems, agricultural runoff and storm water runoff.

A previous study of FIB concentrations in the Conwy estuary revealed that the abundance of FIB and other potentially pathogenic bacteria in sediments had a positive correlation with finer sediment fractions such as clay (<4 µm) and silt (4 µm - 63 µm) and organic matter content (Perkins *et al.*, 2014). Fine sediment particles of < 63 µm are easily mobilised due to their large surface area and geochemical composition (Droppo & Stone, 1994), thus agitation of microbial contaminated sediments by various processes such as tidal fluctuations and water level flux, can release microbes back into the water column (Jamieson *et al.*, 2005) and subsequent particulate matter (Wetz & Yoskowitz, 2013). Complex processes in aquatic systems make it difficult to assess the fate of FIB, and little is known about the survival and persistence of estuarine FIB in relation to varying SPM concentrations in the water column. The aim of this study was to investigate the decay rates of FIB (*E. coli*, enterococci and coliforms) derived from sheep faeces and crude human sewage in relation to three SPM concentrations (low, high and extreme) in microcosms containing brackish water from the Conwy estuary and freshwater.

5.2 Materials and methods

5.2.1 Sample collection

Estuarine (brackish) sediment and water samples were collected on the 04/08/2014, the day before the start of the experiment, from the Conwy estuary, North Wales, UK, during the flood tide to ensure saline conditions. Freshwater sediment and water samples were collected on the 26/04/2015, the day before the start of the experiment from the river Llugwy (Betws-y-Coed, North Wales). Crude wastewater influent (untreated) was collected on the day of the experiment from a local wastewater treatment plant (Treborth Wastewater Treatment Works, Caernarfon, North Wales). In addition, on the day before the start of the experiment 10 independent samples of fresh sheep faeces were collected from a farm located on Anglesey for the freshwater experiment and a farm located on the banks of the Conwy estuary for the brackish water experiment. The 10 independent samples of sheep faeces were pooled to ensure inclusion of microbial variation. Samples were incubated overnight at 4°C to minimize changes in microbial populations.

5.2.2 Microcosm setup

Water and sediment samples from each environment (fresh and brackish) were mixed and homogenised by shaking. To determine the total SPM concentrations Whatman® glass microfibre filters grade GF/F pore size 0.8µm (Sigma-Aldrich, Gilligham, Dorset, UK), were hardened by ignition at 500°C, soaked in distilled water for 5 min to remove any soluble matter then dried at 80°C for 1 hour and weighed. 10 ml of each water sample (fresh and brackish) were filtered under vacuum through the pre-dried, pre-weighed filter. Filters were then dried for 1 hour at 80°C and reweighed. Total SPM concentrations were calculated as the difference between the pre and post weights. Using the excess collected water samples that had been allowed to stand over night to allow the particulate matter to settle out, dilutions were performed to give a suspended particulate matter content of 16 mg/l for low particulate matter (LPM) treatment, 160 mg/l for high particulate matter (HPM) treatment, and 1600 mg/l for extreme particulate matter (EPM) treatment for freshwater microcosms and 16.5 mg/l for LPM 165 mg/l for HPM and 1654 mg/l for EPM for brackish water microcosms. The LPM and HPM SPM concentrations were analogous to the range of concentrations found over a previous 12-month survey of the

Conwy estuary which determined SPM concentrations ranging from 5 mg/l to 178 mg/l and of a freshwater site at Dolgarrog, North Wales, UK where concentrations ranged from 1 mg/l to 482 mg/l.

250 ml sterile opaque bottles (Nalgene® Sigma-Aldrich Company Ltd., Gillingham, Dorset) were utilised for the microcosms. Control microcosms that were not inoculated with sewage or sheep faeces were set up in triplicate. Freshwater control microcosms contained a 100 ml of each SPM concentration (16 mg/l, 160 mg/l and 1600mg/l) and brackish water control microcosms contained 100 ml of each SPM concentrations (16.5 mg/l 165 mg/l and 1654 mg/l). Experimental microcosms containing 95 ml of brackish water were set up in triplicate for each SPM concentration for the inoculation of sewage. Microcosms containing 98 ml of estuarine water were set up in triplicate for each SPM concentration for the inoculation of sheep faeces. A composite sample of sheep faeces was obtained by mixing 5 g of each sample, which was then suspended in brackish water to obtain a (1:2 w/v) dilution. Faecal suspensions were vortexed at maximum speed for 1 min then pulse centrifuged to pellet solids. Two ml of resultant supernatant was then added to 98 ml of brackish water containing each SPM concentration to give a starting volume of 100 ml. 5 ml of crude sewage was added to 95 ml of brackish water containing each SPM concentration to give a starting volume of 100 ml (Although the amount of inoculate may seem unrealistically high when compared to the dilution effect of natural aquatic systems, it was necessary to generate countable CFUs over 5 consecutive days so that bacterial decay rates could be determined). Microcosms were incubated at 10°C in the dark on a shaking platform to ensure constant agitation of the contents.

5.2.3 Isolation and enumeration of FIB from microcosms

To determine the decay rates of *E. coli*, enterococci and total coliforms, FIB were enumerated by transferring 100 µl from each microcosm onto agar plates containing a selective medium for each group (Table 2.1). Plates were inverted and incubated according to the manufacturer's recommendations (Table 2.1). The resulting colony forming units (CFUs) were counted. Samples were taken from each of the microcosms at the beginning of the experiment (T0) and every 24 ± 2 hrs for 5 days (T5). Dilution series were performed where needed to obtain countable CFUs throughout the experiment.

5.2.4 Statistical analysis

In most cases, the data in this study did not meet the assumption for equal variances for a two-way factorial analysis of variance (ANOVA) to be truly valid. However, distinct differences between bacterial decay rates and between treatments were observed throughout this study. ANOVA tests were therefore performed at the 0.05 level of significance to determine if there was at least one mean that was significantly different from the rest. If a significant difference was reported the post hoc Tukey test was performed to report pairwise comparisons at the 0.05 level of significance. Although the statistical results cannot be reported as official values, they are reported to enhance the obvious observed differences between bacterial decay rates and varying SPM concentrations. In addition, the p-values (usually < 0.001) were extremely low, thus giving extra confidence that the indication of difference can be acknowledged even if not reported as statistically different. There are potential reasons why the assumption of equal variances were not met within the data set, such as a low number of replicates which could be rectified by increasing the amount of replicates; although it would have been difficult to process any more replicates within the time constraints of this experiment. However, such limitations should not stop us from inferring from the obvious observed trends displayed within this study.

5.3 Results

5.3.1 Decay rates of *E. coli* and enterococci derived from sheep faeces in brackish water with low, high and extreme SPM concentrations

The enumeration of *E. coli* derived from sheep faeces over 5 consecutive days in brackish water containing low, high and extreme concentrations of SPM revealed that overall there was an increase in their concentrations (Table 5.1).

Table 5.1 The overall trend in decay rates of FIB from sewage and sheep faeces in fresh and brackish water.

FIB	Freshwater	Brackish water
<i>E. coli</i> from sheep faeces	↑	↑
Enterococci from sheep faeces	↓	↓
<i>E. coli</i> from sewage	↓	↓
Enterococci from sewage	↓	↓
Total Coliforms (excluding <i>E. coli</i>) from sewage	↓	↓

Enumeration of sheep faeces derived *E. coli* in brackish water revealed that there was an observed difference between *E. coli* concentrations between all 3 SPM concentrations over time. There was a large increase in *E. coli* concentrations from T0 to T1 for all 3 concentrations (Fig. 5.1). However, the percentage increase varied with SPM concentration. *E. coli* concentrations increased with decreasing SPM concentrations. The mean percentage increase for LPM was 6170%, HPM was 4286% and EPM was 1210% (Fig. 5.1).

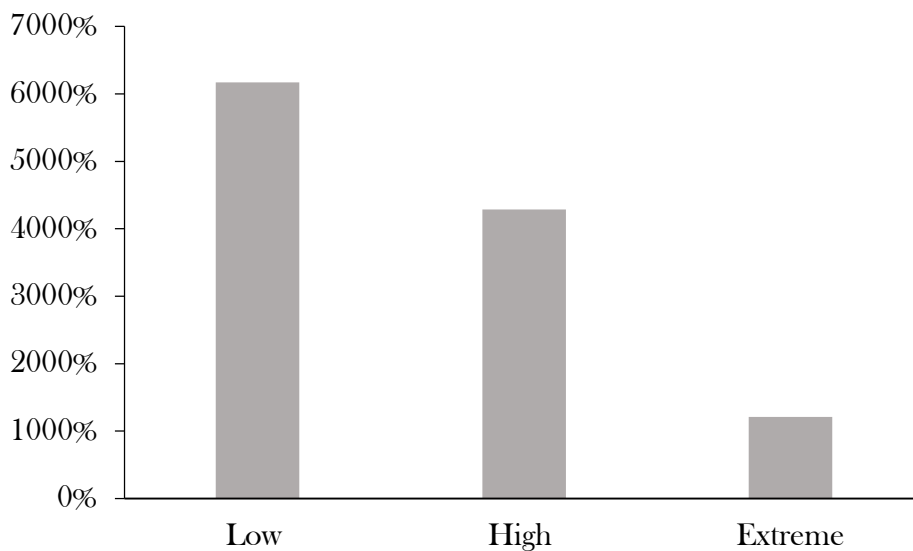


Fig. 5.1 Percentage increase in CFUs per 100 ml from T0 to T1 in sheep faeces derived *E. coli* concentrations in brackish water with LPM, HPM and EPM concentrations.

Furthermore, another increase in concentrations of *E. coli* for all SPM concentrations was observed from T3 to T4 for all SPM concentrations (Fig. 5.3). The reported data from a post hoc Tukey test revealed that there were differences between LPM and HPM ($p = 0.003$), LPM and EPM ($p < 0.001$) and HPM and EPM ($p < 0.001$) based on *E. coli* concentrations in brackish water over time. CFU counts taken at T5 showed that after the initial increase of *E. coli* between T0 and T1 there was an increase in decay rates with a decrease in SPM, the percentage decrease from T0 to T5 was 44% for LPM 39% for HPM and 37% for EPM (Fig. 5.2). Despite the observed increase in the decay rates of *E. coli* in LPM concentrations CFU counts remained higher in LPM than HPM and EPM concentrations throughout the experiment (Fig. 5.3).

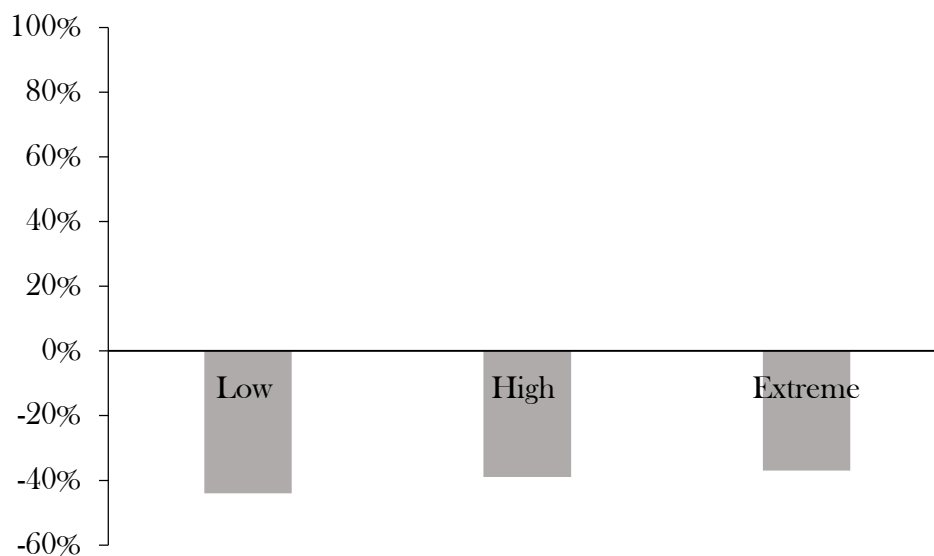


Fig. 5.2 Percentage decrease in CFUs per 100 ml from T1 to T5 of sheep faeces derived *E. coli* in brackish water with LPM, HPM and EPM concentrations.

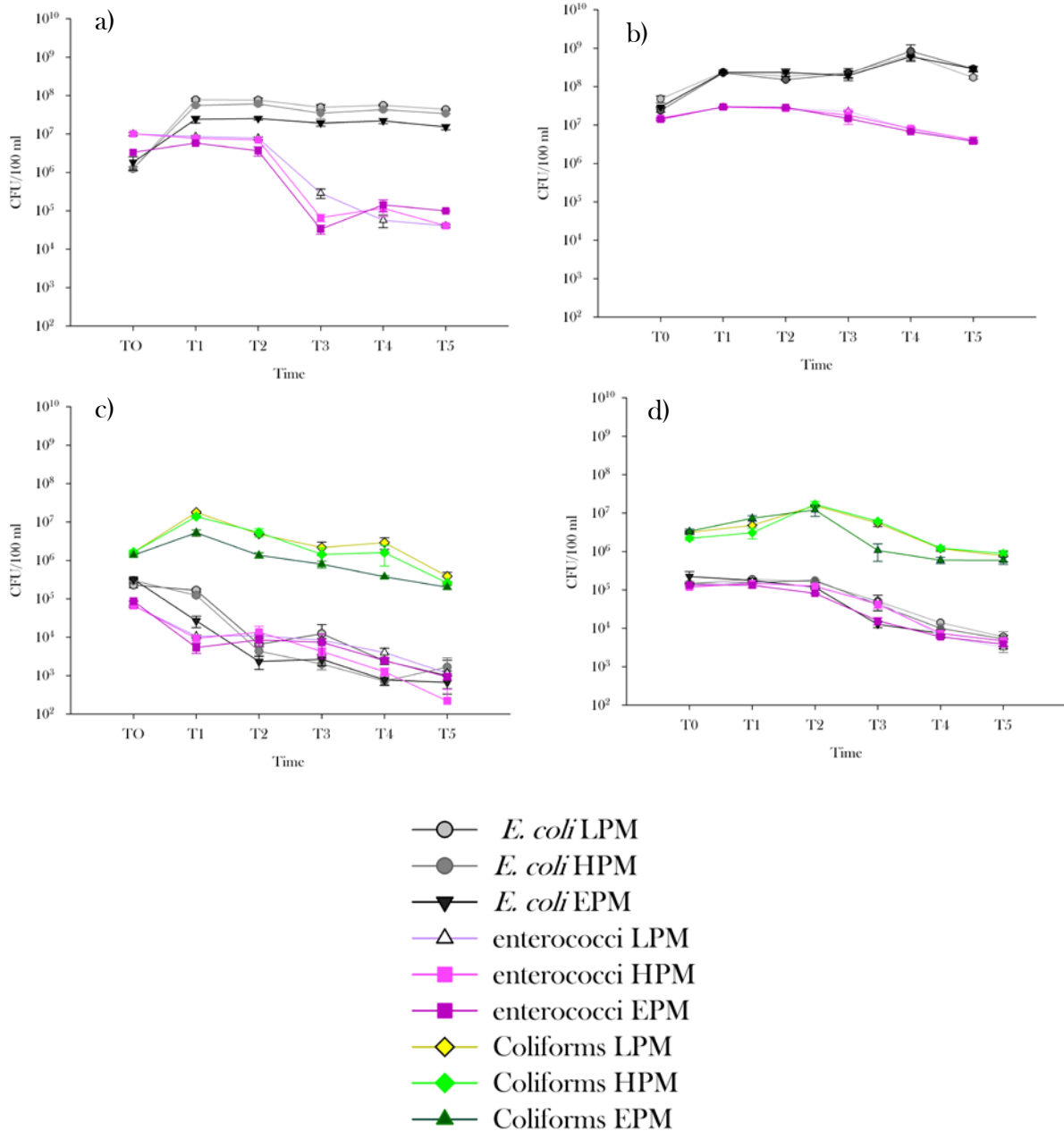


Fig. 5.3 a) Decay rates of *E. coli* and enterococci from sheep faeces in brackish water. (b) Decay rates of *E. coli* and enterococci from sheep faeces in freshwater. (c) Decay rates of *E. coli*,

enterococci and coliforms from sewage in brackish water. (d) Decay rates of *E. coli*, enterococci and coliforms from sewage in freshwater.

The enumeration of enterococci derived from sheep faeces over 5 consecutive days in brackish water containing low, high and extreme concentrations of SPM revealed that overall there was a decrease in their concentrations (Table 5.1).

The enumeration of enterococci derived from sheep faeces in brackish water containing LPM, HPM and EPM concentrations revealed that there was observed differences between enterococci concentrations between all 3 SPM concentrations over time (Fig. 5.3). Concentrations of enterococci at T0 cannot be completely relied upon, as enumeration by selective media resulted in only one countable replicate for both high and extreme SPM concentrations. Consequently, the increase in enterococci concentrations observed for EPM from T0 to T1 may therefore be unreliable. However, a decrease in concentrations of 11% for LPM, 16% for HPM and 31% for HPM were determined at T3 (Fig. 5.4). Decay rates of enterococci derived from sheep faeces increased with an increase in SPM concentrations (Fig. 5.4). A post hoc Tukey test reported a difference between all three SPM concentrations ($p < 0.001$) based on enterococci concentrations over time. In addition a post hoc Tukey test revealed a reported difference between the decay rates of *E. coli* compared with enterococci over time for all SPM concentrations ($p < 0.003$).

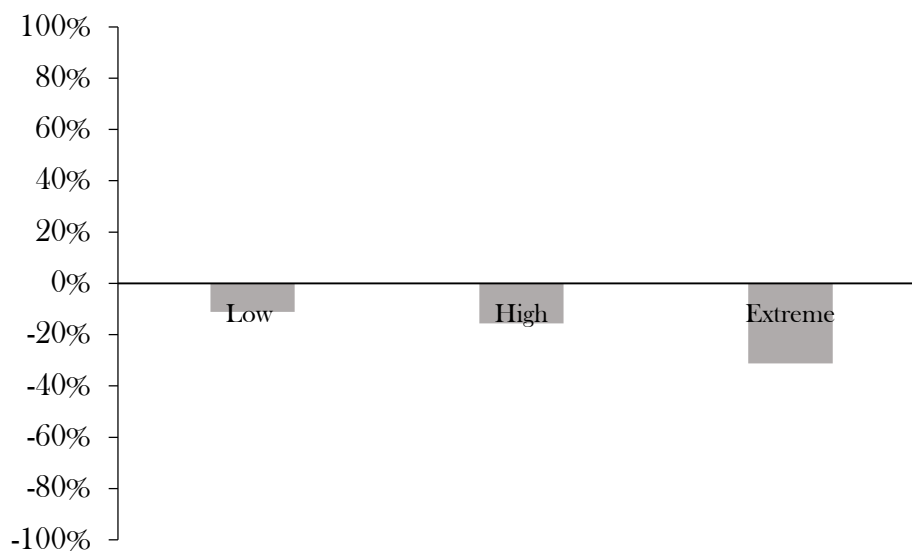


Fig. 5.4 Percentage decrease in CFUs per 100 ml from T1 to T3 of sheep faeces derived enterococci in brackish water with LPM, HPM and EPM concentrations.

*5.3.2 Decay rates of *E. coli*, enterococci and total coliforms derived from human sewage in brackish water with low, high and extreme SPM concentrations*

The enumeration of *E. coli*, enterococci and total coliforms derived from human sewage over 5 consecutive days in brackish water containing low, high and extreme concentrations of SPM revealed that overall there was a decrease in their concentrations (Table 5.1). The decay rates of human sewage derived *E. coli* displayed a different trend over time to that of sheep faeces derived *E. coli* in brackish water. In comparison, levels of sewage derived *E. coli* did not show any initial increase from T0 to T1 but exhibited a reduction of 28% for LPM, 59% HPM and 92% for EPM concentrations (Fig. 5.5). These initial reductions reveal decay rates increased with increasing SPM concentrations.

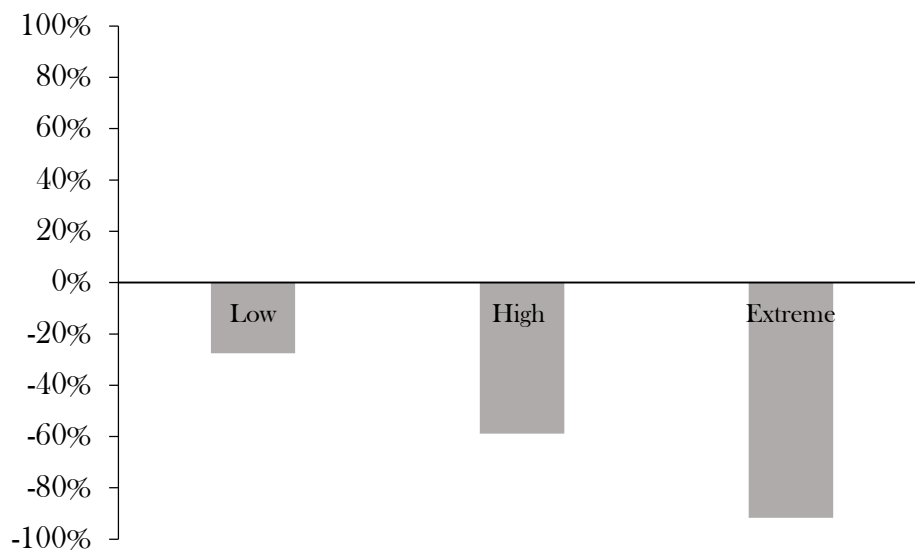


Fig. 5.5 Percentage decrease in CFUs per 100 ml from T0 to T1 of sewage derived *E. coli* in brackish water with LPM, HPM and EPM concentrations.

The enumeration of enterococci in human sewage revealed the same initial trend in decay rates from T0 to T1 as displayed by *E. coli* from human sewage. However, the differences between SPM concentration and the reduction in enterococci from T0 to T1 were much smaller compared with the reduction in *E. coli* concentrations over the same time period. Enterococci concentrations reduced by 84% for LPM, 86% for HPM and 94% for EPM concentrations from T0 to T1 (Fig. 5.6). However, an increase between T1 and T2 was observed. Concentrations increase by 3% for LPM, 45% for HPM and 57% EPM concentrations.

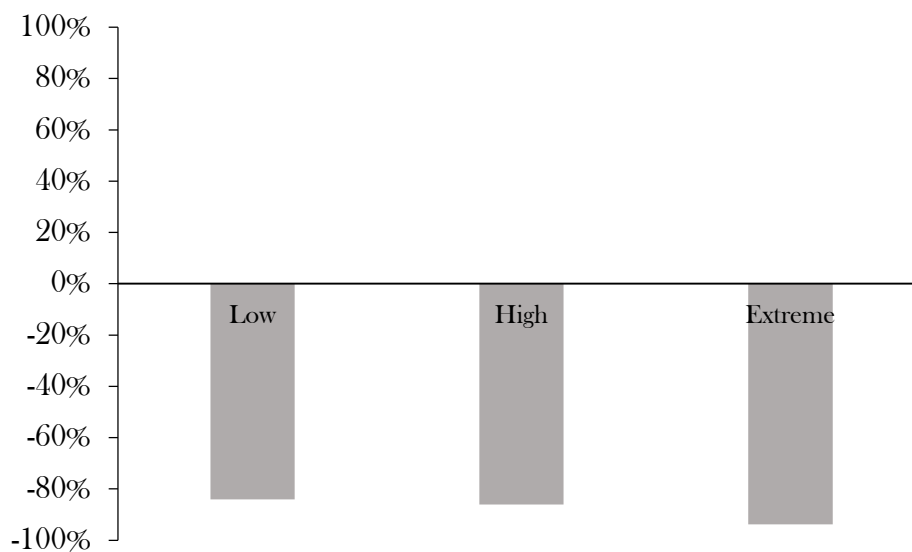


Fig. 5.6 Percentage decrease in CFUs per 100 ml from T0 to T1 of sewage derived enterococci in brackish water with LPM, HPM and EPM concentrations.

The percentage decrease in *E. coli* concentrations at T5 based on the initial counts at T0 revealed an overall reduction of 100% for LPM, 99% for HPM and 100% for EPM. The percentage decrease in concentrations of enterococci determined at T5 and based on counts at

T0 revealed an overall reduction of 98%, 100% and 99% for LPM, HPM and EPM, respectively (Fig. 5.3). The total decay rates based on CFUs taken at T0 and T5 have to be reported with caution, due to the proliferation of certain bacterial species at certain time points during this five-day study, these results do not necessarily indicate total eradication from the water column. Despite the differences in *E. coli* levels between varying SPM concentrations, a post hoc Tukey test reported no difference between SPM concentrations over time. In addition, a post hoc Tukey test also revealed no difference between the decay rates of enterococci over time between all 3 SPM concentrations. There was a difference reported between HPM and EPM concentrations ($p < 0.047$) but due to the extremely high level of significance, combined with the violation of assumptions, this is not considered to be a reflection of difference.

The enumeration of total coliforms from human sewage revealed an increase in concentrations from T0 to T1. Coliform concentrations increased with a decrease in SPM concentrations. Results revealed the percentage increase was 987% for LPM, 743% for HPM and 281% for EPM (Fig. 5.7).

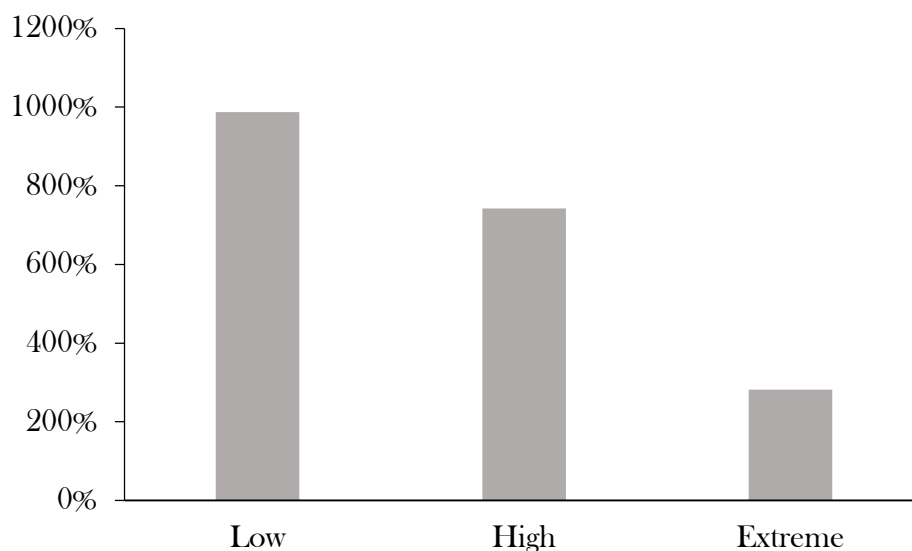


Fig. 5.7 Percentage increase in CFUs per 100 ml from T0 to T1 of sewage derived coliform bacteria in brackish water with LPM, HPM and EPM concentrations.

The increase of coliform bacteria at T1 was followed by a rapid reduction where concentrations dropped by 88% for LPM, 90% for HPM and 85% for EPM at T3. With the exception of T2, coliform concentrations were consistently higher in LPM compared with concentrations in HPM and EPM. A post hoc Tukey test revealed that there was no difference between *E. coli* and enterococci concentrations over time for all three SPM concentrations but there was a difference between *E. coli* and total coliform concentrations ($p < 0.001$) and enterococci and total coliforms concentrations ($p < 0.001$) for all SPM concentrations.

5.3.3 Decay rates of E. coli and enterococci derived from sheep faeces in freshwater with low, high and extreme SPM concentrations

The enumeration of *E. coli* derived from sheep faeces over 5 consecutive days in freshwater containing low, high and extreme concentrations of SPM revealed that overall there was an increase in their concentrations. In comparison, there was an overall decrease in the concentrations of enterococci derived from sheep in freshwater (Table 5.1).

Enumeration of sheep faeces derived *E. coli* in freshwater revealed an initial increase from T0 to T1 of 399% for LPM, 851% for HPM and 686% for EPM concentrations (Fig. 5.8).

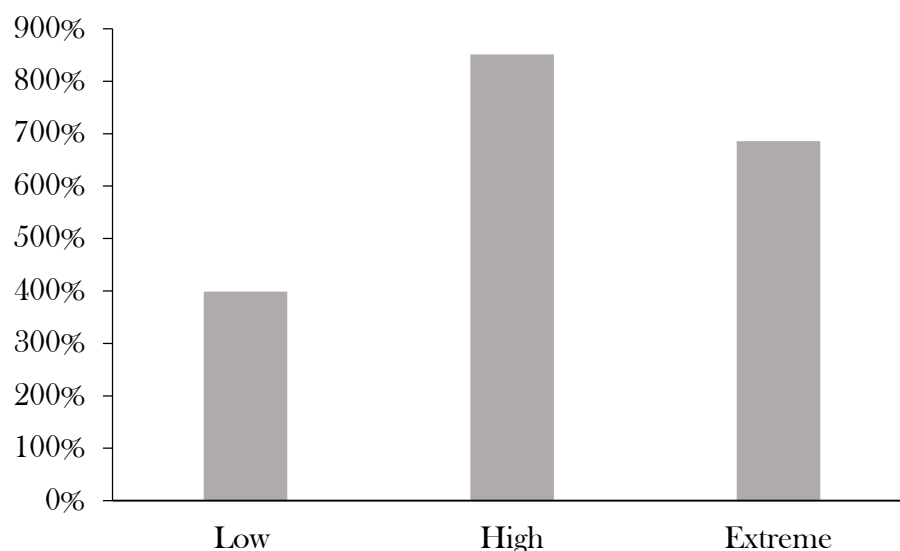


Fig. 5.8 Percentage increase in CFUs per 100 ml from T0 to T1 of sheep faeces derived *E. coli* in freshwater with LPM, HPM and EPM concentrations.

A second substantial increase in *E. coli* levels was also observed between T3 and T4 of 184% for LPM, 286% for HPM and 211% for EPM concentrations. At T5 concentrations of *E. coli* were still higher than the initial concentrations at T0. CFU counts of *E. coli* determined at T5 still showed an increase overall from T0 of 260% 1118% and 888% for LPM, HPM and EPM concentrations respectively. However, the percentage decrease in *E. coli* concentrations calculated from the initial increase at T0 and up to T5 varied between SPM concentrations. CFU counts had decreased by 28% for LPM but had displayed another further increase of 28% for HPM and 26% for EPM (Fig. 5.9). Results revealed decay rates increased with a decrease in SPM concentrations.

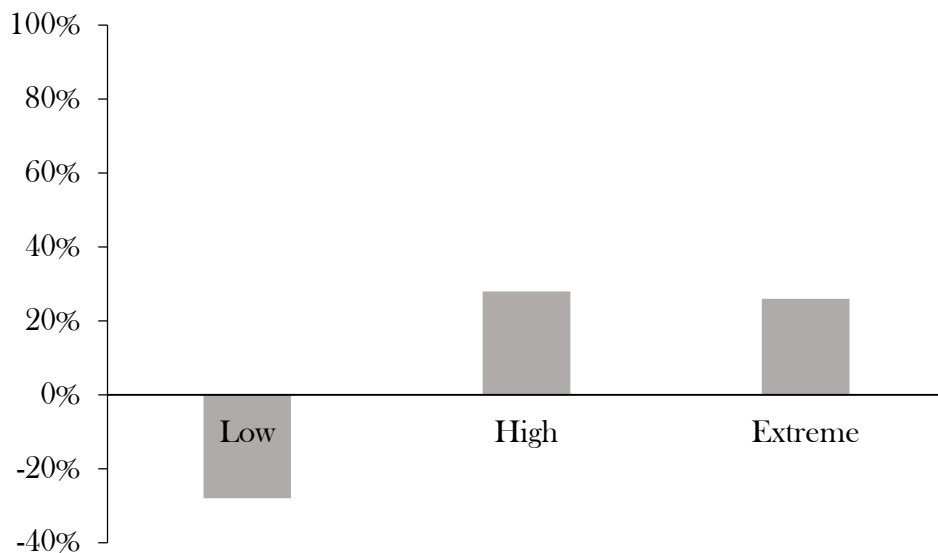


Fig. 5.9 Percentage increase/decrease in CFUs per 100 ml from T1 to T5 of sheep faeces derived *E. coli* in freshwater with LPM, HPM and EPM concentrations.

Despite these differences, the results from a post hoc Tukey test indicated no significant difference between SPM concentrations over time. However, based on the percentage increase of *E. coli* between T0 and T1 and the percentage increase between T3 and T4 it would seem that *E. coli* concentrations increase at a higher rate in freshwater with HPM concentrations. Enumeration of sheep faeces derived enterococci also exhibited an initial increase from T0 to T1 of 104% for LPM, 95% for HPM and 112% for EPM concentrations. CFU counts continued to increase throughout the time between T1 and T2 but exhibited a decline in concentrations thereafter, which continued throughout the experiment. At T5, enterococci levels had dropped

by 88%, 86% and 87% for LPM, HPM and EPM respectively. Enterococci concentrations displayed the same trends in decay rates for all three SPM concentrations. This was supported by results from a post hoc Tukey test that revealed no difference between the decay rates of enterococci in relation to varying SPM concentrations.

5.3.4 Decay rates of *E. coli*, enterococci and total coliforms derived from sewage in freshwater with low, high and extreme SPM concentrations

The enumeration of *E. coli*, enterococci and total coliforms derived from human sewage over 5 consecutive days in freshwater containing low, high and extreme concentrations of SPM revealed that overall there was a decrease in their concentrations (Fig. 5.1).

Enumeration of sewage derived *E. coli* in freshwater revealed that *E. coli* concentrations were similar between all 3 SPM concentrations over time (Fig. 5.3). However, initially levels of *E. coli* in LPM and HPM concentrations increased from T0 to T1 by 30% and 5% respectively, compared with a decrease of 21% for EPM concentrations (Fig. 5.10).

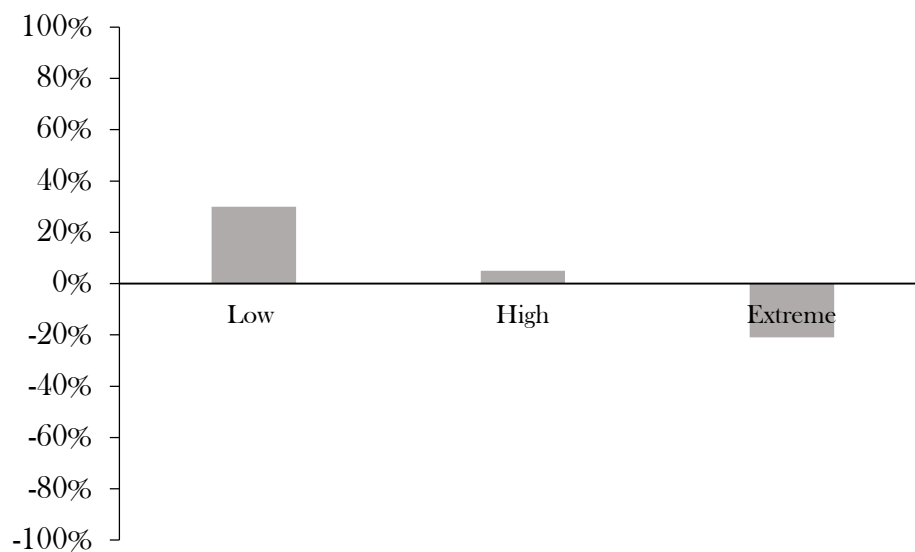


Fig. 5.10 Percentage increase/decrease in CFUs per 100 ml from T0 to T1 of sewage derived *E. coli* in freshwater with LPM, HPM and EPM concentrations.

A second increase was observed between T1 and T2 for *E. coli* in HPM concentrations. Although there was a difference in decay rates from T0 to T1, the overall percentage decrease

in concentrations from T1 to T5 was 97% for all three SPM concentrations, showing the same trend in decay rates over time. This similarity was also supported by results from a post hoc Tukey test that indicated no difference between all 3 SPM concentrations in relation to enterococci decay rates over time. The enumeration of human sewage derived enterococci in freshwater with varying SPM concentrations revealed a similar trend to that displayed by *E. coli* from T0 to T1. An increase in enterococci concentrations was observed for LPM and HPM concentrations in comparison to a decrease for EPM concentrations (Fig. 5.11). The percentage increase in enterococci CFU counts for LPM and HPM concentrations at T1 were extremely similar at 23% for LPM and 14% for HPM whilst a reduction of 1% was observed for EPM. Subsequent CFU counts remained similar throughout the duration of the experiment, whilst levels of enterococci declined at a much quicker rate in EPM concentrations over time.

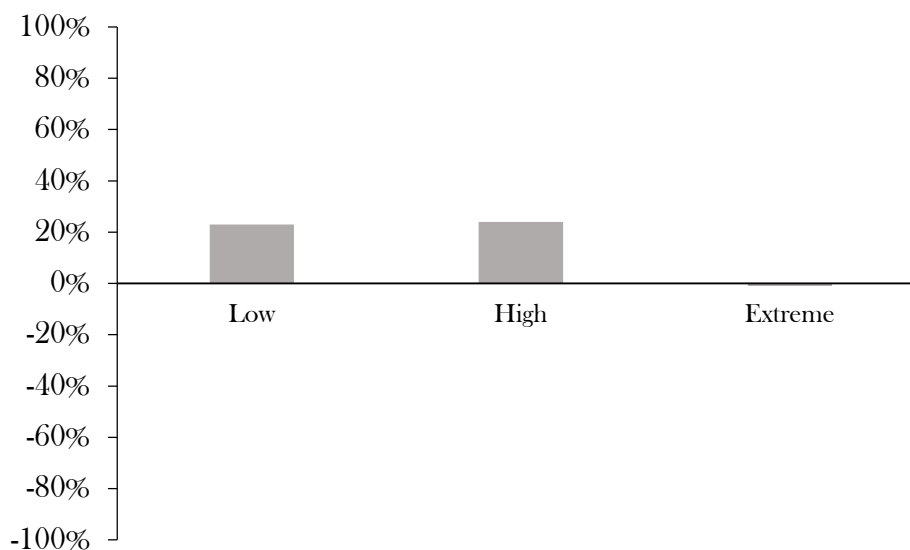


Fig. 5.11 Percentage increase/decrease in CFUs per 100 ml from T0 to T1 of sewage derived enterococci in freshwater with LPM, HPM and EPM concentrations.

Results from the post hoc Tukey test indicated that there was a difference between enterococci decay rates between LPM and EPM ($p < 0.001$) and HPM and EPM ($p = 0.006$). These data indicate that the decay rate of sewage derived enterococci in freshwater is similar for low and high concentrations of particulate matter but increases with extreme concentrations. The enumeration of total coliform concentrations derived from human sewage revealed an increase in concentrations from T0 to T2 for all three levels of SPM (Fig. 5.3). An increase of 53% for LPM 40% for HPM and 119% for EPM concentrations was observed from T0 to T1 (Fig. 5.12),

followed by a further increase of 227% 448% and 64% respectively from T1 to T2. A decline in total coliform concentrations was observed from T2 to T5 for all SPM concentrations (Fig. 5.3). At T1 coliform concentrations had increased with an increase in SPM, however at T2 the increase was consistent with a decrease in SPM showing a total increase between T0 and T2 of 401%, 670 and 260% for LPM, HPM and EPM.

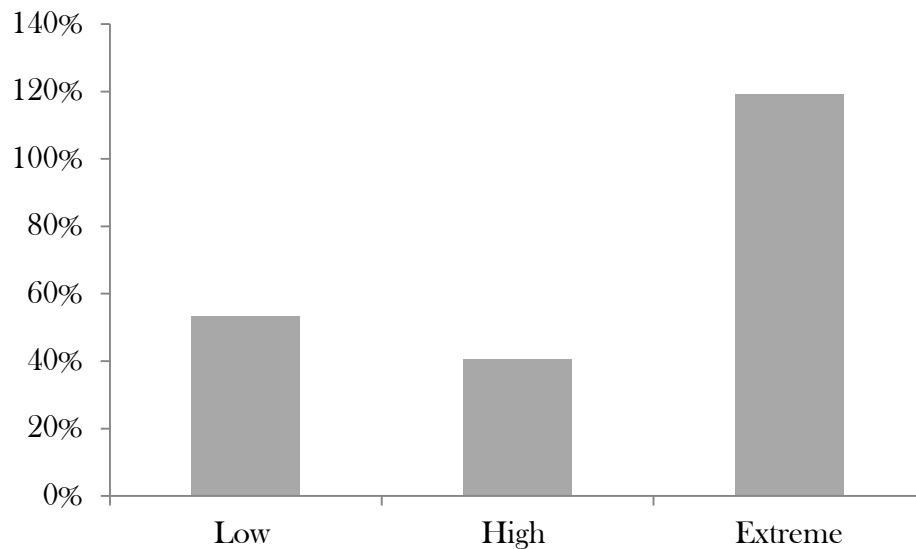


Fig. 5.12 Percentage increase in CFUs per 100 ml from T0 to T1 of sewage derived coliform bacteria in freshwater with LPM, HPM and EPM concentrations.

A decline of 95% in total coliform concentrations was observed from T2 to T5 for all SPM concentrations. These data indicate that the proliferation of sewage derived coliform bacteria in freshwater is similar for low and high concentrations of particulate matter but increases with extreme concentrations. However, despite this increase a post hoc Tukey test reported no difference between coliform decay rates for all SPM concentrations over time. A post hoc Tukey test reported no difference in the decay rates of *E. coli* and enterococci but reported a difference between *E. coli* and total coliform concentrations ($p < 0.001$) and enterococci and total coliforms concentrations ($p < 0.001$) for all SPM concentrations over time.

5.3.5 Decay rates of *E. coli* derived from sheep faeces in fresh and brackish water

Substantial differences were observed between the decay rates of *E. coli* from sheep faeces in fresh and brackish water, although similar trends over time were observed (Fig. 5.3). There was an initial increase from T0 to T1 of sheep derived *E. coli* for all SPM concentrations, in both fresh and brackish water. However, the percentage increase in the levels of *E. coli* across all three SPM concentrations was substantially higher in brackish water compared with freshwater. *E. coli* concentrations in brackish water increased from T0 to T1 by 6170% 4286% and 1210% for LPM, HPM and EPM respectively, compared to an increase of 399%, 851% and 686% for LPM, HPM and EPM respectively in freshwater. An additional increase in *E. coli* concentrations was observed from T3 to T4, 13% for LPM, 23% for HPM and 15% for EPM in brackish, compared with an increase of 184%, 286% and 211% for LPM, HPM and EPM respectively in freshwater (Fig. 5.13). CFU counts in freshwater exceeded those observed at T1 by 173% for LPM, 270% for HPM and 159% for EPM concentrations. Based on the initial increase at T1, *E. coli* concentrations taken at T5 showed a reduction of 28% for LPM but an increase of 28% and 26% for HPM and EPM concentrations, respectively, in freshwater.

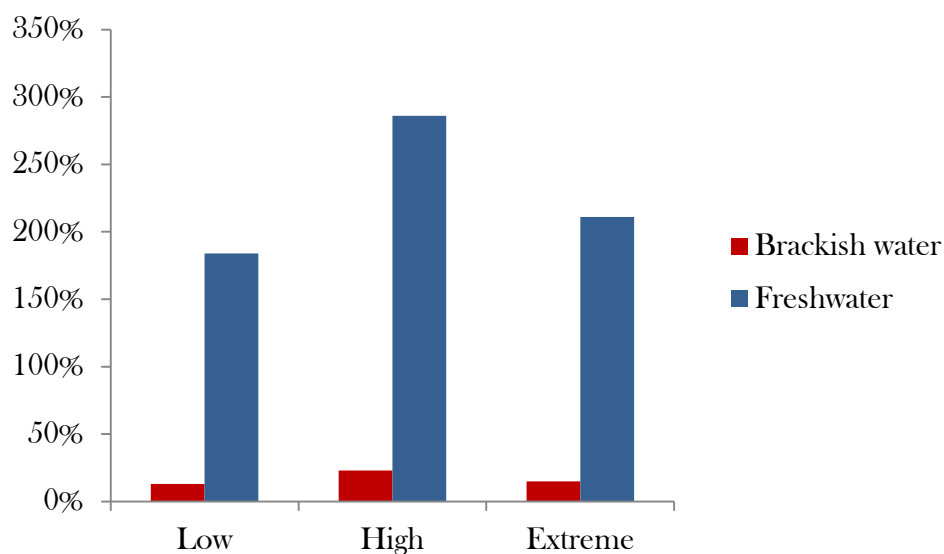


Fig. 5.13 Percentage increase in CFUs per 100 ml from T3 to T4 of sheep derived *E. coli* in brackish and freshwater with LPM, HPM and EPM concentrations.

5.3.6 Decay rates of enterococci derived from sheep faeces in fresh and brackish water

Only one replicate out of three produced countable CFUs for the enumeration of enterococci at T0 for concentrations of EPM in brackish water, which are therefore reported with caution and not considered in this comparison. Enterococci levels in freshwater increased from T0 to T1 for all 3 SPM concentrations, then made a gradual decline (Fig. 5.3). In comparison, enterococci levels in brackish water gradually reduced from T0 to T2, then rapidly declined from T2 to T3 by 96%, 99% and 99% for LPM, HPM and EPM respectively. However an increase in CFU counts was observed between T3 and T4 of 75% for HPM and 330% for EPM concentrations compared to a continued reduction of a further 80% for LPM concentrations.

5.3.7 Decay rates of *E. coli* derived from waste water in fresh and brackish water

The decay rate of *E. coli* derived from human sewage showed a different trend in freshwater compared with brackish water (Fig. 5.3). There was an initial increase from T0 to T1 in *E. coli* concentrations in freshwater of 53% for LPM, 40% for HPM and 119% for EPM concentrations followed by a decline. In comparison, a decrease of 28% for LPM, 59% for HPM and 92% for EPM was observed for sewage derived *E. coli* in brackish water. *E. coli* concentrations in HPM in freshwater continued to increase, but along with the other SPM concentrations declined after T2 and continued to decline throughout the duration of the experiment. Although an increase in *E. coli* levels was observed from T2 to T3 in LPM and EPM concentrations in brackish water (Fig. 5.3).

5.3.8 Decay rates of enterococci derived from waste water in fresh and brackish water

There was a large percentage reduction of sewage derived enterococci from T0 to T1 of 84% for LPM, 86% for HPM and 94% for EPM in brackish water compared with a small percentage increase of 23% and 24% in LPM and HPM concentrations respectively in freshwater. Enterococci levels declined after T1 for all SPM concentrations in freshwater (Fig. 5.3). There was an increase of enterococci levels between T1 and T2 in EPM concentrations in brackish water but a decline followed and continued throughout the duration of the study (Fig. 5.3).

5.3.9 Decay rates of coliform bacteria derived from waste water in fresh and brackish water

A large percentage increase was observed for coliform concentrations from T0 to T1 of 987%, 743% and 281% for LPM, HPM and EPM concentrations respectively in brackish water, compared with a smaller percentage increase of 53% 40% 119% for LPM, HPM and EPM concentrations in freshwater (Fig. 5.14). However, coliform concentrations continued to increase to T2 for all 3 SPM concentrations in freshwater, at levels of 227%, 448% and 64% for LPM, HPM and EPM concentrations respectively, followed by a decline. In comparison, levels of coliform bacteria in brackish water decreased from T1 for all 3 SPM concentrations but another increase for LPM and HPM of 34% and 11% respectively was observed between T3 and T4.

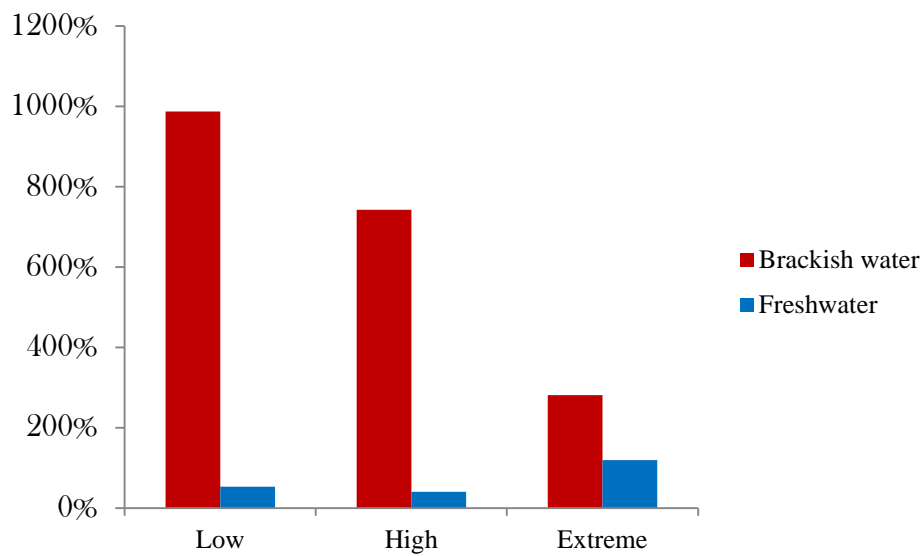


Fig. 5.14 Percentage increase in CFUs per 100 ml from T0 to T1 of sewage derived coliform bacteria in brackish and freshwater with LPM, HPM and EPM concentrations.

All control microcosms demonstrated negligible background concentrations of the target bacteria. Average concentrations did not exceed 254 CFU/ml for coliform bacteria, 133 CFU/ml for *E. coli* and 2 CFU/ml for enterococci concentrations, data not shown.

5.4 Discussion

To address the paucity of information regarding the role of SPM concentrations in the survival of FIB, the decay rates of three FIB groups (*E. coli*, enterococci and coliforms) derived from sheep faeces and crude human sewage in both freshwater and brackish water microcosms were determined for three different SPM concentrations (low, high and extreme). Results from this study show that SPM concentrations influence the proliferation and decay rates of FIB in brackish waters but has minimal influence in freshwater. Furthermore, the different trends observed in decay rates were source and species dependant. In general, decay rates in brackish water increased with an increase in SPM concentrations. In addition, *E. coli* exhibited an initial increase in levels which reduced with an increase in SPM concentrations. In comparison, both enterococci and *E. coli* exhibited an initial increase in CFU counts in freshwater, but no difference in decay rates in relation to SPM concentrations were reported over time. It is important to note that although a post hoc Tukey test reported no difference in decay rates overtime there was a 100 % increase in *E. coli* levels from LPM to HPM and a 50% increase from LPM to EPM suggesting that initially, levels of HPM provide more favourable conditions for the proliferation of sheep derived *E. coli* in freshwater.

Overall, concentrations of sheep faeces derived *E. coli* increased at a much higher rate in both brackish and freshwater compared with sheep faeces derived enterococci. Although *E. coli* from sheep faeces exhibited an increase in CFU counts in both brackish and freshwater, the initial percentage increase was much greater in brackish water. High concentrations of *E. coli* were then sustained in brackish water for the duration of the experiment compared to fluctuating concentrations of *E. coli* in freshwater. In comparison, enterococci from sheep faeces decayed much quicker in brackish water than freshwater. Results show that SPM concentrations impact the proliferation of sheep derived *E. coli* and enterococci in brackish water, but has minimal impact in freshwater over time. Sheep faeces derived FIB not only proliferated in both brackish and freshwater matrices, but they displayed much slower decay rates than sewage derived FIB. Furthermore, a post hoc Tukey test reported no difference between the decay rates of *E. coli* and total coliform bacteria derived from sewage in relation to SPM concentrations in freshwater and no difference between *E. coli* and enterococci decay rates in brackish water. In contrast,

decay rates of sewage-derived enterococci in freshwater and total coliform bacteria in brackish water generally displayed a decrease with a decrease in SPM concentrations. These findings support a similar study by Korajkic *et al.*, (2013) which determined the differential decay rates of *E. coli* and enterococci originating from cattle manure and human wastewater effluent in marine and freshwater under ambient light and dark conditions with and without exposure to the indigenous aquatic microbiota. It was concluded that whilst both sunlight and the indigenous aquatic microbiota contributed to the decay rates of FIB under varying conditions, the faecal source seemed to be the most important factor that influenced FIB decay rates. In addition, manure-derived FIB persisted longer than sewage derived FIB and extended survival of cattle manure FIB, and this was particularly distinct for *E. coli* in freshwater and enterococci in marine water (Korajkic *et al.*, 2013). A study that determined the inactivation rates of *E. coli*, enterococci and total coliform bacteria from various sources of sewage (sewage influent and effluent and storm drain run-off) in relation to different environmental factors in both seawater and freshwater revealed that SPM concentration (concentrations varied from 100 mg l⁻¹ described as medium levels to between 400-500 mg l⁻¹ (described as high levels) were not a significant factor for the inactivation of FIB in both water matrices. It was also stated that inactivation rates of FIB in fresh and seawater were not source dependant (Noble *et al.*, 2004). In contrast, we show that SPM concentrations do impact on decay rates of FIB in both brackish and freshwater, and the difference in trends overtime is source dependant. Previous studies have suggested that enterococci survive longer in brackish water compared with *E. coli* (Jin *et al.*, 2004). Results from this study reveal no difference between the decay rates of *E. coli* and enterococci from sewage in brackish and freshwater. Furthermore, *E. coli* from sheep faeces persisted at much higher concentrations in both water matrices compared with enterococci from sheep faeces.

It is generally considered that the association of FIB with particles in environmental waters can enhance survival, and in particular, provide protection from the bactericidal effects of sunlight. Walters *et al.*, (2014) performed *in vitro* experiments to determine the effect of total suspended solid (TSS) concentrations on the UV inactivation of *E. coli* and enterococci from municipal water in river water. They observed that as TSS concentrations increased inactivation rates of *E. coli* and enterococci decreased, but tailed off as SPM concentrations reached 100 mg/l, concluding that bactericidal effect of sunlight is severely reduced in environmental waters with concentrations of TSS exceeding 100 mg/l (Walters *et al.*, 2014). It was also noted that under high rainfall, TSS concentrations in river water can quite often substantially exceed 100 mg/l. In addition, the release of sewage into environmental waters from combined sewage overflow systems in particular is associated with higher sediment loads. However, this study determined

that in the absence of UV light, SPM concentrations impact on proliferation and decay rates of sheep faeces-derived FIB and sewage-derived total coliform bacteria in brackish water and sewage derived enterococci in freshwater. Whilst some studies have determined that UV inactivation plays a significant role in reducing FIB in environmental waters (Burkhardt *et al.*, 2000; Pommepuy *et al.*, 1992) another study has indicated that UV inactivation of FIB is severely reduced after SPM concentrations exceed 100 mg/l (Walters *et al.*, 2014). This is of particular importance under extreme weather conditions which can lead to high SPM concentrations in environmental waters (Wetz & Yoskowitz, 2013; Wong *et al.*, 2010).

Inactivation of FIB in the environment is an extremely complex process, however in general the initial response of FIB entering the water column (T0 to T1) was either proliferation or inactivation at a much smaller rate compared with subsequent time periods. These data suggest that conditions are more favourable at this earlier time point. One explanation could be that the decay rates of FIB are influenced by the actual attachment of FIB to particulate matter in the water column. In this study we consider that at the initial addition of FIB to the microcosms they have not come in to contact with environmental flocs/suspended sediments. Flocs are aggregates of different materials floating in the water column these include organic detritus and inorganic materials such as clay and silt (Malham *et al.*, 2014). An important component of these aggregates is the accumulation of exopolymers or extracellular polymeric substances (EPS) that are exuded from organisms in the aquatic environment (Wotton, 2004). Exopolymers mainly comprise carbohydrates, which aid in the flotation of the aggregates, they provide a good attachment site and are also utilised as a food substance (Wotton, 2004). It is well documented that bacteria in the water column preferentially attach to suspended material (Malham *et al.*, 2014; Kiorboe & Jackson, 2001). Kiorboe & Jackson, (2001) suggested that these tiny aggregates (termed marine snow for marine aggregates) are extremely nutrient rich and can support bacterial production, in addition to attracting protozoa that predate on bacterial cells. Thus the association of FIB with flocs in the water column can result in competition for nutrients and the subsequent grazing by protozoa. Many studies have attributed the decay of enteric bacteria in environmental waters to predation from protozoa (Barcina *et al.*, 1997; Iriberry *et al.*, 1994; Gonzalez *et al.*, 1992; Gonzalez *et al.*, 1991). However, the susceptibility to grazing can differ between bacteria (Iriberry *et al.*, 1994).

Kiorboe & Jackson, (2001) also showed that the constant movement of these highly productive aggregates results in a plume of dissolved organic matter (DOM) in the surrounding water column. It was suggested that the release of DOM from the aggregates could have a positive impact on the growth of bacteria in the surrounding water column (Kiorboe & Jackson, 2001).

The proliferation of **FIB** in the early stages of this study (between T0 and T1) supports the idea that the growth of bacteria is greater in the water column and subsequent predation occurs upon attachment to **SPM**. This is also highlighted in concentrations of **EPM** where decay rates tend to be much greater. **FIB** derived from sheep faeces exhibited an increase under certain conditions and persisted at higher levels compared with their sewage counterparts. One theory behind this decrease in decay rates is that **FIB** from sheep faeces are already present in a tight consortium bound together by faecal matter, this tight consortium may protect the contained **FIB** and the high nutrient levels contained in the faeces may sustain their survival. These data suggest that **SPM** influences the survival of **FIB** in the water column by the possible facilitation of predation. However, further studies are needed to confirm this. These data support the theory behind bacterial proliferation and predation in aquatic environments as proposed by Kiorboe & Jackson, (2001).

This study suggests that catchment-based risk assessments of **FIB** pollution need to take into account the source of the **FIB**, the influence of **SPM** concentration on survival and the hydrodynamic properties of the environment, all of which will influence the decay rate or proliferation of **FIB** in aquatic environments. The inactivation of **FIB** in aquatic systems is tremendously complex and this study demonstrates the need for further investigations to fully understand the factors influencing their survival and persistence so that human health may be safeguarded.

5.5 Acknowledgements

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CHAPTER 6

General Discussion

6 Discussion

This thesis is presented as a series of manuscripts for publication in peer reviewed scientific journals. Therefore, the aim of this final chapter is to summarise the data presented, and to relate the findings from the previous chapters to the aims and objectives as outlined in chapter 1. Briefly, the five key aims of this thesis are;

1. To investigate the role of sediments as a reservoir for FIB and other potentially pathogenic bacteria.
2. To examine the spatial ecology of FIB in relation to sediment composition (grain size and organic matter content)
3. To investigate the influence of point and diffuse pollution sources on the abundance of FIB, other potential pathogens and the indigenous microbial community in marine and estuarine sediments
4. To investigate the abundance of FIB and human pathogenic bacteria in estuarine sediments using both culture-based and molecular methods.
5. To investigate the influence of suspended particulate matter (SPM) on the survival of FIB in both fresh and brackish water.

6.1 Spatial and temporal distribution of faecal indicator bacteria in marine sediments surrounding an offshore sewage outfall pipe

Sewage effluent is often discharged untreated into marine waters. Consequently, many studies have only focused on FIB decay rates in marine waters (Rozen & Belkin, 2001; Fujioka *et al.*, 1981). However, information on the abundance of FIB in marine sediments is limited, and very few studies have addressed the influence of sewage effluent on the abundance of *E. coli*, total coliforms, enterococci (FIB), *Vibrio* spp. and the indigenous microbial community in sediments surrounding a sewage outfall.

In chapter 2, the abundance of culturable FIB, *Vibrio* spp. and heterotrophic bacteria was determined in marine sediments surrounding an offshore sewage outfall pipe. Results revealed that marine sediments surrounding the outfall pipe were a reservoir for FIB and *Vibrio* spp. Sediment samples were analysed for organic matter content and the grain sizes were determined by laser diffraction. Spatial variation of bacterial abundance was influenced by sediment composition. The finer sediment fractions (<125 µm) contained higher proportions of

organic matter and higher proportions of all bacterial groups isolated. In addition, the spatial variation in sediment type and bacterial abundance reflected hydrodynamic model predictions of the sewage plume, as sediment organic matter content and FIB abundance was greater in sampling points below the outfall pipe and in an east-west direction either side of the outfall.

The findings from this study determine that the release of sewage effluent into marine waters not only had an influence on the abundance of FIB and the indigenous microbial community, but on the composition of the surrounding sediments. These data have important implications for human health if marine sediments are resuspended and transported to nearshore waters. Furthermore, this study corroborates that of Thevenon *et al.*, (2011) who demonstrated that the release of sewage into aquatic environments can influence sediment composition. The alteration of sediment composition poses an added threat as finer sediment grains are more easily mobilized. Therefore, FIB discharged in sewage effluent in marine environments may potentially be transported to nearshore waters.

Further investigations into the fate and transport of FIB associated with deposits surrounding an offshore sewage outfall pipe are needed to safeguard public health.

6.2 Sediment composition influences the spatial variation in the abundance of human pathogen indicator bacteria in estuarine sediments

The enumeration of FIB in water samples has been the paradigm for estimating water quality in coastal zones, but there is an emerging view (supported by the data from Chapter 2) that sediments are a poorly studied and yet a significant reservoir of FIB. Consequently, almost all previous studies have only investigated FIB concentrations in the water column of aquatic systems (Quilliam *et al.*, 2011; Bordalo *et al.*, 2002; Rozen & Belkin, 2001). More recently, attentions have turned to the presence of FIB in aquatic sediments (Thevenon *et al.*, 2012; Chandran *et al.*, 2011). However, there is still a paucity of information on the presence and abundance of FIB and other potentially pathogenic bacteria in estuarine sediments, and their ecology in the coastal zone. To address this, in chapter 3 culture-based methods were used to determine the abundance of *E. coli*, total coliforms and enterococci (FIB), *Campylobacter*, *Salmonella* and *Vibrio* spp. and heterotrophic bacteria.

Results revealed that estuarine sediments are a significant reservoir for FIB and other potentially pathogenic bacteria, as the number of culturable *E. coli*, coliform and *Vibrio* spp. in sediments and the water column revealed that their abundance was 281, 433 and 58-fold greater in sediments (colony forming units (CFU)/100g) when compared with the water column

(CFU/100ml), respectively. In addition, there were significant positive correlations between the abundance of each bacterial group and all other groups enumerated, indicating that culturable FIB are a good proxy for the presence of other bacterial groups.

Sediment grain sizes and organic matter content were determined for the same sediment samples to investigate the spatial variation of FIB and other potential pathogens in relation to sediment composition. Results determined that the finer sediment fractions of clay, silt and fine sand comprised higher proportions of organic matter and contained significantly higher proportions of all bacterial groups enumerated. Furthermore, this trend was observed for the spatial variation of the indigenous microbial community. These data corroborate with results determined in chapter 2.

Sediment cores were not profiled as all sediment samples were taken from the top 5 cm layer of the sediment (within approximately 5 cm depth). Marine sediments comprise only a thin oxic surface layer, particularly in productive shallow coastal areas where the depth of oxygen penetration is restricted to depths of millimetres compared to cm in oceanic sediments that are situated beneath deep oligotrophic water (Kristensen, 2000). Whilst many bacteria are capable of surviving in environments without oxygen using anaerobic respiration, it is more energetically efficient to utilise oxygen as the electron receptor for aerobic respiration (Kristensen, 2000). Therefore, the thin oxic layer of surface sediments provides more favourable conditions for the persistence and growth of FIB. In addition, the fine surface sediments that are typical of tide dominated estuarine systems (Wells, 1995), typically contain higher proportions of organic matter compared with coarser sediment fractions (Perkins *et al.*, 2014), also contributing to the survival and persistence of FIB.

Sample sites were chosen in close proximity to potential point and diffuse sources of pollution. However, results were significantly correlated to sediment composition and not reflective of source.

Findings from this study therefore confirm that estuarine sediments are a reservoir for FIB and other potentially pathogenic bacteria. Furthermore, the positive association of FIB with finer sediment particles that are more readily resuspended back into the water column has significant implications for catchment management, as the resuspension of FIB bound to fine sediments into the water column could transport microbial pollution to recreational or commercial waters. Faecal contamination of a valuable ecosystem such as the Conwy estuary that supports a commercial shellfish bed and impacts on bathing waters can have significant socioeconomic consequences. Here, we show that the finer sediment fractions <125 µm are “hotspots” for microbial contamination. Therefore, current sampling strategies that rely solely

on the enumeration of FIB in the water column may severely underestimate FIB concentrations in the environment. In addition, the place and time of sampling could have an influence on the results based on sediment resuspension and transport. Therefore, sediments should be considered in routine protocols to assess microbial contamination of environmental waters. This study also highlights the difficulty in identifying the source of microbial contaminants in an estuarine system, that is subject to point and diffuse sources of pollution. Subsequently, further studies are needed to determine the origin of microbial contaminants in the Conwy estuary. In addition, culture-based methods used in this study to determine bacterial abundance in the sediments do not distinguish between pathogenic and non-pathogenic bacteria. Molecular-based methods that target specific virulence genes of known human pathogens represent one method to overcome this limitation, and were therefore utilized in chapter 4 to determine actual pathogen abundance of estuarine sediments.

6.3 Detection and quantification of FIB and human pathogenic bacteria in estuarine sediments using culture-based, PCR and qPCR methods

Whilst many studies have investigated FIB concentrations in the water column of an estuarine environment (Quilliam *et al.*, 2011), the pathogen abundance of estuarine sediments is currently under-researched. In this chapter, both culture-based and molecular methods (PCR and qPCR) were used to determine FIB and pathogen abundance in sediments of the Conwy estuary.

Both PCR and qPCR detected the presence of human pathogenic bacteria in sediments that did not yield cultured colonies for the same bacterial species. The use of PCR revealed the possible presence of free-living shiga-toxicogenic bacteriophage in the environment. The ability of bacteriophage to transfer virulence factors between bacteria may cause a significant human health risk by transforming non-pathogenic bacteria into pathogenic bacteria (Martinez-Castillo & Muniesa, 2014). In addition, the rise of new strains may occur from the transference of genes by bacteriophage (Martinez-Castillo & Muniesa, 2014). Results produced by qPCR indicated much higher quantities of FIB in sediments than determined by culture-based methods. These results indicate the possible presence of FIB and other potential pathogenic bacteria in a VBNC state in estuarine sediment. The additional analysis of sediment composition revealed that in general (and in line with data from chapters 2 and 3), the finer sediments of clay, silt and very fine sand (<125 µm) contained higher concentrations of FIB and pathogenic bacteria. However, there was one notable exception to this correlation; the abundance of *Salmonella* as determined by qPCR

had a significant positive correlation with sediments >125 µm. Results revealed that estuarine sediments are a significant reservoir for pathogenic bacteria and the finer sediments harbour significantly greater concentrations of FIB and pathogens. The presence of *Salmonella* in the absence of *E. coli* and enterococci suggests that *Salmonella* may occupy different ecological niches compared with FIB that are currently utilised to monitor their presence in recreational waters. The detection and quantification of FIB and pathogens by PCR and qPCR in the absence of cultured colonies highlights the limitations of culture-based methods that do not detect VBNC cells and cannot distinguish between pathogenic and non-pathogenic types. Whilst the use of PCR overcomes these limitations by the detection of specific species and virulence genes, it does not provide any quantitative data. In comparison, qPCR is highly specific and provides quantitative data. However the use of DNA does not distinguish between living and dead cells and is a proposed area for further research. In addition, the use of PCR and qPCR to detect virulence genes in cultured colonies could have provided extra information on culturable pathogen abundance and is also proposed as future work.

The findings from this study demonstrate that the fine sands of estuarine sediments are a “hotspot” for microbial contamination and therefore sediment type should also be considered in sampling protocols to monitor microbial pollution of aquatic systems. Furthermore, whilst there are advantages with the use of each method utilized in this study, each method also exhibits limitations. Alternative methods which may overcome some of the limitations described in this study include immunological methods that are used for the detection of FIB and utilise either polyclonal or monoclonal antibodies that are specific to certain antigens. IFA (Immunofluorescence assay) and IEA (Immuno-enzyme assay) are commonly used and have the advantage of being able to identify single target cells. This can be achieved by a direct method which utilises an antibody with a fluorescence marker or an indirect method that utilises a primary antibody followed by a second fluorescently labelled antibody which targets the primary antibody. Enumeration of the fluorescence-labelled bacteria can then be attained by either flow cytometry, solid phase cytometry or by using a fluorescent microscope. The efficiency of each enumeration method was tested and it was concluded that results were similar for each technique however solid phase cytometry did show the most accuracy when detecting labelled cells that were integrated with a high volume of unlabelled cells (Lemarchand *et al.*, 2001). Commonly used methods include the ELISA (Enzyme-Linked Immunosorbent Assay) which is based on the immunocapture of antigens or cells by the introduction of antibodies (Rompre *et al.*, 2002). The “sandwich ELISA” incorporates an enzyme labelled antibody for easy detection (Lazcka *et al.*, 2007), this method however does rely on the antibody being highly specific to only the target

cell or antigen to avoid cross reactions with non-target organisms. The ELISA method is simple and can be effective especially when being utilised to monitor drinking water but when applied to naturally contaminated samples detection can be limited (Hanai *et al.*, 1997). Biosensor technology works on biorecognition, and is based on the detection of the physico-chemical change brought on by the interaction of a biological receptor molecule such as a nucleic acid, antibody or enzyme interacting with an analyte such as an antigen. The physico-chemical change that can occur may be a heat change or change in the electrical potential which is then transformed by a transducer into a signal that can be displayed and measured. Often when detecting pathogenic bacteria the enzyme as in the ELISA method will be utilised as a label for DNA probes or antibodies rather than the bacterial recognition component (Lazcka *et al.*, 2007). Speed, sensitivity and specificity are the focus for the improvement of new techniques (Connelly and Baeumner, 2012). Advances in DNA sequencing include next-generation sequencing technologies which utilise instruments that have the capacity to produce millions of sequence reads in one single run (Mardis, 2008). In particular, RNA-seq (RNA sequencing) utilises the capabilities of next-generation sequencing to detect and quantify RNA within a sample. Therefore, by utilising RNA the results would be more representative of living cells compared with the use of DNA which does not differentiate between living and dead cells. Results from this study suggest that the use of a single method may not be sufficient to assess the microbiological quality of environmental samples thus a combination of methods may be more effective to safe guard human health. However, future studies should include the use of the described alternative methods to determine their efficiency as a substitute to enumerate bacteria in estuarine sediments.

Further investigations are also needed to determine *E. coli* and enterococci as efficient indicators for the presence of *Salmonella* in aquatic environments. In addition, the presence of VBNC FIB in estuarine sediments is poorly understood and requires further investigation to fully understand the risk they pose to human health. The transportation of virulence genes between bacteria in the environment should also be further investigated to circumvent the rise of new strains and to avert any potential increase in pathogen concentrations in the environment. Furthermore, sediment resuspension and transport in an estuary is extremely complex, in particular for a tide-dominated system. The influence of suspended sediments on the survival of FIB is currently under-researched and is therefore addressed in chapter 5.

6.4 Decay rates of FIB from sewage and sheep faeces in brackish and freshwater microcosms with contrasting SPM concentrations

Whilst previous studies have ascertained that sediments contain significantly higher proportions of FIB compared with the water column (Perkins *et al.*, 2014) and can be attributed to higher proportions of organic matter (chapters 2, 3 and 4) (Lee *et al.*, 2006), to our knowledge, the survival of FIB in relation to SPM concentrations in the water column has not been investigated. This is particularly important for a tide-dominated estuary that usually incorporates large areas of fine sediments that are easily mobilised (Wells, 1995). To address this, laboratory-based microcosms containing brackish and freshwater with Low, High and Extreme SPM concentrations were inoculated with sheep faeces and crude sewage. The bacterial decay rates were determined over 5 consecutive days.

Results revealed that SPM concentrations had an influence on the decay rates of FIB from both pollution sources in brackish water, but had little influence in freshwater. In general, FIB concentrations increased with a decrease in SPM concentrations. In addition, the survival rates of FIB differed between species and between source, with FIB from sheep faeces proliferating and surviving at much higher concentrations than their sewage counterparts irrespective of water type. Whilst bacterial sorption to particulate matter may have an influence on the results determined in this study the bacterial decay rates were not consistent with particulate matter concentrations across all microcosms and varied with source and system. The survival of FIB in aquatic systems is complex, these findings revealed that the decay rates of FIB in aquatic systems are system specific, species specific, source dependant, and influenced by SPM concentrations.

These data have particular importance for an estuarine environment where levels of SPM concentrations have been recorded at 4 g/l under storm conditions (Liu & Huang, 2009). Furthermore, recent climate data has revealed that extreme weather events such as hurricanes and storms are on the increase (Wetz & Yoskowitz, 2013) which intensify sediment resuspension and transport (Liu & Huang, 2009). Catchment-based management strategies to assess environmental pollution should therefore take in to account the source of pollution the species involved, the hydrodynamics of the environmental system and the influence of SPM concentrations. Further research is needed to fully understand the influence of SPM concentrations on FIB survival and transport in environmental waters.

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APPENDIX I

Sediment composition influences spatial variation in the abundance of human pathogen indicator bacteria within an estuarine environment

Published article

APPENDIX II

The interaction of human microbial pathogens, particulate material and nutrients in estuarine environments and their impacts on recreational and shellfish waters

Published article

APPENDIX III

Using chemical, microbial and fluorescence techniques to understand
contaminant sources and pathways to wetlands in a conservation site

Published article