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Source tracking of faecal indicator bacteria of human pathogens in bathing waters; an evaluation and development

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Source tracking of faecal indicator bacteria of human pathogens in bathing waters; an evaluation and development

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Abstract

Bacterial water pollution is a significant problem because it is associated with reduction in the 'quality' of water systems with a potential impact on human health. Faecal indicator bacteria (FIB) are usually used to monitor the quality of water, and to indicate the presence of pathogens in water bodies. However, enumeration alone does not enable identification of the precise origin of these pathogens. This study aimed to monitor the quality of bathing water and associated fresh water in and out of the 'bathing season' in the UK, and to evaluate the use of microbial source tracking (MST) such as the host-specific based polymerase chain reaction (PCR) and quantitative PCR (qPCR) to recognize human and other animal sources of faecal pollution.

The culture-dependent EU method of estimating FIB in water and sediment samples was performed on beach in the South Sands, Kingsbridge estuary, Devon, UK- a previously 'problematic' site. FIB were present at significant levels in the sediments, especially mud, as well as fresh water from the stream and pond flowing onto South Sands beach. However, the quality of bathing water was deemed to be 'good' and met with the EU bathing water directive 2006. Using MST it was possible to successfully classify the nature of the source from which the bacteria came. PCR was applied to detect the *Bacteroides* species 16S rRNA genetic markers from human sewage and animal faeces. All water and sediment samples displayed positive results with a general *Bacteroides* marker indicating the presence of *Bacteroides* species. Host-specific PCR showed the human *Bacteroides* genetic marker only in the sediment of the stream.

However, limitations in the 'types' of probes available and in the persistence of these markers were identified. Thus, novel dog-specific *Bacteroides* conventional PCR and qPCR primer sets were developed to amplify a section of the 16S rRNA gene unique to the *Bacteroides* genetic marker from domestic dog faeces, and these were successfully used to quantify those markers in water samples at a 'dog permitted' and 'dog banned' beach (Bigbury-on-Sea, Devon, UK).

Generic, human and dog *Bacteroides* PCR primer sets were also used to evaluate the persistence of *Bacteroides* genetic markers in controlled microcosms of water and sediment at differing salinities (< 0.5 and 34 psu) and temperature (10 and 17 ºC). The rates of decline were found did not differ significantly over 14 and 16 days for the water and sediment microcosms, respectively. Beach sediments which were studied in this project may act as a reservoir for adhesive FIB, and this was confirmed using fluorescence *in situ* hybridisation (FISH). The similarity in the persistence of these *Bacteroides* 16S rRNA genetic markers in environmental water and sediment suggests that viable but non-culturable (VBNC) *Bacteroides* spp. do not persist in the natural environment for long. Therefore, 16S rRNA genetic markers can be of value as additional faecal indicators of bathing water pollution and in source tracking. Thus, in this study MST methods were successfully used and in future applications, dog-specific primer sets can be added to the suite of host-specific *Bacteroides* genetic markers available to identify the source(s) of problem bacteria found on failing beaches.

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List of abbreviations

µg	Microgram
µl	Microliter
A/ A	Ratio of absorbance for DNA quality
AE	Elution buffer
AIDS	Acquired immunodeficiency disease syndrome
AFLP	Amplified fragment length polymorphism
AL	Lysis buffer
ALFP	Amplified length fragment polymorphism
ANOVA	Analysis of variance
APHA	American public health association
ARA	Antibiotic resistance analysis
ASL	Lysis buffer
AW	Wash solution for DNA
B. fragilis	*Bacteroides fragilis*
Bac303	*Bacteroides* probe for FISH
Bac32F	Forward general *Bacteroides* primer
Bac708R	Reversed *Bacteroides* primer
BBE	*Bacteroides* bile esculin agar
BLAST	Basic local alignment search
bp	Base pair
BPRM	*Bacteroides* phage recovery medium
BST	Bacterial source tracking
BWD	Bathing water directive
C	Concentration

cDNA	Complementary DNA
CF128F	Forward cow-specific *Bacteroides* primer
CFU	Colony forming unit
cm	Centimetre
Ct	Threshold cycle
CUP	Carbon-source utilization profiling
dATP	Deoxyadenosine triphosphate
ddH_2O	Double-distilled water
DEFRA	Department for environment, food and rural affairs
DF	Dog-specific *Bacteroides* primer
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
E. avium	*Enterococcus avium*
E. coli	*Escherichia coli*
E. coli O157:H7	*E. coli* enterohemorrhagic serotype O157:H7
EcoR1	*E. coli* restriction enzyme
E. durans	*Enterococcus durans*
EDTA	Ethylenediaminetetraacetic acid
EEA	European environment agency
E. faecium	*Enterococcus faecium*
Eff.	Efficiency
E. gallinarum	*Enterococcus gallinarum*
EMB	Eosin methylene blue
EPA	Environmental protection agency
ESAL	Environmental solutions Antigua limited

Esp	Enterococcal surface protein
ETEC	Entertoxigenic *E. coli*
EU	European Union
EUB338	General bacterial probe for FISH
EUBWD	EU bathing water directive
EUUWWTD	EU urban water waste treatment Directive
EUWFD	EU water framework directive
FAME	Fatty acid methyl ester
FC	Faecal coliform
FIB	Faecal indicator bacteria
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
FS	Faecal Streptococci
g	gram
GATC	Genome analysis and technology core
GCRWQ	Guidelines for Canadian recreational water quality
gDNA	Genomic DNA
GESAMP	Group of experts scientific aspects of marine protection
HF183F	Forward human-specific *Bacteroides* primer
HFR	Reverse human-specific *Bacteroides* primer
Hind11	*Haemophilus influenza* restriction enzyme
HoF597F	Forward horse-specific primer
In	Natural $\log_{10}$
IPPC	Integrated pollution prevention and control
IPTG	Isopropyl-β-thiogalcto-pyranoside

l	litre
L4	Plymouth offshore station
LB	Luria-Bertani
LH-PCR	Length heterogeneity PCR
LOD	Limit of detection
Log	Logarithm
LSD	Least significant difference
LT	Heat-labile
LTSB	Lauryl tryptose sulphate broth
m	Mass of plasmid (g)
MAR	Multiple antibiotic resistances
MCL	Maximum composite likelihood
ml	millilitre
MLSB	Membrane Lauryl sulphate broth
mm	millimetre
mM	millimolar
MST	Microbial source tracking
MW	Molecular weight
N	Log_{10}
n	Size of plasmid (bp)
NaCl	Sodium chloride
NCBI	National centre for biotechnology information
NCNERR	North Carolina national estuarine research reserve
NCTC	National collection of type cultures
ng	nanogram

NGS	Next generation sequencing
NJ	Neighbour-joining
nm	nanometre
ºC	Degrees Celsius
OD	Optical density
ºN	Latitude
OTU	Operational taxonomic unit
ºW	Longitude
p value	Statistical probability
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEGE	Pulsed-field gel electrophoresis
PF163F	Forward pig-specific *Bacteroides* primer
PFGE	Pulsed-field gel electrophoresis
pmol	picomole
PVP	Polyvinylpyrrolidone
psi	Pounds per square inch
psu	Practical salinity units
Pvu11	*Proteus vulgaris* restriction enzyme
qBac560F	Generic *Bacteroides* qPCR forward primer
qBac725R	Generic *Bacteroides* qPCR reverse primer
qPCR	Quantitative PCR
r	Pearson's correlation
R^2	Coefficient of correlation
RAPD	Random amplification of polymorphic DNA

rep-PCR	Repetitive element PCR fingerprint
rDNA	Ribosomal deoxyribonucleic acid
R. coprophilus	*Rhodococcus coprophilus*
RFLP	Restriction fragment length polymorphism analysis
RPM	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RS	River sediment
RT-PCR	Real-time PCR
RW	River water
S and B	Slanetz and Bartley medium
SDS	Sodium dodecyl sulphate
SE	Standard error
Sed.	Sediment
SP6	Long primer (promoter)
spp.	Species
SPSS	Statistical package for social sciences programme
SS	Sea sediment
ST	Heat-stable
SW	Sea water
t	Time
T7	Long primer (promoter)
TAE	Tris-acetate-EDTA buffer
TE	Tris-EDTA buffer
Temp.	Temperature
TLE	Tris-low EDTA buffer

T-RF	Terminal restrictions fragment
T-RFLP	Terminal restriction fragment length polymorphism
U	unit
UK	United Kingdom
UNEP	United Nations environment programme
USA	United State of America
UV	Ultraviolet
v	Volume
v/ v	Volume/ volume
VBNC	Viable but non-culturable
W	Water
w/ v	Weight/ volume
WHO	World Health Organization
x g	Times gravity
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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KHWAM

Dedication

This thesis is dedicated to the memory of late my beloved mother

(11th June 2011)

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other university award without prior agreement of the Graduate Committee. This study was financed with the aid of the Ministry of the Higher Education and Scientific Research, Baghdad, Iraq. Relevant scientific seminars and conferences were attended at which work was presented and papers have been prepared for publication:

Publications (please refer to Appendix 1)

- Hussein K. R., Bradley G. and Glegg G. 2012. An evaluation of bacterial source tracking of faecal bathing water pollution in the Kingsbridge estuary, UK. In: Kay, D. and Fricker, C. (Eds) .The significance of faecal indicator in water: A global perspective. Royal Society of Chemistry, Cambridge. pp 114-122 (ISBN: 9781849731690). This paper forms the basis of this thesis, Chapter 3.

- Hussein K. R., Nisr R. B., Glegg G. and Bradley G. 2012. New *Bacteroides*-specific sequences, published in NCBI blast for gene bank, 6th August 2012. GenBank accession numbers: **JX431865, JX431866 and JX431867** (Appendix 1).
  http://www.ncbi.nlm.nih.gov/nuccore/JX431865

- Hussein K. R., Waines P., Nisr R. B., Glegg G. and Bradley G. (2014) Development and use of a *Bacteroides* 16S rRNA PCR assay for source tracking dog faecal pollution in bathing waters. Hydrol Current Res, 5 (163): 1-8 (doi:10.4172/2157-7587.1000163). This paper forms the basis of this thesis (Chapter 4).

- Hussein K. R., Waines P. L., Glegg G. and Bradley G. (2014). Survival and persistence of *Bacteroides* species used as faecal indicators and the recovery of 16S rRNA markers under controlled conditions. In Mendez-Vilas, A. (Ed). Industrial, medical and environmental applications of microorganisms: Current status and trends. Wageningen Academic Publishers. Wageningen. ISBN 978-90-8686-795-0. This paper forms the basis of this thesis (Chapter 5).

Platform presentations

- Monitoring of faecal water pollution using 16S rRNA *Bacteroides* specific markers including novel dog-specific primers, BioMicroWorld, V international conference on environmental, industrial and applied microbiology, Madrid, Spain. 2nd-4th October 2013.
- An evaluation of bacterial source tracking of faecal bathing water pollution, UK. Postgraduate society conference, Plymouth University, UK. 14th March 2012.
- Faecal indicator source tracking in bathing waters, School of Biological Sciences, Plymouth University, UK. 17th April 2013.

Poster presentations

- Effects of temperature and salinity on the survival of faecal *Bacteroides* and persistence of PCR recovery. American Society of Microbiology (ASM), San Francisco, USA. 16th- 19th June 2012.
- Polymerase chain reaction (PCR) for the detection of *Bacteroides* spp. specifically from dogs. Society of Applied Microbiology (SfAM), Edinburgh, UK. 2nd- 5th July 2012.

- Microbial source tracking of human pathogenic bacteria from faecal pollution in the Kingsbridge estuary, the UK. Marine conference spirit of discovery, 3rd annual conference, Plymouth University, UK. 20th December 2010.

  http://f1000.com/posters/browse/summary/803

- A bacteriological study and source tracking of water pollution in the Kingsbridge estuary, UK. Environmental pollution: Chemical and biological approaches for protecting organisms, 1st annual conference 2011, (ERIC), Plymouth University, UK. 4th April 2011.

  http://cdn.f1000.com/posters/docs/803

- An evaluation of bacterial source tracking of faecal water pollution in the Kingsbridge estuary, UK. Faecal Indicators: Problem or Solution? An international conference, Edinburgh, UK. 6th-8th June 2011.
- Microbial source tracking of human pathogenic bacteria from the waters in the Kingsbridge estuary. Postgraduate society conference, Plymouth University, UK. 18th November 2010.
- Monitoring of faecal water pollution using *Bacteroides* 16S rRNA specific markers, the centre for agricultural and rural sustainability (CARS), Duchy College, UK. 19th June 2013.
- Survival and persistence of *Bacteroides* species used as faecal indicators and the recovery of 16S rRNA markers under controlled conditions. BioMicroWorld, V international conference on environmental, industrial and applied microbiology, Madrid, Spain, 2nd-4th October 2013.

- Survival and persistence of *Bacteroides* species used as faecal indicators and the recovery of 16S rRNA markers under controlled conditions. The postgraduate society conference series. Plymouth University, 19th March 2014.

Attendance to training courses, conferences and taught sessions is shown in the Appendices.

Professional membership

- Member, Society for Applied Microbiology (SfAM) 2010 to date.
- Member, American Society of Microbiology (ASM) 2012 to date.
- Member, International Postgraduate Society, Plymouth University, 2010 to date.

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Signed ----------

Date ----------

Chapter One

General introduction and literature review

1.1 Introduction

A major public concern around the world is the attainment and maintenance of good water quality. Clean and safe water is essential for the health of humans as well as other living creatures. Achieving a good grade of drinking and bathing water is difficult given the simultaneous need to dispose of sewage and other waste waters which even after sewage treatment may contribute pathogens and other pollutants (Shao *et al.*, 2006). However, numerous disadvantaged communities suffer from health-related problems attributed to poor water quality and hygiene. There may be many possible sources of water system contamination, including absence of adequate waste treatment and disposal services, rapid-paced urban development, and agricultural (livestock and poultry) activities (Graves, 2000). Microbial water pollution leads to the degradation of water quality. The quality of coastal and bathing water is directly related to anthropogenic activities (Touron *et al.*, 2007). A serious ecological and public health concern in marine/ sea coastal zones used for recreation is microbial pollution of bathing water, particularly those situated near highly populated areas (Janelidze *et al.*, 2011). As a result, many states and organizations have issued recreational or bathing water quality guidelines in order to help protect public health in lakes and coastal water (Zhang *et al.*, 2012).

In numerous parts of the world, faecal pollution of the bathing waters in coastal areas may cause restriction in the use of recreational and commercial waterways (Ahmed *et al.*, 2009c). A key source of pollution is faecal pollution from people and other animals and faecal indicator bacteria (FIB) are used as indicators of microbial water quality especially during the

bathing season (Zhang *et al.*, 2012). Many different pathogenic microorganisms are found in water systems, but it is difficult and expensive to monitor bathing water for all these pathogens at the same time. Instead a certain bacterium or a group of bacteria is used as the indicator of these pathogens (Rhodes and Kator, 1991). Human activities can accelerate the rate of polluted material and pathogenic microorganisms into water ecosystems (Janelidze *et al.*, 2011).

Dwight *et al.* (2004) stated that a single exposure event rather than exposure over time has been observed by several epidemiological studies in developing countries. In addition, similarly most previous studies focused on coastal water polluted with domestic sewage, and have identified correlations between faecal water pollution levels and incidence of disease symptoms. Several pathogens are spread by human and animal faeces, which can contain specific enteric pathogens, including *Salmonella typhi*, *Shigella* (Table 1.1), hepatitis A virus, and Norwalk-group viruses (Ray, 1989; Scott *et al.*, 2002; Brooks *et al.*, 2007). Moreover, waste water derived from the farming of domestic animals such as cattle, horses, and poultry may additionally supply pathogens such as *Escherichia coli* (*E. coli*) O157:H7 (Ahmed *et al.*, 2009c). The group of experts on the scientific aspects of marine environmental protection (GESAMP, 2001) stated that in terms of the marine environment, two main routes to exposure of humans to potential pathogens arising from faecal pollution:

i. directly through bathing, surfing and boating activities and
ii. indirectly through the consumption of seafood.

Table 1.1 Primary FIB and pathogens found in water and seafood contaminated by faecal material. Adapted from Brooks *et al.* (2007).

Microorganisms	Main importance in water and seafood
E. coli	Indicator or pathogen
Enterobacter aerogenes	Indicator
Klebsiella spp.	Indicator or pathogen
Enterococcus faecalis	Indicator
Salmonella spp.	Pathogen
Shigella spp.	Pathogen
Vibrio cholerae	Pathogen
Vibrio parahaemolyticus	Pathogen
Campylobacter jejuni	Pathogen
Yersinia enterocolitica	Pathogen
Staphylococcus aureus	Pathogen
Clostridium perfringens	Pathogen
Bacillus cereus	Pathogen
Pasturella tularensis	Pathogen
Mycobacterium avium	Pathogen

1.2 Sources of bathing water pollution

Microbial pollution i.e. increased levels of pathogenic microorganisms, can result from the introduction of faecal material into the aquatic ecosystem. Faecal pollution generally originates from several sources. These include (Figure 1.1) domestic sewage, discharged raw or having passed through sewage treatment plant, sewage discharge from pavements by accident or

design, wastes from domestic farm animals and wildlife (Ely, 1997; Servais *et al.*, 2007).

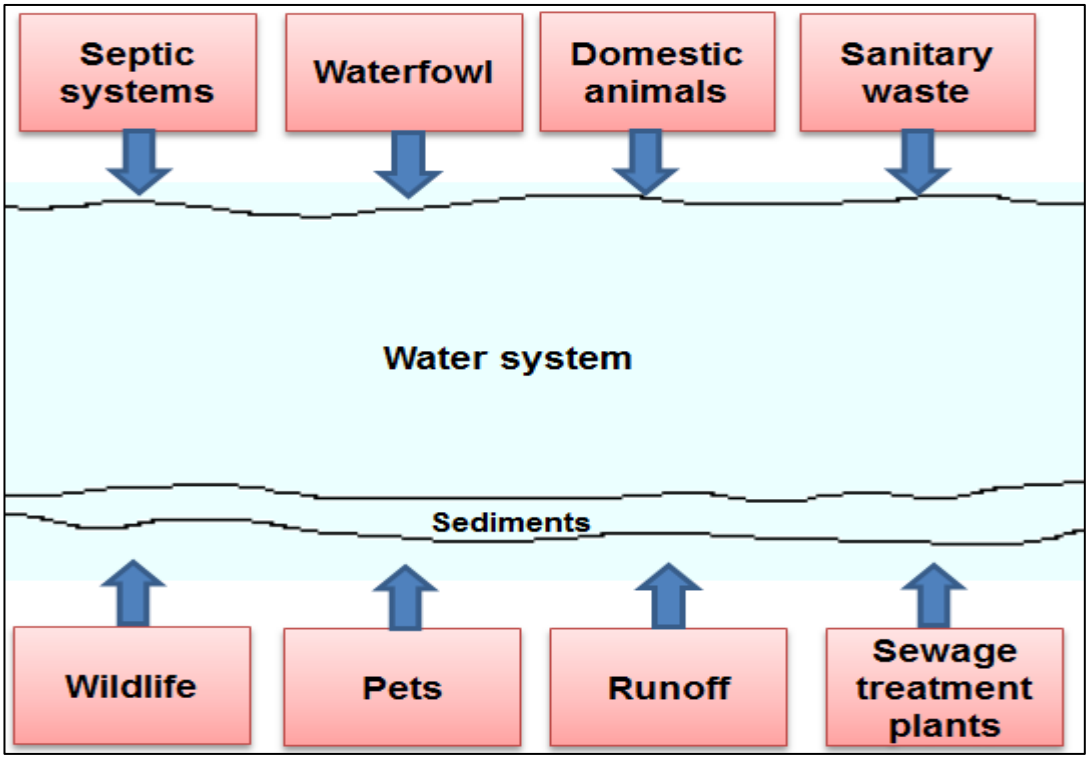


Figure 1.1 Major sources of faecal microorganism contamination in aquatic ecosystems, modified and adapted from Ely (1997).

Rural lands and river catchments are dominated by livestock farming, and the discharges from these farming activities are a noticeable source of water pollution, because the animal population is higher and the faecal output is therefore also high (Vinten *et al.*, 2004). According to the report published by the US environmental protection agency (EPA), most of the faecal bacteria present in storm water runoff are commonly from non-human sources (EPA, 2011). Waste from pets (dogs and cats), birds and rats appear to be a major source of faecal pathogens in urban runoff, collecting through the watershed

and presenting a high risk to public health because they can carry zoonotic pathogens (Crowther *et al.*, 2001; Overgaauw *et al.*, 2009; Atwill *et al.*, 2012; Smith and Whitfield, 2012). Crowther *et al.* (2002) and Gannon *et al.* (2005) have demonstrated that agriculture is an important factor in bathing water quality, as housed animals and pasture runoff can be a significant source of water pollution. Bathing water quality and waterborne diseases are associated with the density of livestock in rural agricultural lands. Furthermore, in the urban areas many sources lead to the pollution of water supplies such as urban runoff and negligence of householders (Ely, 1997). Leaks or damage to sewer pipe connections can occur, allowing sewage to flow into surface waters. Excessive rainfall and trees growing into sewer pipe lines are the common causes of sewer failures and storm overflow. In addition, grease from household and/ or restaurant drains can also block sewer pipes and causes sewage overflow into yards, streets and surface waters as stated by the North Carolina national estuarine research reserve (NCNERR, 2003). Roads, roofs and buildings also prevent rainfall water from infiltrating into the soil. Velocity and volume of surface water increases but the infiltration decreases due to large impervious surfaces, therefore storm water has shown to mobilize faecal bacterial pollutants (Martin, 2012). Figure 1.2 shows the various routes by which humans may be exposure to waterborne pathogens. In developing countries, the lack of water and sanitation infrastructures has serious complex effects on human health (Figure 1.3).

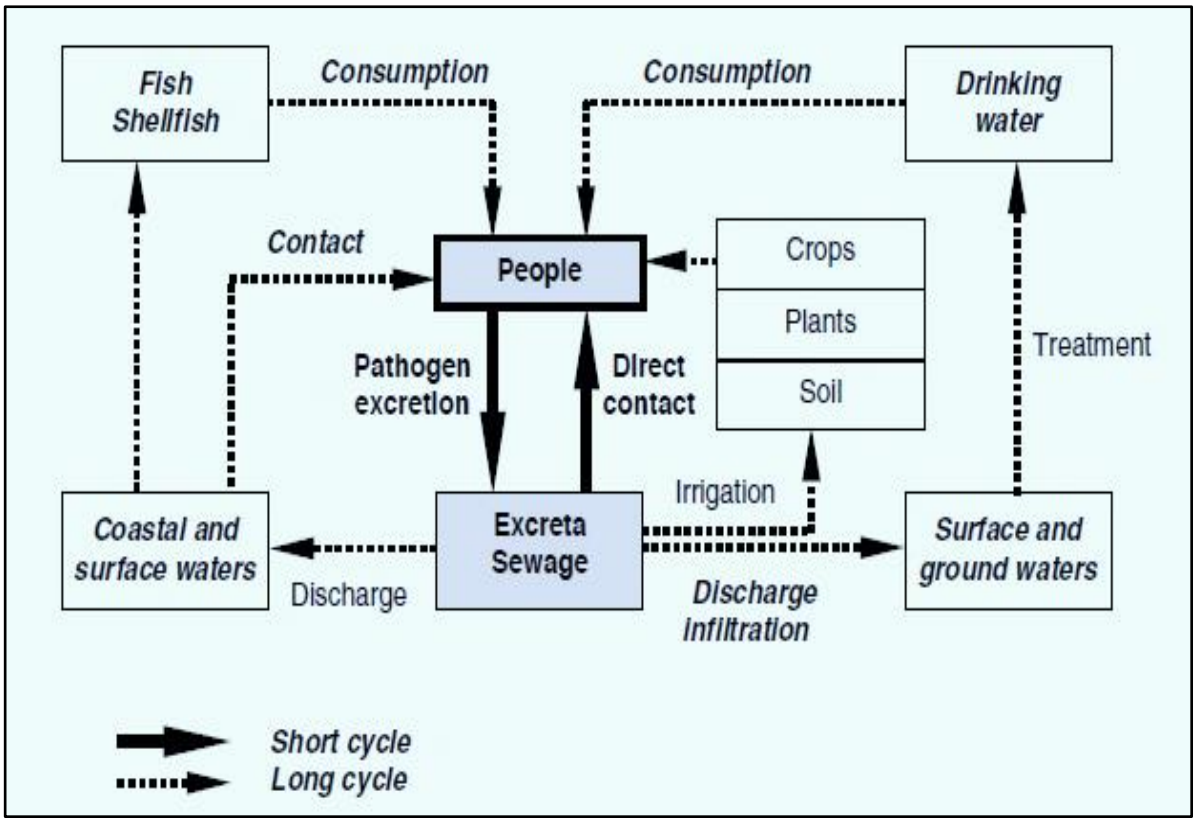


Figure 1.2 Common pathways leading to human exposure to microbial pathogens from the aquatic environment, adapted from Bosch *et al.* (2001).

Kay *et al.* (2008a; 2008b; 2010) have stated that the source(s) of microbial pollution of the aquatic ecosystems can be basically classified into two categories. The first category consists of the point sources, which are widely separated from each other and are often discrete waste discharges through pipelines. Examples include out-flow from yards and animal waste storage facilities on livestock farms, waste water treatment plants, combined sewer overflows and leaking septic tanks. The second category of microbial pollution originates from diffuse sources. These include surface runoff and soil leaching from agricultural land, farm manure, sewage sludge and septic tank waste (Crowther *et al.*, 2001; Okabe *et al.*, 2007).

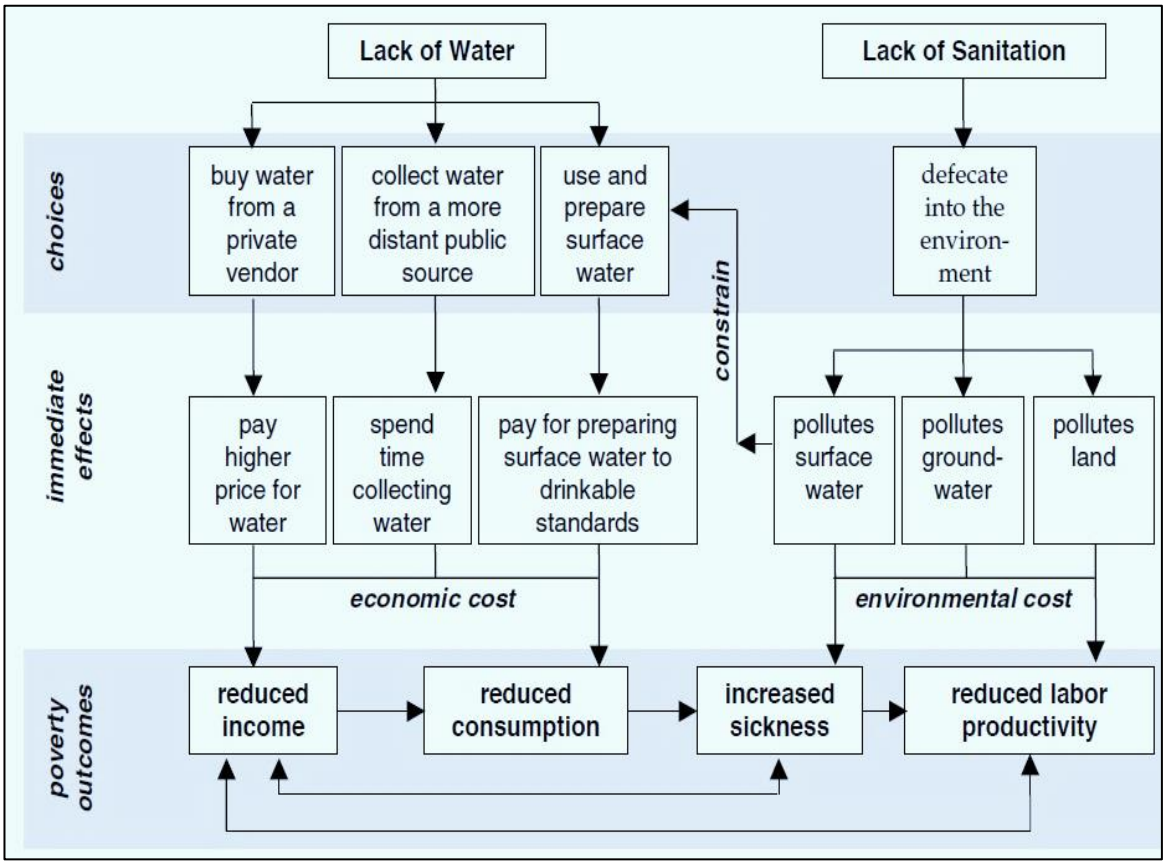


Figure 1.3 The effects of water shortage and sanitation on human health in developing countries, adapted from Bosch *et al.* (2001).

Human waste in urban areas is usually transmitted to a sewage treatment plant either by combined sewer systems or separate storm and sanitary sewer pipes. During or after rainfall or snowmelt, when increased waste water flows can exceed the capability of the transport systems or treatment services, these systems are designed to overflow directly into surface water. These occasions, known as combined sewer overflows, can be major sources of water pollution (Martin, 2012). In rural and suburban areas, septic systems are designed to treat domestic waste water and to prevent microbiological and faecal material pollution from reaching surface water and

groundwater. However, waste disposal systems occasionally fail or do not work properly due to blockage and overloading. This may lead to the discharge of pollutant material and pathogens into aquatic environments (Ahmed *et al.*, 2005; Martin, 2012).

Moreover, bathers are also considered to be a source of bacterial water pollution; human skin provides multiple niches for many microorganisms. Counts of aerobic bacteria in wet areas of human skin such as the axilla can reach 10^7 bacteria per cm^2 and for dry areas such as the forearm up to 10^2 per cm^2 (Leyden *et al.*, 1987). In addition, anaerobic bacteria on human skin also exist with counts up to 10^6 per cm^2 (Fredricks, 2001). Black (2002) stated that an adult human body consist of approximately 10^{14} microorganisms on the skin surface, mucous membranes, and in the digestive, respiratory, and reproductive system passages.

1.3 Faecal indicator bacteria

The American public health association (APHA) is an environmental organization which has worked on all aspects of water and waste water analysis techniques in the USA since 1905. The APHA has established the standard methods for the examination of water and waste water, which define the faecal coliform group as aerobic and facultative anaerobic, bacilli-shaped, Gram-negative, non-spore forming bacteria which ferment lactose with gas production in a suitable culture medium at 44.5 ± 0.2 ºC for 24 ± 3 hours (APHA, 1999). The presence of pathogens in an aquatic ecosystem can be tested based on the isolation and enumeration of FIB (Okabe *et al.*, 2007). Commonly, these FIB are used to determine bathing water quality of public health significance (Kay *et al.*, 2008a). Olivieri (1982) identified that

there certain criteria are necessary when assessing the risk of diseases and bathing water pollution using indicators including the following:

i. ‘The faecal indicator should always be present when the source(s) of the pathogenic microorganisms of concern is present, and absent in clean uncontaminated water.
ii. The faecal indicator should be present in numbers much greater than the pathogen(s), it is intended to indicate.
iii. The faecal indicator should respond to natural environmental conditions of water and waste water treatment processes in a manner similar to the pathogens of interest.
iv. The faecal indicator should be easy to isolate, identify, and enumerate’.

In general, bacterial indicators should die away in the environment and during treatment processes in a manner similar to the bacterial pathogens. However, viruses, protozoan cysts, and parasites are considerably more flexible in the environment and particularly more resistant to disinfection (El-Shaarawi and Pipes, 1982).

The intestines of warm-blooded animals contain abundant indicator bacteria (Table 1.2), and if these bacteria are present in environmental waters, then this are presumed to indicate faecal pollution, and the potential presence of pathogenic microorganisms. However, it has been reported that FIB such as *E. coli* may replicate in the environment (Anderson *et al.*, 2005). A study was carried out by Ahmed *et al.* (2009c) in Australia, who demonstrated that *E. coli* and faecal Streptococci were chosen as standard indicator bacteria, because of their successful use in other studies. They found that these

bacteria were relatively faeces-specific, and that there were new selective methods for their counting.

Table 1.2 The total numbers of *E. coli* and Enterococci calculated per gram of adult faeces of humans and domesticated animals (median values from 10 animals), adapted from NCNERR (2003).

Source	*E. coli*	Enterococci
Human	5.0×10^6	1.6×10^4
cow	2.0×10^4	2.0×10^5
dog	3.2×10^7	4.0×10^7
Horse	1.3×10^4	6.3×10^6
Pig	3.2×10^6	2.5×10^6
Sheep	3.2×10^6	1.3×10^6
cat	4.0×10^7	2.0×10^8
Chicken	4.0×10^6	3.2×10^7

Moreover, Payment *et al.* (2000) and Touron *et al.* (2007) have previously stated that *E. coli* and Enterococci have been traditionally been used, rather than anaerobic bacteria such as *Clostridium* spp. and *Bacteroides* spp., as indicators for the concentration of faecal input and the probability of the presence of pathogens. The identification and enumeration of FIB, rather than the actual pathogens, has several advantages. In extreme conditions FIB will live only for a short period of time which is advantageous because they can be used to indicate recent faecal pollution incidents. They can also be used to indicate pollution from a range of sources including humans and

other animals. However, these methods fail to identify the sources of faecal pollution (Toze, 1999; Scott *et al.*, 2002). Detection of the source of faecal pollution is requisite for the effective and efficient management of an aquatic environment, it also assists in reducing the time and cost of remedial measures (Okabe *et al.*, 2007).

Some bacteria, that are normally non-pathogenic such as *E. coli, Pseudomonas* spp.*, Aeromonas* spp.*,* and most of *Staphylococcus* spp. may behave as opportunistic pathogens in appropriate conditions (Niewolak and Opieka, 2000; Wiśniewska *et al.*, 2007). Unlike primary pathogens these are particularly invasive amongst susceptible individuals such as the elderly, new-borns, infants, and patients with acquired immunodeficiency syndrome (AIDS). These infections may be caused by aerosol inhalation and from body contact with contaminated recreational or irrigation water (Geldreich, 1996).

Total coliform bacteria were first used as an indicator of faecal water pollution in the 1890s (Edberg *et al.*, 2000), but after a few decades of research it has been become increasingly apparent that these bacteria are a poor indicator of the risks of water pollution (Feachem, 1975). Several researchers have therefore concentrated on faecal coliforms and faecal Streptococci as the most useful indicators of pathogens in bathing waters (Geldreich, 1970; Feachem, 1975). The amount of faecal material in water is measured by enumerating non-pathogenic faecal bacteria for which reasonably accurate microbiological techniques are available. The basis for this is the assumption that if the water is contaminated with the faeces of a large number of people, the person to person variation in excretion of pathogens and indicators will be averaged out and there will be a more or

less stable ratio of indicators (Pipes, 1982). In addition, Toze (1999) noted that in spite of the fact that most bacterial pathogens will grow easily, there are several difficulties associated with attempts to identify and enumerate them in water and waste water samples. These difficulties relate to the direct culture of specific pathogens and the time taken to identify them.

Another problem may occur in environmental strains, namely that viable bacteria can enter a dormancy step where they become non-culturable or viable but non-culturable (VBNC). This can cause an inability to isolate the pathogenic bacteria from environmental water (Porter *et al.*, 1995). Thus, the purpose of indicator microorganisms is to avoid the need to detect every pathogen that may be present in water. Furthermore, FIB are non-pathogenic, rapidly detected, easily enumerated, have similar survival points to those of the pathogens, and can be significantly correlated with the presence of pathogenic microorganisms (Scott *et al.*, 2002; 2004). They must also have qualities like the ability to survive in the intestines of warm-blooded animals as normal microflora do and be absent in clean waters. Similarly, a good faecal indicator should be unable to multiply in the environment and requires the same environmental conditions as do the pathogens (Bradley *et al.*, 1999; Gerba, 2000; Ishii and Sadowsky, 2008). Therefore, several microorganisms have been studied for use as indicators of water pollution including faecal coliforms, *E. coli*, Enterococci, *Bacteroides* spp*.* and *Clostridium perfringes* (Bradley *et al.*, 1999; Scott *et al.*, 2002; Griffith *et al.*, 2003; Ahmed *et al.*, 2008c), and these are discussed in more detail in subsequent sections.

1.3.1 Faecal coliforms

Faecal coliforms (FC) are thermo tolerant Gram-negative bacilli. They are facultatively anaerobic, non-spore forming, oxidase negative and able to ferment lactose. FC bacteria include members that originate in faeces of animals and humans such as *E. coli* and some other members not of faecal origin and capable of growing under the same conditions as for example *Klebsiella* spp., *Serratia* spp., *Erwinia* spp., *Enterobacter* spp., *Yersinia* spp. and *Citrobacter* spp. (Figure 1.4) . Faecal coliform bacteria can enter water systems directly with waste from mammals, birds, agriculture, treatment plants and sewage overflow (Zeckoski *et al.*, 2005; Doyle and Erickson, 2006). The enumeration of FC bacteria in water samples is usually given as the number of colony forming units per 100 millilitres (CFU 100 ml^{-1}).

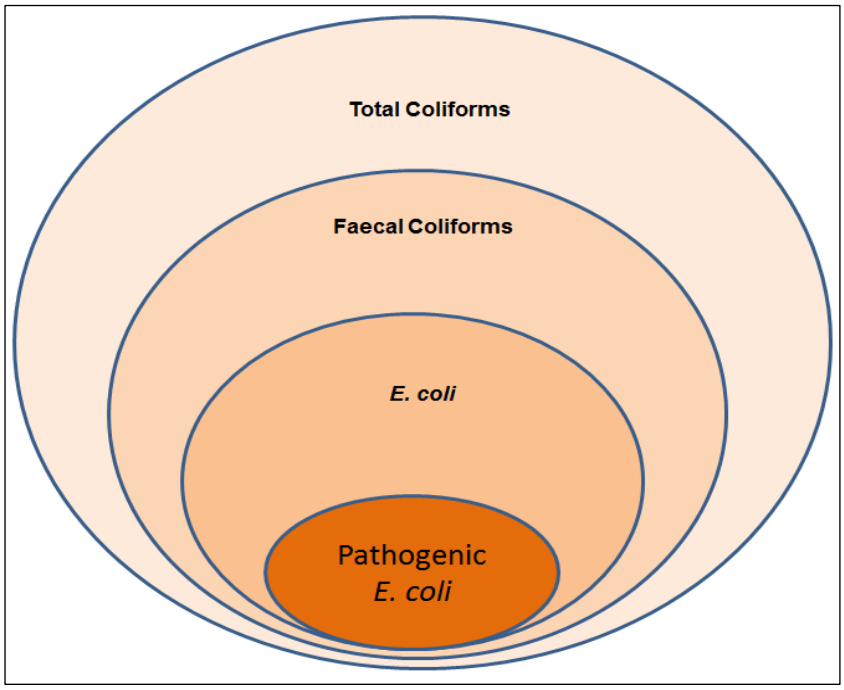


Figure 1.4 Chart depicting the relative abundance of coliform groups in the aquatic environment. Cycle size reflects the relative bacterial numbers. Modified and adapted from Wutor *et al.* (2009).

The FC group is the principal indicator of the suitability of water for domestic, industrial and other uses. The concentration of these bacteria is a significant criterion for the degree of pollution and sanitary quality (Gerba, 2000). Moreover, FC are present in the gut of warm-blooded animals in concentrations ranging from 10^6 to 10^9 cells per gram in human faeces, 10^6 to 10^7 cells per gram in animal faeces. With suitable temperature and availability of nutrients in aquatic environments, faecal coliform populations may be self-sustaining and may survive for a long time (Figure 1.5), but many factors affect this survival of these bacteria such as temperature, nutrients, moisture, solar radiation, predation, etc. (Geldreich and Litsky, 1976; Ishii and Sadowsky, 2008).

1.3.2 *Escherichia coli*

E. coli is a short, motile, and Gram-negative rod, lactose fermentative and Indole positive reaction. It grows as non-viscous flat colonies with a metallic sheen on selective media such as eosin methylene blue (EMB) agar. *E. coli* is a major member of the faecal coliforms and is a member of the family Enterobactereceae. *E. coli* is useful as a faecal indicator because it is not normally pathogenic to humans and is found at higher levels than the pathogens for which it acts as an indicator for faecal pollution (Brooks *et al.*, 2007; Ishii and Sadowsky, 2008). However, a new strain of *E. coli* O157:H7 which causes severe diseases that can be lethal for children was recently reported in the USA, Canada and Australia (Scott *et al.*, 2002; Ahmed *et al.*, 2008c; 2009c). About 10^6 *E. coli* cells are present in one gram of colonic content and may be released into the environment and water through defecation (An *et al.*, 2002).

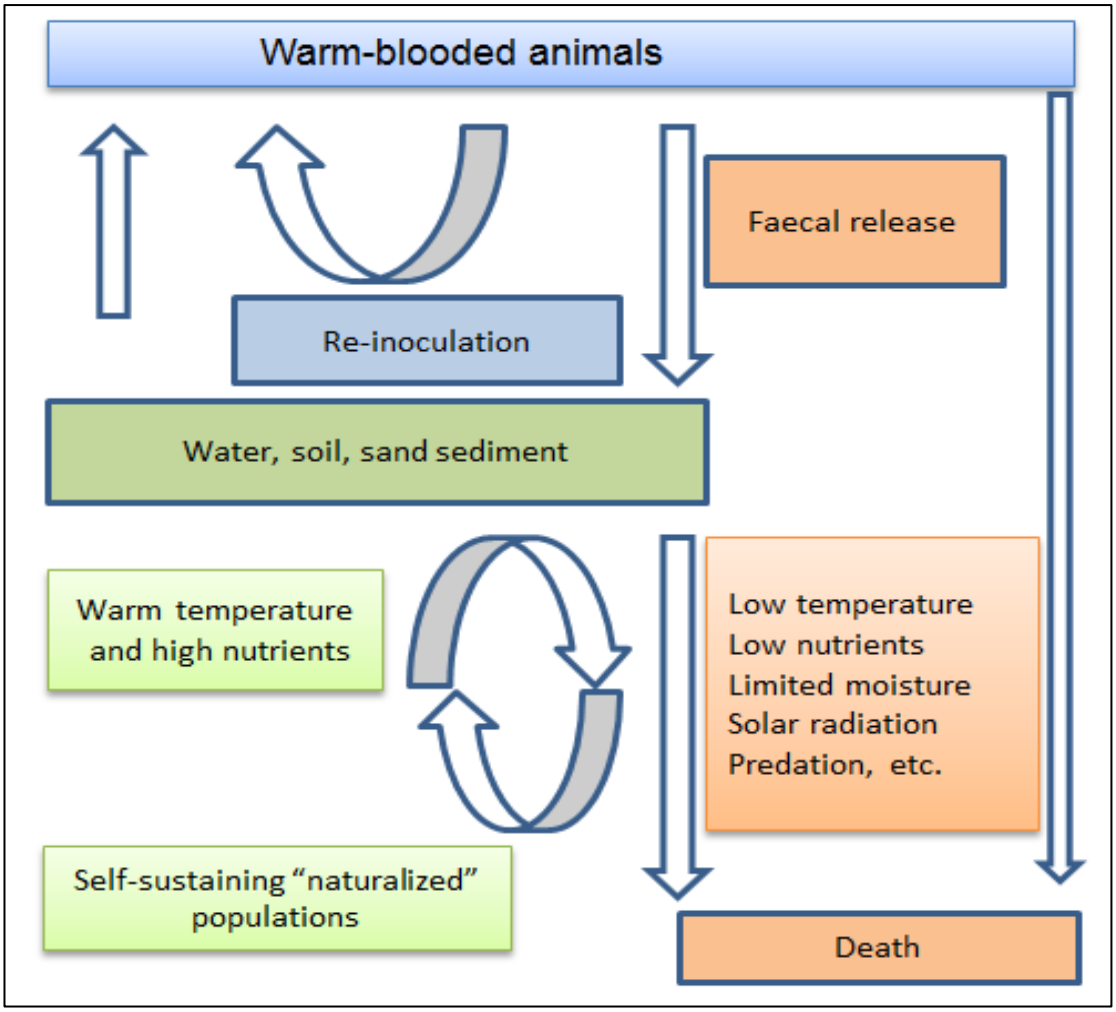


Figure 1.5 Schematic diagram illustrates the life cycle of faecal coliforms in the aquatic environment. Modified and adapted from Ishii and Sadowsky (2008).

1.3.3 Faecal Streptococci (Enterococci)

Enterococci or group D Streptococci are Gram-positive cocci which occur singularly or in pairs and are non-spore forming bacteria. They are a subgroup of the faecal Streptococci which includes many species such as *Enterococcus faecalis, E. faecium, E. durans, E. avium and E. gallinarum* (Pinto *et al.*, 1999; Whitman and Parte, 2012). They are commonly found in the intestinal tract of warm-blooded animals and humans. *Enterococcus*

faecalis and E. faecium are the most frequently found species in human. Enterococci are facultative anaerobic bacteria and may be differentiated from other Streptococci by their ability to grow in 6.5 % NaCl, high pH (6-9) and at a high temperature (45 ºC) in the presence of 40 % bile and on standard azide media (Collins *et al.*, 1995; Brooks *et al.*, 2007). They have been used as indicators of faecal pollution, and they are reliable indicators of health risks in marine environments and bathing waters (Cabelli *et al.*, 1982; Scott *et al.*, 2002; Scott *et al.*, 2005). The life cycle of Enterococci is similar to the life cycle of *E. coli*; they can be released into the environment through defecation, but Enterococci are more resistant to disinfectants than *E. coli* (Collins *et al.*, 1995).

1.3.4 *Bacteroides* species

Bacteroides species are obligate anaerobic, Gram-negative pleomorphic bacilli. They are non-motile and non-haemolytic on blood agar. They constitute a large component of the normal colonic bacterial flora in the intestines of animals and humans (10^{11} *Bacteroides* bacteria per gram of faeces, compared with 10^{8} per gram facultative anaerobic bacteria), but have a shorter survival rate outside of the intestinal tract (Bradley *et al.*, 1999; Brooks *et al.*, 2007). *Bacteroides* spp. can behave in many different ways. For example, when grown in a medium containing bile or ox bile, *Bacteroides* spp. tends to hydrolyse esculin and develop gentamycin resistance. These bacteria also produce succinate and acetate through fermentation, unlike other anaerobic groups. The growth of *Bacteroides* spp. is usually stimulated by adding haemin and vitamin K to the medium (Stokes *et al.*, 1993; Bradley *et al.*, 1999; Brooks *et al.*, 2007; Whitman and Parte, 2012).

Nosocomial infections, intra-abdominal abscesses, pleuropulmonary, female urogenital tract and soft tissue infections can be caused by *Bacteroides* spp., particularly when these areas are polluted with the contents of the colon (Brooks *et al.*, 2007; Hampson *et al.*, 2010). In addition, Bradley *et al.* (1999) and Ahmed *et al.* (2008a; 2008c) have discussed the beneficial effect of using these bacteria as a possible indicator of faecal pollution. Unlike *E. coli* and Enterococci, *Bacteroides* spp. are highly host-specific in distribution, with different *Bacteroides* spp. found in a variety of species of mammals such as humans, ruminants, horses, dogs, sheep etc. Moreover, Field and Samadpour (2007) stated that the use of *Bacteroides* spp. bacteria for routine monitoring is limited because of difficulty in cultivation. However, recent advances in molecular methods have resulted in the easier identification of *Bacteroides* spp. The survival of faecal *Bacteroides* in environmental waters and sediments is lower than the survival of other indicator bacteria such as faecal coliforms and Enterococci (Cabral, 2010).

1.4 Waterborne disease transmission

The majority of waterborne pathogens are introduced into the water system with human or animal faeces and do not grow in clean water and initiate infection. Some of these pathogens survive and thrive in water polluted with untreated or inadequately treated sewage. Indirect faecal-oral transmission happens mainly by the spread of pathogens from human and animal faeces (Black, 2002). In many developing countries, waterborne diseases have become an epidemic or endemic problem where the access to basic amenities, especially clean and safe water, is lacking. For instance, in Ghana, typhoid was recognized as the most common water-associated

disease in some areas because of inadequate drain and open waste dump sites which may also serve as breeding grounds for harmful insects such as mosquitoes (Geldreich and Litsky, 1976). Moreover, Maxwell *et al.* (2012) reported on a cholera outbreak relating to waterborne diseases in Accra resulting in 4.19×10^3 cases with 36 deaths.

Waterborne diseases are the main cause of infection and death around the world, particularly in developing countries suffering from a cycle of poverty and poor health areas (Cabral, 2010). More than 2.5 billion people struggle to obtain access to safe water, and more than 1.5 million children die each year because of waterborne diseases (Cabral, 2010). More than 5×10^6 human beings die every year due to waterborne diseases, lack of sanitation, unclean domestic environment and improper sewage disposal (Geldreich and Litsky, 1976). According to Black (2002), 3×10^4 people die every day in developing countries due to a lack of clean water. Moreover, Dorion *et al.* (2012) reported that the main five water-related diseases among children in Niger were diarrhoea, parasitosis, conjunctivitis, respiratory tract infections and skin infections (Figure 1.6).

In the aquatic environment, pathogen passage to susceptible water users is generally through the ingestion of contaminated water, body contact with recreational water, or by consumption of shellfish raised in contaminated harvesting beds and salad crops irrigated with polluted water. Major contributors to the spread of various waterborne pathogens are sewage, polluted surface water and storm water runoff (Geldreich, 1996).

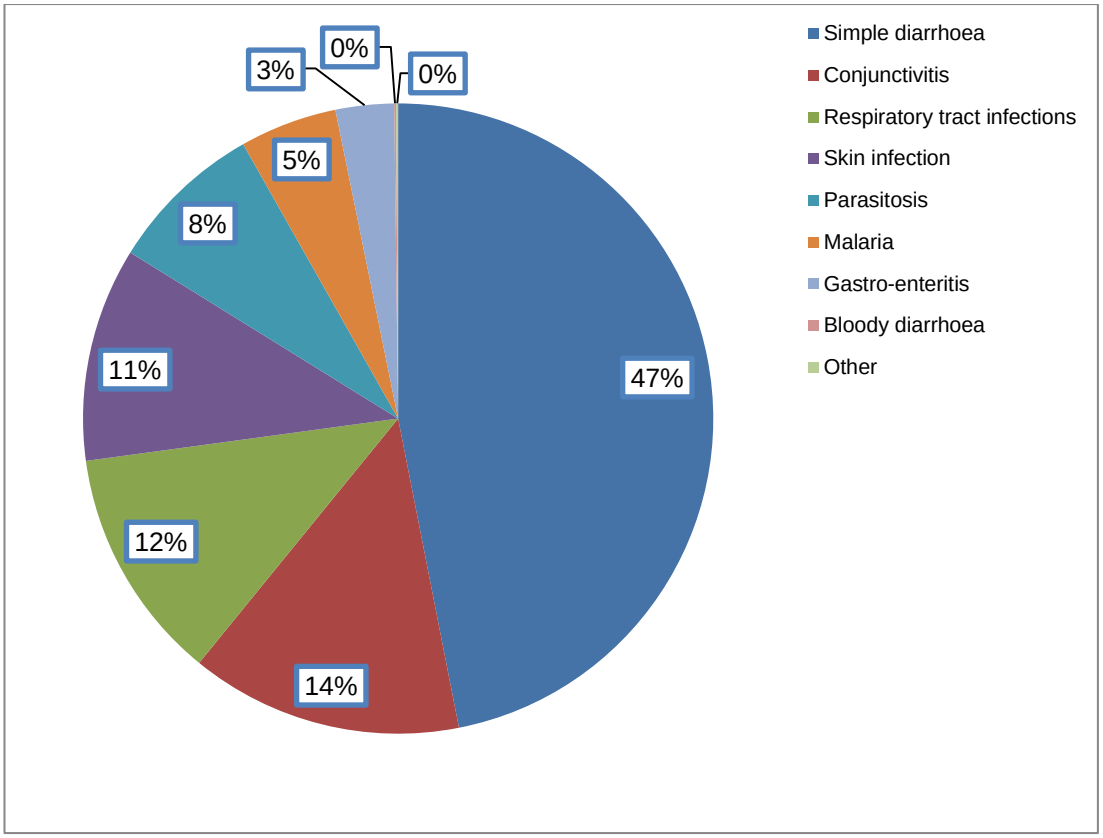


Figure 1.6 Pie chart showing the main infectious diseases among children from 20 study villages in Niger, relating to poor water quality, adapted from Dorion *et al.* (2012).

Developed countries also suffer from microbial waterborne diseases. In the USA, the overall microbiological quality of water has decreased. It has been estimated that 5.6×10^5 humans are infected annually with severe waterborne diseases in the USA (Medema *et al.*, 2003; Field and Samadpour, 2007). Maxwell *et al.* (2012) described four categories of water related diseases:

i. waterborne diseases caused by drinking water polluted with waste from humans and/ or animals, including cholera, typhoid, amoebic and bacillary dysentery and diarrhoea,

ii. waterborne diseases caused by bacteria in bathing waters for personal hygiene or recreational activities and skin or eye contact with polluted water such as scabies and trachoma,
iii. waterborne diseases cause by parasites in contaminated water such as schistosomiasis and helminths,
iv. waterborne diseases cause by insect vectors mainly mosquitoes that breed in water such as malaria and yellow fever.

There is strong evidence that faecal bacteria such as *E. coli,* faecal Streptococci and some anaerobic bacteria such as *Clostridium* spp., present in coastal waters and originating from mammalian hosts, can be responsible for gastrointestinal disease outbreaks (Shanks *et al.*, 2007). A variety of diseases have been related to exposure to recreational waters (Prüss, 1998) essentially in contaminated waters (Shuval, 2003) such as:

i. infectious diseases related to swimming/ surfing in coastal water contaminated with faecal and waste materials,
ii. infectious diseases associated with the consumption of seafood collected from polluted water.

Tibbetts (2005) stated that approximately 850 billion gallons of untreated human waste water and storm water is discharged into sea surface waters around the USA each year. Worldwide, there has been an annual incidence of an estimated 1.2×10^8 cases of gastrointestinal infections and in excess of an estimated 5×10^7 cases of severe respiratory infections related to bathing, surfing and swimming in coastal waters polluted with sewage and waste water (Shuval, 2003; Abdelzaher *et al.*, 2013). Since the middle of the last century, epidemiological studies have been used to determine the

relationship between the source of pollution of bathing water and health problems (gastrointestinal, respiratory, eye, ear, skin and throat infections), and they have found that diseases were higher in bathers compared to non-bathers (Prüss, 1998; Abdelzaher *et al.*, 2013).

Contaminated bathing waters and seafood are significant contributors to the human global burden of disease (GBD). GBD is defined as a comprehensive assessment of death or disability from certain disease and risk, which is estimated by combining:

i. loss from premature death, which is defined as the difference between actual age of death and life expectancy in a low mortality population,
ii. increased mortality resulting from severity of infections (Shuval, 2003).

1.5 Correlation between FIB and pathogens

To be effective, FIB should be present in aquatic environments at the same time as pathogenic microorganisms and in concentrations that are in ratio to the concentration of pathogens (Tamplin, 2003). However, no clear association was found between the presence of FIB with several faecal pathogens such as *Salmonella* spp., *Campylobacter* spp., *Giardia*, adenoviruses, enteroviruses and hepatitis (Lemarchand and Lebaron, 2003; Jiang and Chu, 2004; Kemp *et al.*, 2005). A limited number of studies have reported similarly unclear results between the presence of faecal indicators and *Campylobacter* spp. However, other studies have found a significant correlation between the presence of *Cryptosporidium* and faecal coliforms in sewage effluent, *Cryptosporidium* and faecal Enterococci in river water (Lamendella, 2006; Lamendella *et al.*, 2013). In a review of several studies,

Hörman *et al.* (2004) found no correlation between the presence of *Clostridium* spp. and the presence of both *Cryptosporidium* spp. and *Giardia* spp. Craig *et al.* (2003) reported that the relationship between *Salmonella* spp. and most FIB is complicated. In coastal water the number of culturable *Salmonella* spp. did not correlate with counts of Enterococci and faecal coliforms. However, in river water, when number of the Enterococci and faecal coliforms exceeded EU guidelines, *Salmonella* spp. were more likely to be recovered (Walters and Field, 2009; Lamendella *et al.*, 2013).

1.6 Economic implications of water pollution

Faecal water pollution not only impairs ecosystems and causes risks to human health, but also causes serious economic losses, the cumulative effect of which can become relatively large on a national budget from closures of failing beaches and shellfish farming, loss of work and productivity due to diseases, medical expenses and the management processes to deal with these issues (Walters and Field, 2009). In addition, beach leisure activities are considered a robust point of economic growth. The British tourist authority estimated the total income from the tourism sector in the UK in 2012 to be worth more than £134 billion per year and of this £3,035 million is related to recreational activities (Hirst, 2013). Additionally, according to the report published by the marine management organisation (MMO, 2012), UK sea fisheries were worth £1.37 billion in 2011. Indeed, commercial fishing industries are particularly susceptible to economic hardship resulting from faecal water pollution (Pugh and Skinner, 2002). Devine (2013) demonstrated that in 2012, bathing related recreational activities were worth approximately $35 to the USA economy for each visitor

daily. However, it is estimated that a $37,030 economic loss was incurred per day from closing Lake Michigan beach in the USA due to bacterial pollution. A high number of FIB tends to increase the risk of gastrointestinal illness from eating contaminated shellfish which may also have significant impacts on the local economy (Walters, 2007). Bacterial pollution in surfing waters that threatens human health is a common reason for beach closures and public warnings on many coasts. The level of pollution at any one beach is dependent on (Defeo *et al.*, 2009):

i. the suitability of waste water management processes,
ii. the timing and intensity of local rainfall events and
iii. subsequent runoff.

1.7 EU environmental legislations for water quality

Bathing water pollution is an important social problem around the world. Many countries have developed management techniques to reduce social and health problems associated with faecal pollution of water systems. In developed countries including the UK, strict regulatory measures apply to keep people safe. Monitoring of the water in the UK is undertaken by the environment agency (EA) under the auspices of Department for Environment, Food and Rural Affairs (DEFRA, 2011). The EA then have to report to the EU to demonstrate compliance with their standards. The key directives of relevance to pathogens in coastal and fresh water are:

i. EU water framework directive (EUWFD, 2000/ 60/ EC, EU, 2000),
ii. EU bathing water directive (EUBWD, 2006/ 7/ EC, EU, 2006),

iii. Integrated pollution prevention and control (IPPC, 96/ 61/ EC, IPPC, 2000) and

iv. EU urban waste water treatment directive (EUUWWTD, 91/ 271/ EEC, Jacobsen, 1999).

The main purposes of water quality monitoring for pathogens are (Figure 1.7) (Field and Samadpour 2007):

i. ensure compliance with regulations and

ii. facilitate identification of possible sources

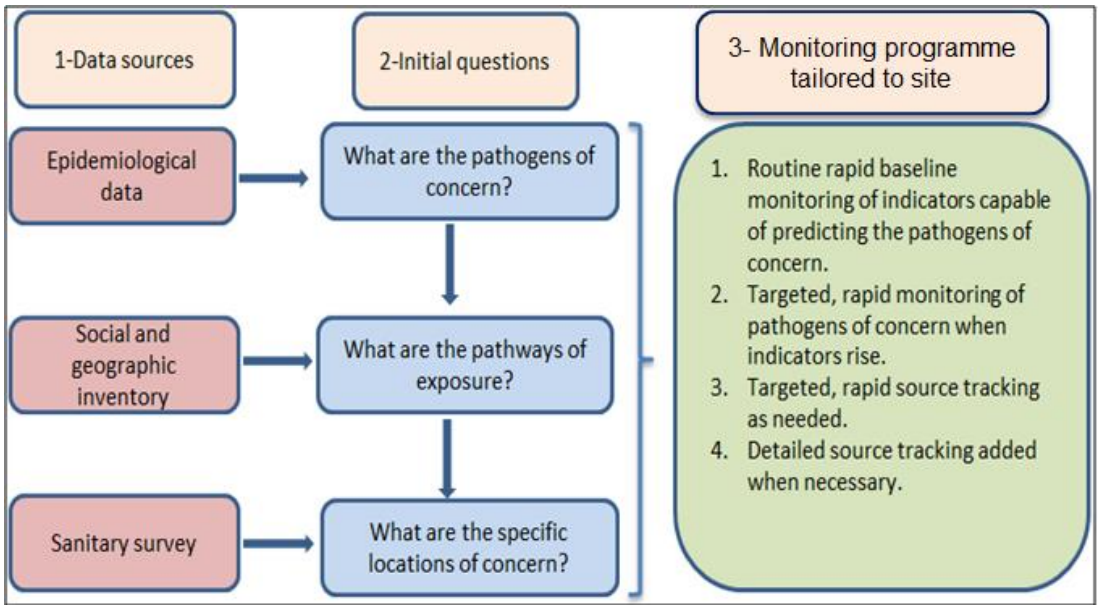


Figure 1.7 Schematic diagram illustrating rational approaches for protecting water quality. These approaches focus on potential pathogens and not on faecal indicator bacteria in water systems. Modified and adapted from Field and Samadpour (2007).

1.7.1 EU bathing water directive

The EU bathing water directive (EUBWD) is mainly focused on the protection of the environment and the public health of bathers and those involved in coastal recreation. It also aims to decrease the risk and to maintain the beauty of beaches. The EUBWD legislation is based on the concentration of FIB in faecal pollution as it impacts on bathing waters (Kay *et al.*, 2008b). The first directive was published in 1976 (EU, 76/ 160/ EEC) and subsequently an updated directive was introduced in 2006 (2006/ 7/ EC, EU, 2006, Kay *et al*., 2008b). These stipulate that bathing waters are monitored for indicators of pathogens throughout the bathing season and that the findings are compared to the given standards. Nineteen parameters for the quality of the bathing water consisting of both imperative and guideline instructions are defined by the EUBWD. The frequency of sampling and the method of analysis provide an idea of the level of FIB and the effectiveness of monitoring methods (EU, 2006). In the UK, the bathing season runs from the beginning of May to the end of September and water is monitored every two weeks (Rees *et al.*, 1998; Howarth and McGillivary, 2001). Crowther *et al.* (2002) and Kay *et al.* (2004) presented the mandatory and recommended standards for coastal waters set by EUBWD (Table 1.3).

Table 1.3 Bathing water directive standards 76/ 160/ EEC for FIB, colony forming units (CFU) per 100 ml. Adapted from Kay *et al.* (2004). Percentage values refer to the minimum percentage of 20 samples required to meet the standards to gain compliance.

FIB	Imperative[a] (Mandatory)	Guide (Recommended)
Total coliforms	1×10^5 (95 %)	5×10^2 (80 %)
E. coli	2×10^3 (95 %)	1×10^2 (80 %)
Faecal Streptococci	-	1×10^2 (90 %)

[a]Member states must achieve the imperative parametric values with the compliance level specified in the brackets and should strive to achieve the guide values at identified bathing waters.

Subsequently another directive was written in 2006, named 2006/ 7/ EC, which introduces three categories for water quality (‘excellent’, ‘good’ and ‘sufficient’) and applies to inland as well as coastal waters. This directive reduced the acceptable concentration of FIB; it also provided mechanisms for closing beaches failing to comply and required improved public information dissemination. According to EU 2006/ 7/ EC article 2, ‘water is a scarce natural resource, the quality of which should be protected, defended, managed and treated as such. Surface waters in particular are renewable resources with a limited capacity to recover from adverse impacts from human activities’.

The mandatory and guideline standard directives 2006 for FIB in all bathing water (inland and coastal) are shown in Table 1.4. From this Table it can be seen that to be defined as being of ‘excellent’ quality, inland waters should

contain less than 2×10^2 CFU of Enterococci per 100 ml in 95 % of the samples, and less than 5×10^2 CFU per 100 ml of *E. coli* in 95 % of the samples. Also, the 'sufficient' standard for Enterococci requires less than 3.3×10^2 CFU per 100 ml in 90 % for the samples, while the 'sufficient' standards for *E. coli* should be less than 9×10^2 CFU per 100 ml in 90 % of the samples over the previous four bathing seasons. The EU bathing water directive 2006 will come to an end after the 2015 bathing season; thereafter all European beaches are expected to be at the minimum sufficient category regarding to water quality (EU, 2006).

In addition, Table 1.4 shows that the 'excellent' quality standards for Enterococci from coastal waters require a less than 100 CFU 100 per ml in 95 % of the samples, while the 'excellent' quality standards for *E. coli* should be less than 2.5×10^2 CFU per 100 ml in 95 % of the samples.

Table 1.4 The maximum numbers of FIB allowed by the EU bathing water directive standards 2006/ 7/ EC use CFU per 100 ml, modified from EU 2006.

FIB/ categories	Excellent (*)	Good (*)	Sufficient (**)
Inland waters			
Enterococci	2×10^2	4×10^2	3.3×10^2
E. coli	5×10^2	1×10^3	9×10^2
Coastal waters			
Enterococci	1×10^2	2×10^2	1.85×10^2
E. coli	2.5×10^2	5×10^2	5×10^2

*Based upon a 95 percentile evaluation
**Based upon a 90 percentile evaluation

1.7.2 EU water framework directive

The EU water framework directive (EUWFD, 2000/ 60/ EU, EU, 2000) standards deal with water quality management; it was established in 2000 and is currently being implemented. It provides a framework for the protection of groundwater, inland surface waters, estuarine waters, and coastal waters (Borja *et al.*, 2006; Howarth, 2009). The EUWFD pursues environmental targets via a chain of steps that are set out in the directive. These standards include (Borja *et al.*, 2006; Howarth, 2009):

i. rearrangement of obligations in national law,
ii. undertaking initial inspections of waters and compiling registers of protected areas,
iii. undertaking monitoring programmes, identifying important issues,
iv. identifying competent authorities, and
v. packages of measures to realise the achievement of good environmental studies.

The major aim of EUWFD is to eliminate faecal pollution of water and to achieve good quality for all water systems by 2015 (Howarth, 2009). This will include elimination of faecal pollution.

1.7.3 EU urban waste water treatment directive

The EU urban waste water treatment directive (EUUWWTD, 91/ 271/ EEC, EU, 1991) was firstly established in 1991 and amended in 1998 (98/ 13/ EC). The major objective of this directive is to preserve aquatic ecosystems from the risk of untreated urban waste water as well as insufficient treatment of waste water (Zabel *et al.*, 2001). The EUWWTD describes the standards of treatment required by urban communities dependent upon population size.

Human and animal wastes become useful nutrient substrates for microorganisms which are at a significant concentration in waste water, domestic waste water and environmental waters (Al-Safady, 2011). Untreated waste water causes significant harm to human health and the aquatic ecosystems as a result of the high biological oxygen demand, the high concentrations of suspended solids and the litter associated with it (Zhang *et al.*, 2010). Treatment of waste water is an important process in removing or reducing faecal microorganisms to a harmless level alongside the removal of solids and oxygen consuming substances. It consists of three stages; primary (physical), secondary (biological) and tertiary treatment. The primary stage involves sedimentation, meaning that the incoming waste will be reduced up to 20 % before discharge. The secondary stage involves biological degradation which reduces organic matter in waste up to 70-85 % before discharge. The final stage is tertiary treatment performed by many disinfection processes, which removes faecal microorganisms as well as reducing the nutrient load in the effluents of water (Zabel *et al.*, 2001; Kay *et al.*, 2008b). Disinfection processes include exposure to ultraviolet light or addition of ozone to the waste water. Figure 1.8 demonstrates the change in concentrations of bacteria as result of the various treatment processes (Gleeson and Gray, 1997) .

1.8 Microbial source tracking

Microbial source tracking (MST) methods are modern techniques which are used for qualitative assessment of the origin of faecal microorganisms present in the aquatic ecosystem. MST is an essential tool for the proper management associated with bacterial water pollution (Kay *et al.*, 2008b).

These methods are potentially powerful tools that are widely used and show much promise for identifying the nature of water quality issues across the world. Identifying the source(s) of faecal pollution in water systems has been termed MST or bacterial source tracking (BST). Key factors to be considered when applying on MST approach include specificity of source, level of experience required, cost, and time of analysis (Field and Samadpour, 2007; Campos *et al.*, 2012).

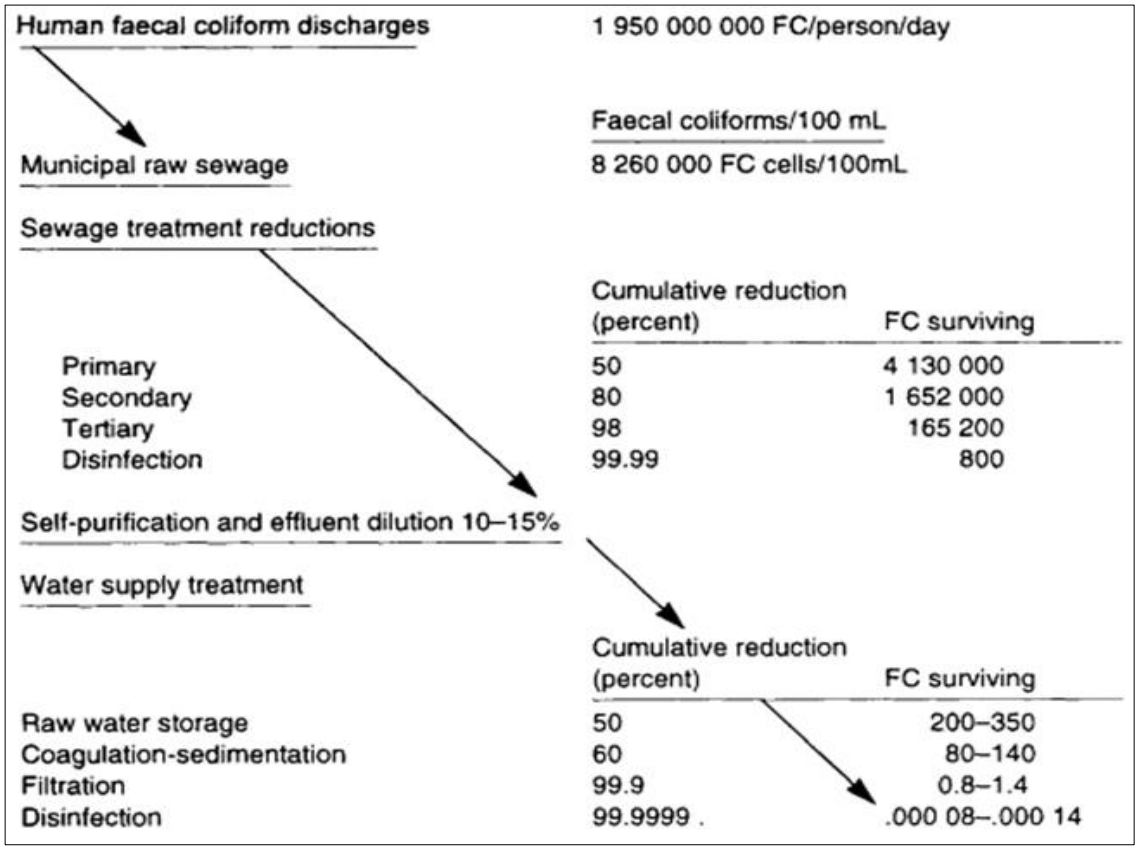


Figure 1.8 The control of pathogens in water systems using the barriers method, adapted from Gleeson and Gray (1997).

The first reason for undertaking MST is to detect the source of high concentrations of FIB. A second reason is to investigate particular pathogens; because some sources might be associated with a particular pathogen, for example *E. coli* O157:H7 with ruminant faeces. A third reason is to evaluate the risk to human health of exposure to faecally contaminated water (Field and Samadpour, 2007).

In recent years changes such as the EUUWWTD have reduced point source pollution which has highlighted the impact of diffuse pollution. Recently, it has been identified as an important cause of surface water quality degradation. However, determining the amount and source of diffuse pollution requires huge amounts of information (Kim *et al.*, 2005; Novotny, 2007). The qualitative assessment of FIB sources in environmental waters can be provided by MST (Kay *et al.*, 2008a). Coastal waters are facing a wide variety of different stresses (affecting both the ecosystem and human health) via domestic waste water treatment and disposal practices that may lead to the introduction of high levels of nutrients and enteric human pathogens. Moreover, several coastal region authorities are currently trying to treat water quality problems associated with primary non-point sources of pollution (Lipp *et al.*, 2001). Kay *et al.* (2012a; 2012b) have identified the four main sources of microbial contamination of river and coastal waters as:

i. human sewage disposal systems known as ‘point source’,
ii. livestock-derived microbial fluxes generally termed ‘diffuse-source’ pollution,
iii. wildlife populations in catchment systems and
iv. further sources such as urban diffuse microbial pollution.

The microbial quality of coastal and estuarine waters is directly related to human activities and animal waste according to the study carried out in the river Seine, France, by Touron *et al.* (2007). The high prevalence and concentration of potential pathogens along with the concentration of one or more faecal indicators indicates a poor level of microbial quality of surface water, and could represent a significant health risk to bathers. This is common after heavy rainfall (Ahmed *et al.*, 2009c).

Field and Samadouor (2007) and EPA (2011) have stated that the main different methods used in MST including:

i. culture-dependent, library-dependent methods,
ii. culture-dependent library-independent methods and
iii. culture-independent library-independent methods (Figure 1.9).

Overall, library-dependent methods rely on fingerprint databases of culturing microorganisms, whereas, library-independent methods are normally performed by nucleic acid amplification that does not need culturing of microorganisms (Field *et al.*, 2003; EPA, 2005).

The majority of library methods are culture-dependent, and involve the growing and isolation of microorganisms from environmental water samples and the creation of a reference library. Reference libraries are made using isolates taken from known faecal sources. However, most libraries have been built using isolates from possible source(s) in the target study area. Identification of the source(s) of bacterial pollution happens by a comparison between fingerprints from the library and environmental isolates. It includes the evolution of both phenotypic and genotypic analysis (Field and Samadpour, 2007). The library-dependent culture-dependent method is

commonly used for detection of *E. coli* and Enterococci, but a culture-independent library-independent method is commonly used for detection of anaerobic bacteria, and includes a host-specific polymerase chain reaction (PCR) (Bernhard and Field, 2000b; Field and Samadpour, 2007).

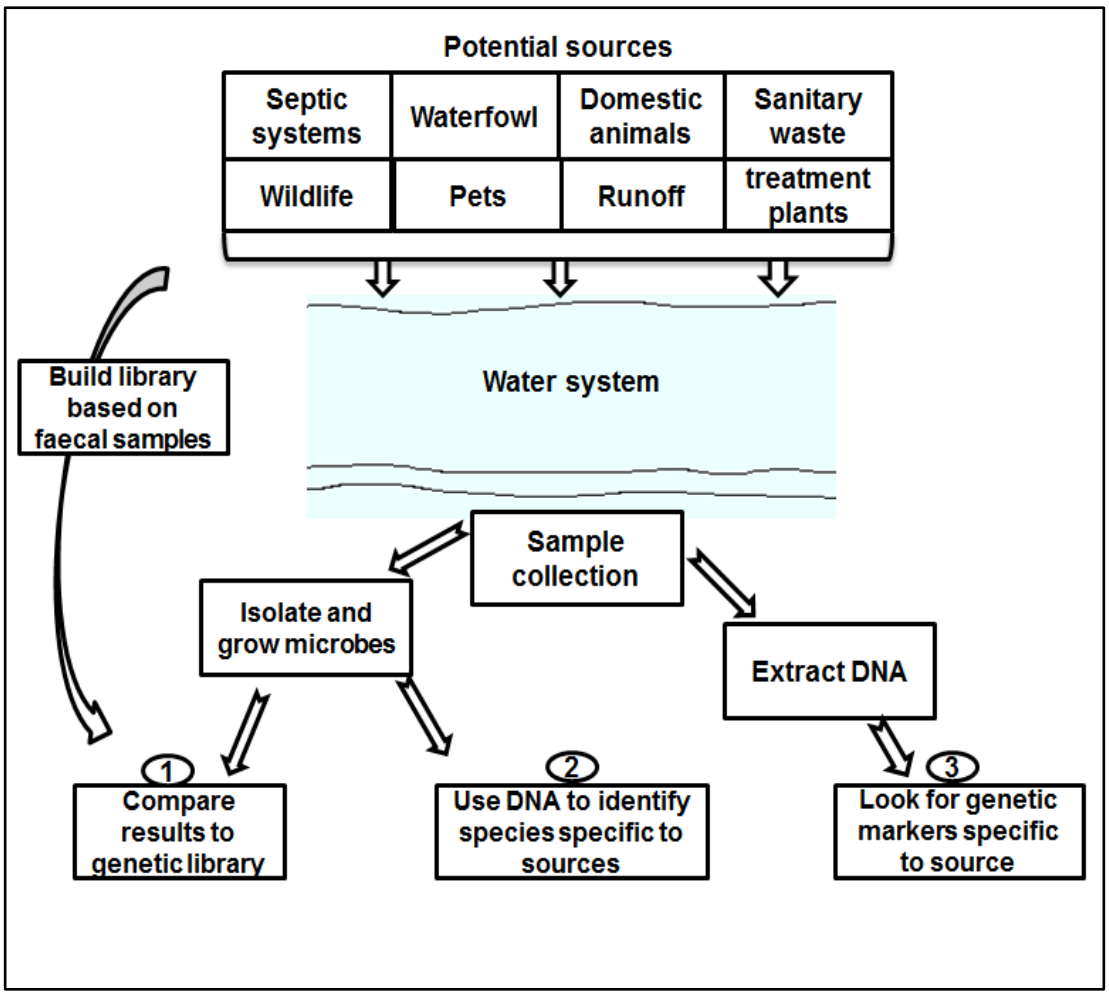


Figure 1.9 Summary of the MST methods from collection the environmental samples to obtaining specific results. Types of MST methods are 1: library-dependent and culture-dependent, 2: library-independent and culture-dependent, 3: library-independent and culture-independent. Modified and adapted from EPA (2011).

1.8.1 Culture-dependent library-independent methods

Culture-dependent library-independent methods are based on the presence or absence of the target microorganism or their 16S rRNA genetic marker, thus a source library is not required. These methods apply when the target for MST is found in low numbers and requires the growing of the source-specific microorganisms. How culture-dependent library-independent methods have been widely applied as MST tools and are discussed in more detail in subsequent sections.

1.8.1.1 Culture-dependent library-independent methods using phages

Bacteroides fragilis bacteriophage (Hsp40) produces only from human sewage. Bacteriophage source discrimination has been tested in Europe and South Africa and was considered a useful test in comparative studies (Hamilton *et al.*, 2006), but has not been investigated with blind samples (Field and Samadpour, 2007). Bradley *et al.* (1999) stated that many kinds of bacteriophages can be used as appropriate faecal indicators for viral pathogens. Bacteriophages have been classified into three groups depending on their similarities to human enteric viruses, nucleic acids and source frequency in polluted water: F^+ RNA coliphage, *Bacteroides fragilis* phages and somatic coliphages. The two main types of coliphages are somatic coliphages and male-specific coliphages (Kazama *et al.*, 2011). In addition, somatic coliphages can be grouped in four families: Podoviridae, Microviridae, Myoviridae, and Siphoviridae (Lee, 2009). Large numbers of *E. coli* bacteriophages are present in human faeces and sewage and also have been investigated as possible indicators of viral pollution in environmental samples. The coliphage faecal pollution test is for F^+ RNA coliphage; it is a

simple, inexpensive and can distinguish human and animal wastes, but requires overnight incubation. Coliphage method did well in blind comparative studies to identify human sewage, but was not successful in all human waste samples (Shanks *et al.*, 2006b). Field and Samadpour (2007) stated that the phage methods are limited to discriminating between human and non-human waste sources.

1.8.1.2 Bacteriological methods

The ratio between faecal coliforms and faecal Streptococci (FC: FS) is used as a method for distinguishing the source of faecal water pollution. If the ratio is greater than 4.0 this indicates a human source of faecal pollution, but if the ratio is less than 0.7 it indicates a non-human or animal waste source (Feachem, 1975, Scott *et al.* 2002). However, Silkie and Nelson (2009) demonstrated that the FC: FS ratio method has become unreliable because of the different survival rates of faecal Streptococci species and faecal coliforms meaning the ratio changes over time. Changes in the ratio are associated not only with human/ non-human sources of pollution, but also with the age of these bacteria.

In addition, several methods for MST are based on culture-dependent host-specific bacteria, such as *Bifidobacterium adolescentis* and sorbitol fermenting *Bifidobacteria* for human waste and *Rhodococcus coprophilus* for grazing animal waste. The main limitations of these bacteria as an MST tool are a short survival life (50 % reduction within 10 hours) and that they cannot be detected at high temperatures (summer) and are found at lower concentration in human faeces than *Bacteroides*. This method has been

used in the European Union, USA and South Africa, but not with blind samples (Bonjoch *et al.*, 2005; Shanks *et al.*, 2006a).

1.8.2 Culture-independent, library-dependent methods

1.8.2.1 Community fingerprinting

This method of microbial community fingerprinting from environmental samples is based on the construction of 16S rRNA gene clone libraries. Culture-independent MST methods of microbial community analysis examine biochemical markers taken directly from environmental samples (Field and Samadpour, 2007). These methods include terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE) and length heterogeneity PCR (LH-PCR) (Martin, 2012). T-RFLP fingerprinting of the 16S rRNA gene is a popular method and is used as a semi-quantitative and highly reproducible technique suitable for presuming bacterial diversity (Pandey *et al.*, 2007). The T-RFLP method comprises five steps: (i) DNA isolation and purification, (ii) PCR amplification and restriction enzyme digestion, (iii) separation and detection, (iv) data analysis and (v) clustering analysis (Applied Biosystems, 2005). However, phylogenetic identification of T-RFLP peaks based exclusively on comparison of terminal restriction fragment (T-RF) lengths with database information is misleading because of incomplete sequence databases, inaccurate size of peaks and the possible presence false T-RF (Saito *et al.*, 2010). Another method that can be used to compare of T-RFLP templates of *Bacteroides-Prevotella* faecal DNA from faeces of human and different types of animals, but there is overlap among those host species making of ineffective (Field and Samadpour, 2007). DGGE is a genetic analysis method used to separate

multiple DNA sequences based on their movement and melting behaviour when run on a polyacrylamide gel containing increasing concentrations of denaturing chemicals; it provides information about the different species present in the community. Separation of PCR products by DGGE is imperfect, and the method is considered to be less sensitive than T-RFLP (Pandey *et al.*, 2007; Saito *et al.*, 2010).

Culture-independent methods have many possible advantages as MST over library-dependent and cultured isolate-based methods, but they have been exposed to limited testing. Culture-independent methods are potentially faster (Field *et al.*, 2003). Hagedorn and Liang (2011) demonstrated that the host-origin database (library) of isolates from known microbial sources is essential information for library-dependent methods. This known information is applied to provide a group of fingerprint patterns that permit a direct comparison with other fingerprints of unknown microorganisms with the support of statistical classification algorithms. It is one of the more expensive and time-consuming methods. The combination of many molecular biological techniques requires the creation of clone libraries. These techniques include nucleic acid extractions, PCR, DNA ligation, bacterial transformation, and plasmid extraction. Thus, DNA sequence analysis of clone libraries creates a huge electronic data archive (EPA, 2005).

1.8.3 Culture-dependent library-dependent methods

The majority of library methods are culture-dependent, and involve the growing and isolating of microorganisms from environmental water samples. They also require the creation of a reference library which is made using isolates taken from known faecal sources. However, most libraries have

been built using isolates from possible source(s) in the target study area (Stoeckel and Harwood, 2007). Identification of the source of bacterial pollution happens by a comparison between fingerprints from the library and environmental isolates, and includes the evaluation of both phenotypic and genotypic analyses (Field and Samadpour, 2007). These methods are mainly based on a standard host origin database (library). Library databases consist of a group of bacterial isolates from samples of known origin, and are also used to refer to material isolated from the unknown samples (Stoeckel and Harwood, 2007).

1.8.3.1 Antibiotic resistance methods

These methods are based on multiple antibiotic resistances (MAR) patterns and are also called antibiotic resistance analysis (ARA). The MAR method performs a test for FIB against a group of antibiotics to differentiate between faecal sources from humans or animals, because FIB vary in the type and grade of antibiotic resistance profiles (Scott *et al.*, 2004; EPA, 2005; Campos *et al.*, 2012). Antibiotics are used to prevent and treat most infectious diseases in humans and domestic animals, but most of the bacteria generate resistance via plasmids. Antibiotic resistance profiles were developed as a method for MST based on the observation that bacteria from sources exposed to antibiotics will create resistance to those antibiotics (Field *et al.*, 2003). MAR has been used widely because it is rapid, comparatively simple, requires minimal training and is inexpensive, but the main limitation is the transfer of antibiotic resistance genes between bacteria (Choi *et al.*, 2003; EPA, 2005; Campos *et al.*, 2012). Ebdon and Tylor (2006) stated that antibiotic resistance was not stable geographically and temporally. Also,

many of the comparative studies have mentioned that the MAR methods are not very specific and have performed poorly in identifying blind samples.

1.8.3.2 DNA fingerprinting and related methods

The molecular fingerprinting tools are used to differentiate specific microorganisms. These methods require a reference library that is divided into two divisions direct analysis of genome and indirect analysis after PCR (Field and Samadpour, 2007). PCR methods have been commonly used in many MST approaches because they are highly sensitive, rapid, need a small amount of starting DNA, use bacterial cells without performing nucleic acid extraction and achieve accurate fingerprinting comparing with other methods (EPA, 2005). Genotypic techniques are based on some characteristics of DNA sequences of microorganisms, whereas phenotypic techniques are based on attributes that are expressed. Genotypic methods that have been applied for MST include:

i. Ribotyping is a fingerprinting method consisting of bacterial DNA digestion and DNA fragment separation using gel electrophoresis and probe hybridizations (EPA, 2011).
ii. Whole genome restriction fragment length polymorphism analysis (RFLP) using pulsed-field gel electrophoresis (PFGE). PFGE involves DNA extraction, DNA purification, DNA digestion and separation by gel electrophoresis (EPA, 2011).
iii. Repetitive element PCR fingerprint (rep-PCR) uses PCR primers for amplification of repetitive segments of bacterial DNA, followed by DNA separation by gel electrophoresis (EPA, 2011).

iv. Random amplification of polymorphic DNA (RAPD) is a type of PCR where the DNA fragment amplifications occur randomly (Kumar and Gurusubramanian, 2011).

v. Amplified fragment length polymorphism (AFLP) is based on gDNA restriction followed by PCR amplification with specific primers (Savelkoul *et al.*, 1999).

Most of these methods require selective culturing of indicator bacteria from environmental samples as well as from expected faecal sources (EPA, 2005). Moreover, the protocol of fingerprint methods is necessary, because sometimes there more than one protocol. For example, the ribotyping method of DNA fingerprinting uses oligonucleotide probes after treatment of genomic DNA with restriction endonucleases (Scott *et al.*, 2002). This method has two different protocols, the first using restriction enzymes EcoR1 and PvuII and the second using restriction enzyme HindIII (Nikolausz *et al.*, 2005). The method of analysis and the size of the library are particularly important as, depending on the size of the library, many environmental isolates cannot link to faecal isolates (Pugh and Skinner, 2002). Field and Samadpour (2007) stated that ribotyping, PFGE, and rep-PCR have been considered as comparative methods.

1.8.3.3 Carbon-source and fatty acid methods

The carbon-source utilization profiling (CUP) method also called biochemical or phenotypic fingerprinting, is focused on differences in the utilization of several carbon and nitrogen substances by different microorganisms (Field and Samadpour, 2007). It is used as a MST tool because it is rapid, simple and requires little technical skill, and it used successfully for the identification

of clinical Gram-negative bacteria (Holmes *et al.*, 1994). However, Blanch *et al.* (2004) and Field and Samadpour (2007) demonstrated that the carbon-source utilization method did poorly in two comparative studies.

Table 1.5 Library-dependent methods as tools of the MST, modified and adapted from Domingo (2005).

Method	Target	Major costs	Time require
MAR*	*E. coli*	Antibiotic discs	4-5 days
	Enterococci	96-well microplates	
CUP	*E. coli*	Microplates	2-5 days
	Enterococci		
rep-PCR	*E. coli*	PCR reagents	1 day
		PCR disposable	
		Gel electrophoresis	
RAPD	*E. coli*	PCR reagents	1 day
		PCR disposable	
		Gel electrophoresis	
AFLP	*E. coli*	DNA extraction kit	5 days
		AFLP kit	
PFGE	*E. coli*	Plug prep reagents	2-4 days
	Enterococci	Restriction enzymes	
		Gel electrophoresis	
Ribotyping	*E. coli*	DNA purification reagents	1-3 days
	Enterococci	Gel electrophoresis	
		Restriction enzymes	
		Hybridization/ detection solution	
		Labelled gene probe	

*MAR: Multiple antibiotic resistance, CUP: Carbon utilization profiling, rep-PCR: repetitive element PCR, RAPD: Random amplification of polymorphic DNA, AFLP: Amplified fragment length polymorphism, PGGE: Pulsed-field gel electrophoresis.

In addition, the fatty acid methyl ester (FAME) profiling method involves by a reaction between fatty acid and methanol. Microorganisms have specific FAME profiles. The FAME method has been mentioned among the available MST tools, but there are few published work on the application and accuracy of the FAME method for predicting source(s) of faecal water pollution (Duran *et al.*, 2006). Table 1.5 illustrates the summary of library-dependent methods.

1.8.4 Culture-independent, library-independent methods

Culture-independent, library-independent methods are mainly based on nucleic acid techniques originating from the molecular microbial ecology field. In the 1980s, molecular microbial ecology was developed for the first time based on microbial ribosomal RNA (rRNA) gene sequences (Dick *et al.*, 2005a). Nowadays, most organisms can be classified from the Kingdom to the genus-species rank based on their rRNA gene sequences. These methods are discussed in more detail in subsequent sections.

1.8.4.1 Chemical methods

In the aquatic environment many chemical substances are present, that could be used as indicators for human and non-human sewage such as caffeine, faecal sterols or coprastanols, bile acids, laundry brighteners, surfactants including linear alkyl benzenes, fragrances and pesticides (Field and Samadpour, 2007). Caffeine is present in many soft drinks and pharmaceutical products. Caffeine is ingested by humans and discharged in the urine. The presence of caffeine in the environmental samples indicates the presence of human waste in these samples. Waste water treatment can remove the caffeine from water (Scott *et al.*, 2002).

In addition, Campos *et al.* (2012) demonstrated that the faecal sterol biomarkers can be used as an alternative method for detecting sewage pollution in environmental sediment. There is variance in the persistence of faecal sterol in aerobic and anaerobic conditions; the decay may happen within 14 days in water but in sediment extremely slowly. Some of these biochemical markers are stored in sediment for long time (Field and Samadpour, 2007). Moreover, Scott *et al.* (2002) stated that there was no direct association between the presence of these chemical materials in aquatic areas and pathogenic microorganisms.

1.8.4.2 Development of host-specific genetic marker methods

i. Suppression subtractive hybridization method

Suppression subtractive hybridization (SSH) method is commonly used for differentiating between DNA molecules to discriminate two closely related DNA samples. The method includes two main suppression subtraction applications: complementary DNA (cDNA) and genomic DNA (gDNA) (Lukyanov *et al.*, 2007). SSH is a highly competent PCR - based method for differentiating between expressed genes, and for simultaneous subtraction and normalization of both target cDNA and gDNA (Rebrikov *et al.*, 2000). Researchers have used the subtractive hybridization method to develop new host-specific 16S rRNA genetic markers from *Bacteroides* species as a tool for MST. Human and cattle host-specific *Bacteroides* genetic markers have been developed by Shanks *et al.* (2006b; 2007) based on the subtractive hybridization method. Dick *et al.* (2005a; 2005b) have also used subtractive hybridization and developed horse, elk, dog and pig host-specific

Bacteroides genetic markers. A limitation of this method is the generation of false positive clones that may give a signal in the primary screening procedure (Rebrikov *et al.*, 2000).

ii. Microarray method

This method is based on immobilizing single-stranded DNA fragments of known nucleotide sequences that are used particularly in the identification and sequencing of DNA targets and studying gene expression (Seefeld *et al.*, 2012). DNA microarrays use specific probes to identify specific sequences which can be utilized for determining target microorganisms by observing the nucleotide polymorphisms within the target DNA (Berthet *et al.*, 2013). Vuong *et al.* (2013) have used a DNA microarray method to identify the presence of human wastes and 27 other animal wastes representing possible sources of faecal water pollution in some Canadian water systems. Edge *et al.* (2006) and Soule *et al.* (2006) have also evaluated a microarray method developed from *E. coli* and *Enterococcus* spp. from known hosts to distinguish potential host-specific probes. However, genetic markers that have been developed by the microarray method showed less specificity and had a narrow geographic range (Hamilton *et al.*, 2006; Field and Samadpour, 2007).

1.8.4.3 Host-specific genetic marker methods

The genetic markers are tested by molecular methods, either directly from a water sample or from nucleic acid extracted from a water sample and do not require a culture-dependent step. Specific genetic markers are tested using PCR, for that reason they also called host-specific PCR. They allow access

to novel genetic markers that would be difficult to detect by growth assay (Field and Samadpour, 2007). These methods include:

i. **Viral detection methods**

Monitoring of faecal viral pollution in environmental water samples is performed directly without culturing, but may need more than 100 ml water. Researchers have used nested-PCR to increase the sensitivity of these methods. Nested-PCR uses two sets of PCR primers which are used for amplification of the DNA fragment. The first set (forward and reverse) of PCR primers is similar to conventional PCR primers, but the second set binds inside the first PCR fragment to allow amplification of the second PCR fragment which is shorter than the first one. Generally, nested-PCR has more specific PCR amplification than conventional PCR (Pérez de Rozas *et al.*, 2008). In addition, real-time PCR techniques have been used to quantify some enteric viruses and adenovirus (McQuaig *et al.*, 2012). The performance of viral detection methods was good in detecting human sewage and these methods also important because they can detect viral pathogens which are not well linked with FIB. Therefore, these methods give some information on viral pathogen status that is not provided by FIB counts (Dick *et al.*, 2005a; Hamilton *et al.*, 2006). However, assays for testing and typing of F^+ specific coliphages, adenovirus and enteroviruses were applied in comparative MST studies with blind samples (Field and Samadpour, 2007).

ii. **Anaerobic bacteria methods**

Anaerobic faecal bacteria such as *Bacteroides* spp. and *Bifidobacterium* spp. have host-specific distributions. Faecal anaerobic bacteria form the majority of faeces content at much higher densities than other FIB, and around 10^3 fold greater than *E. coli* in human faeces (Fiksdal *et al.*, 1985). However, they were not commonly used as indicator bacteria until the beginning of the molecular technique era, because of the difficulties associated with the detection and identification of faecal anaerobic bacteria. Ribosomal 16S RNA (rRNA) genes and host-specific genes from anaerobic bacteria have been used for detecting the source of faecal pollution (Shanks *et al.*, 2007). Host-specific *Bacteroides* genetic markers based on uncultured anaerobic microorganisms can identify and distinguish human *Bacteroides* 16S rRNA genetic markers from other animal *Bacteroides* genetic markers using host-specific PCR primers (Bernhard and Field, 2000b; Dick *et al.*, 2005a). Related methods have targeted other anaerobic bacteria such as *Bifidobacterium* species. These species are known to be animal specific with variable results for specificity; however the survival of these bacteria is limited in environmental conditions. The molecular technique of Bifidobacteria for MST has undergone comparative testing in the EU, but has not been examined using blind samples (Field and Samadpour, 2007).

iii. Toxin and virulence gene method

Host-specific toxicity genes in *E. coli* and Enterococci have been widely studied. Enterotoxigenic *E. coli* (ETEC) produce two types of

enterotoxin: heat-labile (LT) and heat-stable (ST) enterotoxin. The most common enterotoxins found in ETEC are ST types Ia (STIa), Ib (STIb) and LT type h (LTh). STIb toxin for *E. coli* is specific for human and LTIIa toxin for *E. coli* from cattle (Abe *et al.*, 1990; Chern *et al.*, 2004). Moreover, Enterococcal surface protein (Esp) is a virulence element from *Enterococcus faecium* which is used as a human-specific PCR target (Scott *et al.*, 2005; Ahmed *et al.*, 2008c). Leavis *et al.* (2004) have designed the human-specific assay to amplify a human Esp gene from *E. faecium.* According to Scott *et al.* (2005), 97 % of human sewage tested positive for this Esp marker, but bird or livestock samples were negative. The limitations of the *E. coli* enterotoxin method are failure to detect ST products by a large number of *E. coli* strains, and there appeared to be a correlation between ST product and the animal species. Also, chemical structure variations of ST enterotoxin have been observed in different strains (Gyles, 1979; Picken *et al.*, 1983).

iv. ***Rhodococcus coprophilus* detection method**

R. coprophilus bacterium has been used in MST, because it is present at a high level in faeces of different animals such as cattle, sheep, pigs, horses, geese, ducks and hens. However, this bacterium is absent in human faeces, which qualifies it as a good indicator for animal faecal pollution. Detection of *R. coprophilus* marker by conventional PCR and quantification by real-time PCR is specific and sensitive (Savill *et al.*, 2001; Field and Samadpour, 2007; Wicki *et al.*, 2012).

v. Host-mitochondrial sequence method

The host-mitochondrial sequence molecular method is based on the detection of mitochondrial DNA from many animal species to track the source of faecal water pollution and differentiate between human and non-human sewage. The method developed by Martellini *et al.* (2005) is based on PCR primers targeting host mitochondrial genes. Caldwell *et al.* (2007) stated that the labelled probes for human, pig and bovine mitochondrial genes were specific and largely identified in blind samples. However, the amount of these genetic markers in faeces is low and it may require larger amounts of water samples to detect them.

Table 1.6 (overleaf) shows examples of culture-independent and library-independent methods.

Table 1.6 The types of culture-independent and library-independent methods of the MST, adapted from Domingo (2005).

Method	Target	Cultivation required	Major costs	Time required
Phage typing	F^+ coliphage	Individual Isolates	Hybridization/ detection solutions Labelled gene probe Phage specific antigen	1-3 days
Gene specific PCR	*E. coli* toxins	Sample enrichment	PCR reagents PCR disposables	2 days
Total community analysis	16S rRNA	None	Filtration units PCR reagents PCR disposable DNA sequencing	6-8 hours
Host-specific PCR	*Bacteroides* *Bifidobacterium* spp. Enterococci *Rhodococcus* spp. F+ coliphage Enterovirus Adenovirus	None	Filtration units PCR reagents PCR disposable	6-8 hours

1.8.5 Pros and cons of culture-dependent methods as MST tools

The culture-dependent methods for FIB require a relatively low level of technical expertise, also are inexpensive and simple techniques. The method works by isolating single and pure colonies, making it widely used and available. This method usually uses traditional FIB with some information about their survival, transport, and correlation with diseases and pathogenic microorganisms (Stoeckel and Harwood, 2007). However, the disadvantages of using the culture-dependent method are that it does not provide the diversity, host-specific source tracking of many pathogens and some FIB, and the composition of microbial communities may change quickly (Field and Samadpour, 2007).

1.8.6 Pros and cons of culture-independent methods as MST tools

The advantages of these methods are the ability to sample the whole community which is present in the sample, without needing cultivation. They are simple and more rapid than the culture-dependent method. These methods usually perform well in detecting the source and nature of the FIB community in blind samples such as sewage of humans and animals. They may only need a few hours to determine FIB and identify the source(s) of faecal water pollution (Field and Samadpour, 2007). Moreover, a library is not required; through validation is needed when these methods are applied in new areas. An additional limitation of the culture-independent methods is that specific markers for a limited animal species are available with some other species are poorly represented. In addition, more and different gene targets are required (Field and Samadpour, 2007; Stoeckel and Harwood, 2007).

1.9 Host-specific genetic markers using PCR as MST tools

Detection of genetic markers can be used to assay a water sample by DNA extraction without culturing. This method is widely used for assessment of bacterial pollution because rapid results can be obtained when compared with other methods, as stated in Table 1.6 (Scott *et al.*, 2004; Field and Samadpour, 2007; Lee *et al.*, 2008). PCR is a widely used methodology which has enabled major steps in biological and biomedical research throughout the world as a tool for amplifying specific nucleotide sequences in target samples (Fu and Li, 2013). Host-specific PCR based on the 16S rRNA gene is used in most MST methods by many researchers, as a genetic marker approach to determining faecal source of pollution (Lee *et al.*, 2008; Layton *et al.*, 2013). The principle of PCR method (Figure 1, Appendix 1) is to make a huge copies of DNA from few fragments of target DNA *in vitro* (Domingo, 2000; Roche, 2006).

Bernhard and Field (2000b) tested the sensitivity of host-specific primers using serial dilutions of cow faeces and raw sewage, and the DNA which is formed from each dilution tested, against genetic markers of host-specific PCR. They have designed host-specific primers to identify specific genetic markers in the case of *Bacteroides-Prevotella* 16S rRNA gene, which is used as a genetic marker. This genetic marker is capable of identifying human- and ruminant- specific *Bacteroides-Prevotella*. Furthermore, Ahmed *et al.* (2009b) have studied five sewage-associated genetic markers, which demonstrated high specificity (99 %) and were able to differentiate between human and non-human waste. In addition, sewage-associated *Bacteroides*

genetic markers have been used to detect human faecal pollution including human-specific marker (Bernhard and Field, 2000b; Gawler *et al.*, 2007).

1.9.1 Advantages of host-specific PCR

Host-specific PCR has several advantages for example; it is culture-independent and does not require any cross referencing with bacterial databases. Host-specific PCR is a sensitive and rapid method, and can detect target genetic marker in complex environmental samples (Field and Samadpour, 2007). The use of host-specific PCR for detecting *Bacteroides* genetic markers has emerged as a potential tool for MST in environmental studies. In the UK, USA and Japan, environmental agencies have highly recommended this method for PCR detection of *Bacteroides* genetic markers as an important tool in MST (Gawler *et al.*, 2007; Okabe *et al.*, 2007). Host-specific PCR is a reliable and reproducible technique and some of the applications discussed reflect the rationale behind the improvement of this methodology (Fu and Li, 2013).

1.9.2 Limitation of host-specific PCR

Despite the many advantages of host-specific PCR, it may be affected by many factors, such as thermocycling conditions and template/ primer concentrations etc. Another factor which may ultimately affect PCR success is the efficiency of DNA sample preparation from target cells. The preparation of the PCR template includes various steps from cell lysis to DNA extraction and purification and it may lead to both loss and pollution of the sample (Marty *et al.*, 2012). Markey *et al.* (2010) demonstrated that the quality of conventional PCR is mainly restricted by both spatial constraints and a slow thermal ramp rate between temperature steps caused by a large

thermal programme. Moreover, other limitations related to the use of host-specific PCR as a tool of MST (Traub *et al.*, 2004; Field and Samadpour, 2007) include:

i. no specific genetic markers for many wildlife/ domestic animals suspected to be sources of water pollution,
ii. the target gene is sometimes found in low numbers,
iii. the PCR reaction may be inhibited, and
iv. FIB genes, carried on plasmids are capable of being transferred among species.

1.10 Persistence and survival of FIB in the natural environments

For accurate estimation of human health risks related to faecal water pollution, FIB should not reproduce in the aquatic environments but should be as persistent as the pathogens (Tamplin, 2003). The ubiquity of *E. coli* and Enterococci in natural environments has been demonstrated by several studies (Walters and Field, 2009). Moreover, Whitman *et al.* (2003) stated that *E. coli* and Enterococci survived for more than 180 days in dried Cladophora mats at 4 ºC, and re-grew after rehydration of the mats. They can proliferate and survive for a long time in aquatic environments (Field and Samadpour, 2007). According to Haller *et al*. (2009), FIB can still be detected in soil and sediment for up to 90 days. In addition, Power *et al.* (2005) stated that the environmental *E. coli* isolates, which were recognized as derived from animal hosts indicated that they can persist as free-living microorganisms for an extended time, and need not rely on recent faecal input. Therefore, these extensive cases of *E. coli* and Enterococci in the environment reduce their efficiency in use for risk assessment and for

predicting the time of water pollution events (Walters and Field, 2009). Spores of *Clostridium perfringens* persist for longer periods in the aquatic environments than other FIB (Hörman *et al.*, 2004). According to Craig *et al.* (2003) the decay rates of *Salmonella* spp. were greater than that of *E. coli*, and Enterococci (*Salmonella* spp.> *E. coli*> Enterococci) in seawater microcosms.

Non-culture based indicators for identifying the sources(s) of faecal water pollution that have been used in recent years are host-specific *Bacteroides* 16S rRNA genetic markers. However, little information is known about the survival and persistence of these bacteria and their markers in the environment after release (Okabe and Shimazu, 2007). Additionally, the relationship between *Bacteroides* spp. persistence and the survival of other usual microbial indicators should be considered (Balleste and Blanch, 2010). *B. fragilis* can grow in low oxygen concentrations but has a high oxygen tolerance (Baughn and Malamy, 2004; Balleste and Blanch, 2010), whereas, other *Bacteroides* species have variability in their survival in environmental conditions (Wilkins *et al.*, 1978; Walters *et al.*, 2009). Survival of *Bacteroides* spp. and the detection of their molecular have been shown to be affected by some environmental parameters, such as temperature and salinity. Temperature has been studied in most detail; it has been found that an increase in temperature increases the rate of cell decay, and also that nucleic acid degradation of *Bacteroides* occurs more quickly at higher temperatures (Kreader, 1998; Savichtcheva *et al.*, 2005; Seurinck *et al.*, 2005; Okabe and Shimazu, 2007; Bell *et al.*, 2009; Balleste and Blanch, 2010). The environmental profiles have been measured by microcosms,

because they allow for the environmental variables to be examined under highly controlled conditions (Kreader, 1998; Dick and Field, 2004; Okabe and Shimazu, 2007; Bae and Wuertz, 2009b; Walters *et al.*, 2009; Schulz and Childers, 2011).

1.11 Rationale and thesis research aims

The main cause of waterborne diseases is bacteria and viruses which are commonly found in waste water and environmental water of both developing and developed countries (Cabral, 2010). Faecal material may contain various pathogens. Therefore, the more faecal material in the water means a greater risk to the people using it. The risks of a waterborne disease can be measured by the incidence of disease in the humans who have been in contact with the contaminated water (Pipes, 1982; Colford Jr *et al.*, 2007). Although, there has been much investment to improve waste water treatment, point sources of waste water remain extremely problematic in developing countries, while waste water treatment and effluent disposal systems in developed nations can easily be overwhelmed as a result of adverse weather conditions (Kivaisi, 2001). Thus, in spite of the progress that has been achieved in waste water treatment in developed countries, while they strive to minimize the risk from bacteria, they still cannot eliminate them completely. Faecal pollution still affects beaches and coastal ecosystems all over the world (Solo-Gabriele *et al.*, 2011).

In the UK, waste water treatment and receiving water quality is much better but there are commonly still problems on bathing beaches which are not easily explained. The result from diffuse sources such as the overflow of waste water, runoff from agricultural land, runoff from farms and urban

environments and *in situ* animals such as dogs and birds which still contribute to the presence of bacteria in surface waters. Direct evidence of the presence of microbial pathogens can be obtained through monitoring processes. However, many of the pathogens are not easy to detect as they are frequently present in low numbers, but even a low concentration in polluted waters is hazardous to health for humans (Scott *et al.*, 2004).

Epidemiological data have been used to identify pathogens of concern in a specific water body, and then used to target pathogen monitoring coupled with methods based on faecal source tracking as an additional rationale for this approach to try and maintain the water quality (Field and Samadpour 2007). Various microbiological, genotypic and phenotypic methods have previously been proposed to identify FIB for the purpose. Genetic methods can be used for differentiating between the lineages of bacteria within various animal hosts (Scott *et al.*, 2002; 2004).

FIB have been used to detect faecal pollution based on the rationale that these indicators are indigenous to faecal material, and their presence in bathing water or the aquatic environments is indicative of faecal contamination (Goodridge *et al.*, 2009). Microbial processes such as culture-dependent methods are now available to identify the presence of FIB in bathing water but these have problems associated with quickly changing diversity or communities of these FIB. Other methods are available to identify source(s) of bacteria such as MST methods but these are not suitable for all potential sources of faecal pollution, in particular there is no marker for some common sources of faecal contamination such as cats, goats and most wild animals. Thus the rationale for using MST methods here is to identify the

source(s) or host of the FIB from which causes intermittent faecal pollution was derived. Hence, this study explores the development and application of analytical techniques to support improved management of contaminated bathing waters.

1.11.1 The aim of this study

Therefore, in the light of this situation, where routine pollution by human sewage is much reduced but not eliminated, the aim of this study is to evaluate, use and develop better MST techniques for the detection of faecal pollution from humans and other animals which enable determination of the presence and source of such pollution so that monitoring and management can be improved. This work has built on a previous study (Joseph, 2009) carried out to monitor bathing water quality in the Kingsbridge estuary, Devon, UK which has shown intermittent spikes of bacterial pollution and obvious bacterial pollution in water and sediment on beach in Devon. In addition, the identification of the nature and scale of the reservoirs of indicator bacteria in the sediment of some beaches and its influence on bathing water quality was studied.

1.11.2 The objectives

In order to achieve this aim, the following specific objectives were established:

i. To use and assess different methods of tracking faecal water pollution at Kingsbridge estuary, South Sands, Devon, UK, namely current culture based EU methods and PCR-based on *Bacteroides* spp. of human genetic marker and other origins (described in Chapter 3).

ii. To develop new dog-specific conventional PCR and qPCR primer sets to detect dog faecal pollution in bathing water and test at a tourist bathing beach where dogs are permitted access and banned (Bigbury-on-Sea, described in Chapter 4).

iii. To investigate the survival and persistence of *Bacteroides* PCR genetic markers when compared to culture-dependent recovery and to study the possible adherence to sediment under controlled laboratory conditions (described in Chapter 5).

iv. To make recommendation on the application of these new methods for bacterial monitoring.

Chapter Two
General materials and methods

2.1 Introduction

The general materials and methods in this chapter were used unless otherwise indicated. This chapter will deal with the sample collection, media, culture-dependent techniques and molecular methods including DNA extraction, DNA amplification of samples using polymerase chain reaction (PCR) and quantitative PCR (qPCR). Samples of bathing water, coastal sediment and faeces were collected from Devon, United Kingdom. Culture-dependent analysis of water and sediment were conducted for isolation and enumeration of the FIB specifically *E. coli*, Enterococci and *Bacteroides* species.

2.2 Preparation of media and solutions

All media were prepared according to the manufacturer's instructions and stored at 4 ºC for further using. The preparation and ingredients of the media, reagents and chemical solutions are given in Appendices 2 and 3. Reagents containing heat-sensitive compounds were sterilized using filtration through 0.45 µm membranes (Sartorius Stedim, UK), whereas, other reagents and equipment requiring sterilization were autoclaved at 121 ºC for 15 minutes and a pressure 15 pounds per square inch (psi). All laboratory chemical materials were obtained from Sigma-Aldrich, Qiagen, Bioline Biotech and Fisher Scientific, UK unless stated otherwise. Bacterial isolates were obtained from Plymouth University culture collection. The type culture of *Bacteroides fragilis* NCTC 9343 was purchased from the national collection of type cultures (NCTC, UK).

The following outlines in brief, the media that were used in this study. How they were used is described in section 2.4.

2.2.1 Slanetz and Bartley medium

Slanetz and Bartley (Oxoid, UK) is a selective medium which was used to enumerate Enterococci in water and sediment samples. Slanetz and Bartley medium is an improved medium using filter membrane and does not need autoclaving. Enterococci are distinguishable as red or maroon colonies (Slanetz and Bartley, 1964; Fricker and Fricker, 1996).

2.2.2 Rapid sodium chloride medium

Rapid sodium chloride medium is a selective medium for confirmation of Enterococci in water and sediment samples. The slant medium was prepared with 65 g l^{-1} sodium chloride (NaCl) and 20 mg l^{-1} Bromocresol purple as a pH indicator (Qadri *et al.*, 1978). Enterococci were confirmed by their ability to grow in the presence of 6.5 % NaCl. This ability was indicated by growth of Enterococci colonies which were accompanied with a colour change from purple to yellow.

2.2.3 *Bacteroides* bile esculin (BBE) agar

BBE agar is a selective medium for the detection *Bacteroides* spp. BBE medium contains gentamicin and oxgall for inhibition of facultative anaerobic bacteria. Filtered water samples were placed on the surface of this medium, and then incubated at 37 ºC under anaerobic conditions. *Bacteroides* spp. are characterized as brown-black colonies (Livingston *et al.*, 1978).

2.2.4 *Bacteroides* phage recovery medium (BPRM) broth

BPRM broth was used for the cultivation of faecal *Bacteroides* spp. from faecal and environmental samples. BPRM broth (one litre) was prepared as explained in Appendix 3, followed by aliquoting into 100 ml glass bottles and

autoclaving, before addition of hemin, disodium carbonate and glucose. The pH was adjusted to 7 with hydrochloric acid (0.1 M HCL) and stored at 2 to 8 ºC (Tartera *et al.*, 1992).

2.2.5 Membrane Lauryl sulphate broth

Membrane Lauryl sulphate broth (MLS; Oxoid, UK) was used for the enumeration of *E. coli* in water and sediment samples. *E. coli* counts were assessed using known volumes of water with *E. coli* appearing as a yellow colony (Stanfield and Irving, 1981). This medium was modified by adding 1.2 % agar to prepare membrane Lauryl sulphate agar, as this was easier to handle and useful for accurately screening colonies.

2.2.6 Lauryl tryptose sulphate broth

Lauryl tryptose sulphate broth (LTS; Oxoid, UK) is a medium for the confirmation of *E. coli* in water and sediment samples (Mallmann and Darby, 1941). LTS broth was prepared in one litre and distributed in 50 ml glass universal bottles containing an inverted Durham tube per bottle 10 ml and then sterilised by autoclaving.

2.2.7 Brain heart infusion broth

Brain heart infusion broth (BHI; Oxoid, UK) is a versatile infusion medium for the cultivation and storage of a wide variety of bacteria. BHIB was prepared in one litre, mixed well and distributed into 50 ml universal tubes (25 ml), and then sterilized using autoclaving.

2.3 Sample collection

The samples of water and sediment were obtained from three main locations at South Sands, Salcombe, Devon, UK (Latitude 50.13 ºN, Longitude - 3.47 ºW) and two sites at the beach near Bigbury-on-Sea, Devon, UK (50.28 ºN, - 3.89 ºW). Faecal samples were collected from the farms close to Salcombe, and Plymouth (50.37 ºN, - 4.14 ºW), Devon, UK.

2.3.1 Water sample collection

The water and sediment samples were obtained from the three different points near South Sands, a stream, a pond and the beach and from the two sites at Bigbury-on-Sea, a beach which allowed dogs and one from where they are banned during the bathing season (1st of May to the end of September). The water samples were taken in triplicate in pre-sterilized, wide mouth and labelled plastic containers (500 ml), at a depth of approximately 30 cm below the surface of the water. The bottles were then packed in an ice box to keep cool, and these samples returned to the microbiological laboratory within six hours for further analysis. Once in the laboratory, the samples collected from the same point were mixed and analysed as a single sample. Salinity and pH levels of the water samples were measured using a refractmeter (Extech RF10, UK) and pH meter (Hana, UK), respectively. The water and sediment samples were collected both during the EU bathing season (June, July and August) and out of the EU bathing season (October and February). These samples were analysed for the detection and enumeration of FIB according to the EU bathing water directive 2006. These same samples were also subjected to molecular analysis for microbial source tracking.

2.3.2 Sediment samples

Sediment samples were collected in triplicate in sterile and pre-labelled 50 ml plastic containers whilst minimizing disturbance of the sediments. These sampling containers were kept in an ice box, and returned to the laboratory within six hours for further analysis. In the laboratory, the triplicate samples were obtained from the same location mixed together and analysed as a single sample.

2.3.3 Faecal samples

Animal faecal samples from cows, sheep, horses, deer, pigs, dogs, cats and ducks were obtained from two farms in the South Hams area (Kingsbridge and Salcombe), south Devon, UK. Human faecal samples were obtained from four adult volunteers (age range 18-50 years). The samples were collected with sterile plastic containers (50 ml volume), kept on ice for transport to the laboratory, stored at 4 ºC and processed within six hours.

2.4 Bacteriological methods for isolation and enumeration of FIB

Bacteriological (culture-dependent) methods were used to detect and enumerate *E. coli,* Enterococci and *Bacteroides* spp*.* in the water and sediment samples.

2.4.1 Treatment of water samples

Membrane filtration method was used for detection and enumeration of FIB. This method can give a direct count of FIB within 48 hours incubation. The filtration procedure was carried out using 47 mm (diameter) 0.45 µm (pore size) filter membranes (Whatman, UK). A steel manifold unit was attached to a vacuum pump and sterilized with a Bunsen burner. Filter membranes were

placed on the membrane holder using sterile tweezers. Exactly 100 ml of the water was measured by standard cups and passed through the membrane using vacuum pump. Then, the filter membranes were placed carefully onto the Petri dishes, containing appropriate solid or broth media.

2.4.1.1 Detection and enumeration of Enterococci

Slanetz and Bartley medium (Oxoid, UK) was used to detect Enterococci. Water samples (100 ml) were filtered through 0.45 µm filter membranes (Whatman, UK) as described above, and placed on Slanetz and Bartley agar plates. The plates were incubated at 35 ºC for 4 hours for resuscitation, then at 44 ºC for a further 44 hours. Colonies of Enterococci appeared red or maroon in colour on this medium and the number was expressed as colony forming units (CFU) 100 ml^{-1} as described by Fricker and Fricker (1996). To positively identify these bacteria, pure colonies were inoculated into a 10 ml slant of a rapid sodium chloride media as described in section 2.2.2, and incubated at 35 ºC for 8-24 hours (Qadri *et al.*, 1978).

2.4.1.2 Detection and enumeration of *Escherichia coli*

To detect *E. coli* bacteria, membrane Lauryl sulphate broth (MLS) and/ or agar was used as a selective medium. After the water filtration process, the filter membranes were placed face upward on an absorbent pad previously saturated with 2.5-3 ml MLS broth in small Petri dishes (60 mm × 15 mm), or onto a MLS agar plate. The plates were incubated at 35 ºC for 4 hours for resuscitation, and then incubated at 44 ºC for a further 24 ±3 hours (Eckner, 1998). Yellow colonies indicated the presence of *E. coli*, and the numbers were expressed as CFU 100 ml^{-1} (Barrell, 1992). For the confirmation of *E. coli,* a pure colony from MLS agar was selected and inoculated into Lauryl

tryptose mannitol (LTM) broth (10 ml, Oxoid, UK). The inoculated broths were incubated overnight at 44 ºC. *E. coli* was confirmed by production of gas in the Durham tube and further confirmed using an Indole test (Barrell, 1992; Craig *et al.*, 2002). The ability of *E. coli* to degrade tryptophan was performed using the Indole test. Briefly, the Indole test was performed by adding a few drops of Kovac's reagent (Appendix 2) onto the inoculated broth. A positive reaction was indicated by a cherry red layer (Choi *et al.*, 2003).

2.4.1.3 Detection and enumeration of *Bacteroides* species

To detect *Bacteroides* spp., *Bacteroides* bile esculin (BBE) agar was used (Livingston *et al.*, 1978), which contains gentamycin and oxgall (bile salts); these inhibit facultative anaerobic bacteria. Water samples (100 ml) were filtered using 0.45 µm filter membranes (Whatman, UK), and plated as previously described. The plates were incubated at 37 ºC for 72 hours under anaerobic conditions (MACS 500 anaerobic cabinet, Don Whitley Scientific Limited, UK). The plates were examined for circular brown-black colonies and numbers expressed as CFU per 100 ml. For downstream molecular analyses, pure colonies of *Bacteroides* spp. were inoculated into separate 15 to 25 ml volumes of BPRM broth and incubated at 37 ºC for 72 hours anaerobically.

2.4.2 Processing of sediment samples

Two grams of sediment (wet weight) samples were added to sterile seawater up to a final volume of 20 ml to give a tenfold dilution. Each sample was blended for two minutes in a Stomacher Lab-Blender (Seward Laboratory, UK). The sediment samples were left to settle for 10 minutes at room

temperature, before pipetting the supernatant. The samples were vacuum-filtered through 0.45 µm filter membranes (Whatman, UK), which were then placed on either solid media or an absorbent pad soaked with broth (Craig *et al.*, 2002; Ferguson *et al.*, 2005). The media and incubation conditions used are described in section 2.4.1.1. The number of bacterial colonies was expressed as CFU per gram (wet weight).

2.4.3 Preparation of standard calibration curve for estimation of *Bacteroides* numbers

Based on a specific absorbance or optical density (OD) reading, a standard calibration curve of OD versus CFU numbers was produced for *B. fragilis* NCTC 9343 as shown in Figure 2.1 (Sutton, 2011).

Sterile BPRM broth was placed in five sterile 2 ml Eppendorf tubes (one ml in each tube). One ml of exponentially growing *B. fragilis* NCTC 9343 BPRM broth culture was then added to first tube and mixed well. From the first tube, one ml was then added to a second tube and so on to obtain a twofold dilution series down to $^{1}/_{32}$. The spectrophotometer (Unicam, UK) was blanked using sterile PBRM broth. One ml from each dilution and the undiluted stock was placed in a cuvette and optical density (OD) taken at wavelength of 590 nanometres (nm) using the spectrophotometer. In addition, tenfold serial dilution series (10^{-1} to 10^{-7}) of growing bacteria were prepared using sterile phosphate buffered saline (PBS). From each dilution, 100 µl (triplicate) was inoculated onto BBE agar, and then incubated at 37 ºC for 48 hours anaerobically. The mean number of CFUs was counted, and expressed as CFU ml^{-1} (Morris and Nicholls, 1978; Koch, 1994; Sutton, 2011).

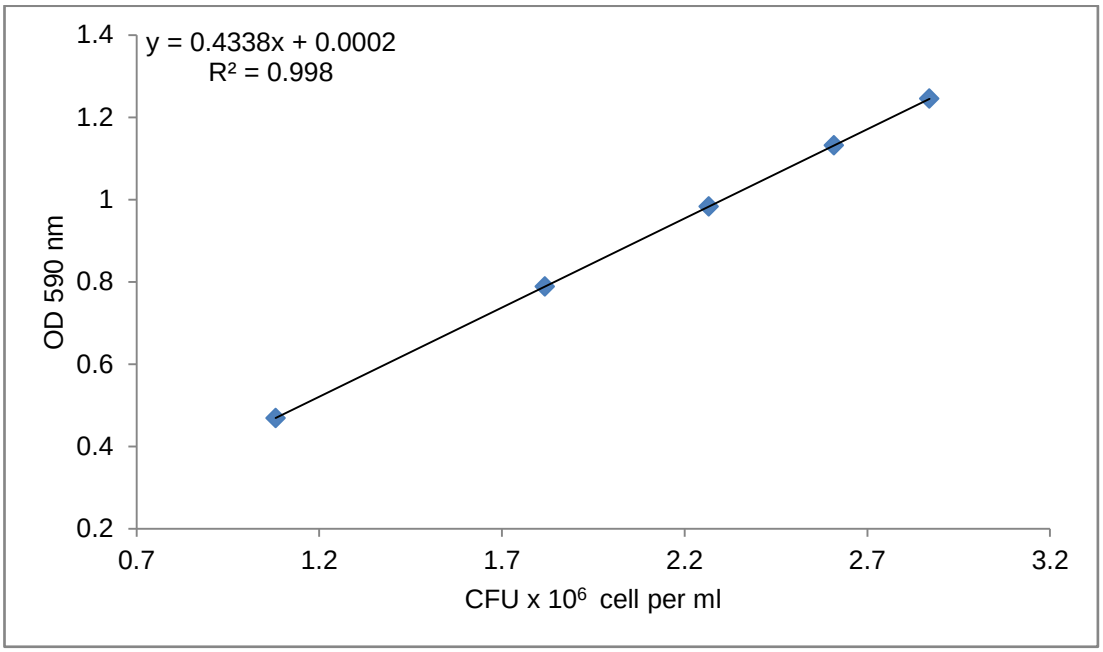


Figure 2.1 Calibration curve showing the relationship between optical density (OD) and the number of *B. fragilis* NCTC 9343 CFU per ml.

2.4.4 Storage of bacteria

Where it was necessary to store bacteria, the first step was to grow a pure culture in broth (15 ml) for 24-48 hours. BPRM broth for *Bacteroides* spp. and brain heart infusion broth for both Enterococci and *E. coli* were used to store for long time. The entire broth volume was centrifuged at 3000 × *g* for 8 minutes, and the supernatant discarded into a container with 2 % Virkon® as disinfectant (Fisher Scientific, UK). Re-suspension of the pellet was then carried out in one ml of 30 % (v/ v) sterile glycerol (Thomas Scientific, USA) in an appropriate broth. Finally, 500 µl of the suspension was placed into a cryovial tube (2 ml, Thomas Scientific, USA), sealed, labelled and stored at -80 ºC (Malik, 1984).

2.5 Molecular methods for detection of *Bacteroides* genetic markers

Molecular techniques using host-specific *Bacteroides* 16S rRNA gene, host-specific primers can be used to distinguish faecal *Bacteroides* spp. from faeces of human and other animals.

2.5.1 Bacterial DNA extraction

Four methods were used for bacterial DNA extraction from the water, sediment, faeces and pure cultures.

2.5.1.1 DNA extraction from water samples

All buffer solution compositions used in the following methods are listed in Appendix 2. DNA was extracted from the water samples by using a QIAamp DNA mini kit (Qiagen, UK) according to the manufacturer's protocol (Figure 2.2 a). This method contained four stages; cell lysis, inhibitor removal, protein removal and DNA clean-up (all centrifugation steps were carried out at 13000 x *g,* unless otherwise stated).

i. **Cell lysis**

Water samples (100 or 300 ml) were filtered through 0.45 µm pore size filter membranes (Whatman, UK). The filter membrane was transferred in 15 ml tube contained 0.5 ml guanidine thiocyanate buffer (Schulz and Childers, 2011). The filter membrane was mixed well with the buffer and stored overnight at - 20 ºC or for longer at - 80 ºC (Ahmed *et al.*, 2008c). The filtered sample (0.5-1 ml) was placed into a 2 ml Eppendorf tube and then ASL buffer (700 µl) added. The mixture was vortexed for one minute, and then heated at 90 ºC for five minutes, followed by mixing and centrifugation for one minute.

ii. **Inhibitor removal**

The supernatant (800 µl) was pipetted into a new 1.5 ml Eppendorf tube; half an InhibitEX® tablet was added to remove inhibitory substances and the mixture vortexed immediately for one minute. The tube was then incubated at room temperature for one minute, followed by centrifugation for three minutes. The supernatant was aspirated and placed into a new 1.5 ml Eppendorf tube, then centrifuged for three minutes.

iii. **Protein removal**

Proteinase K (15 µl) was added into a new 1.5 ml Eppendorf tube along with 200 µl of the centrifuged supernatant. AL buffer (200 µl) was added. After mixing, incubation was carried out at 70 ºC for 10 minutes, before addition of 200 µl of molecular biology grade ethanol (Fisher BioReagents®, UK).

iv. **DNA clean-up**

The entire supernatant was pipetted into a QIAamp spin column/ collection tube and centrifuged for one minute. A QIAamp column was placed into a new collection tube and AW1 buffer (500 µl) added, and then centrifuged for one minute. A QIAamp column was placed into a new tube and AW2 buffer (500 µl) added, and then centrifuged for three minutes. A QIAamp column was placed into a new tube (1.5 ml) and elution buffer (AE, 100 µl) added, and then centrifuged for one minute. DNA pellet was transferred into a new 1.5 ml Eppendorf tube and stored at - 20 ºC for future use.

2.5.1.2 DNA extraction from sediment samples

A SoilMaster™ DNA extraction kit (Cambio, UK) was used for DNA extraction from the sediment samples as described the manufacturer's protocol (Figure 2.2 b). In brief:

i. Sediment samples (200 mg) were weighted and placed into a 2 ml sterile Eppendorf tube. A soil DNA extraction buffer (250 µl) was added, then followed by proteinase K (2 µl).

ii. Soil lysis buffer (50 µl) was added, the mixture vortexed briefly, and incubated at 65 ºC for 10 minutes. The mixture was then centrifuged at 1000 x *g* for two minutes.

iii. Supernatant (180 µl) was transferred into a new 1.5 ml Eppendorf tube; protein precipitation reagent (60 µl) was added and mixed thoroughly by inverting the tube 6-8 times. After that, the mixture was incubated on ice for 8 minutes, followed by 8 minutes centrifugation.

iv. Following centrifugation, 100 µl supernatant was transferred carefully into the spin column, followed by further centrifugation for two minutes at 2000 x *g* in a 1.5 ml Eppendorf tube. The spin column tube was discarded.

v. DNA precipitation solution (6 µl) was added and the mixture incubated at room temperature for 5 minutes. Thereafter, the mixture was centrifuged for 5 minutes and the supernatant decanted carefully. The DNA pellet was washed by addition of 500 µl of pellet wash solution. Finally, 100 µl of TE buffer was added to re-dissolve the pelletted DNA.

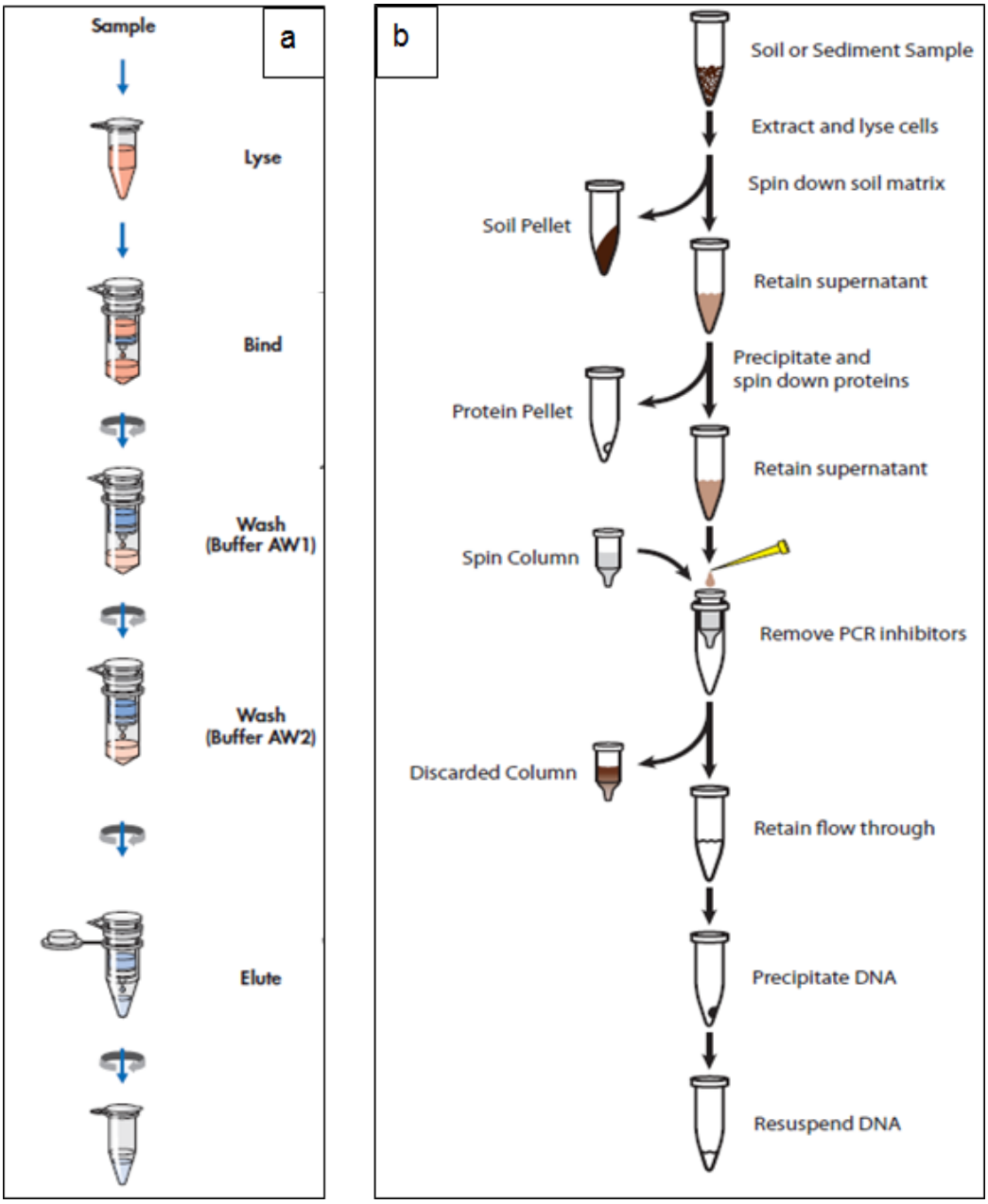


Figure 2.2 The main steps of DNA extraction, (a) water and faecal samples using QIAamp DNA mini kit and (b) sediment samples using SoilMaster™ DNA extraction kit (adapted from literature supplied by Qiagen and SoilMaster™, UK).

2.5.1.3 DNA extraction from faecal samples

Faecal samples (200 mg) from human, cow, sheep, horse, dog, deer, pig, cat and duck were weighed and DNA extracted by using the QIAamp stool DNA kit (Qiagen, UK) as described previously for water samples, with some modifications. Briefly, 500 µl lysozyme solution (Sigma Aldrich, UK; 50 mg lysozyme was dissolved in one ml TE buffer) was added to each faecal sample tube, and the mixture incubated at 37 ºC for 30 minutes. In the final step, 100 µl of the AE buffer was added to suspend the DNA pellet and stored at - 20 ºC for future use.

2.5.1.4 DNA extraction from pure culture of *Bacteroides*

Pure cultures of isolated *Bacteroides* species were grown in BPRM broth (15-25 ml) as described in section 2.4.1.3. DNA was extracted as described below:

i. One millilitre of broth culture was placed in a 1.5 ml Eppendorf tube, centrifuged at 12000 x *g* for 3 minutes, and the supernatant discarded.
ii. The pellet was washed twice by the sterile PBS (500 µl), and then centrifuged at 12000 x *g* for 3 minutes. The supernatant was discarded.
iii. TLE lysis buffer (500 µl) was added, and mixed gently.
iv. The mixture was boiled for 10 minutes, and then centrifuged at 12000 x *g* for 5 minutes.
v. The supernatant was pipetted off carefully and placed in a new 1.5 ml Eppendorf tube.

vi. 500 µl of Phenol-Chloroform-Isoamyl alcohol (25:24:1 v/ v, kept at 4 ºC) was added. The mixture was mixed, and then centrifuged at 12000 x *g* for 5 minutes.

vii. The supernatant containing the bacterial DNA was taken and placed in a new 1.5 ml Eppendorf tube, before storage at - 20 ºC.

Extracted DNA from each sample was processed in aliquots to reduce the effect of repeated freeze-thawing. DNA aliquots were kept in ice between reaction steps.

2.5.2 DNA spectrophotometric assay

The extracted DNA was quantified using a NanoDrop® (ND-1000) UV spectrophotometer (Labtech International, UK). Two microliters of either buffer (AE or TE) or molecular biological grade water was used as a blank to calibrate the spectrophotometer. Thereafter, DNA purity and the concentration was determined using 2 µl of extracted DNA. The ratio of DNA/ protein was measured at A_{260}/ A_{280} nm and the ratio of DNA/ other contaminants (humic acids) measured at A_{260}/ A_{230} nm. The extracted DNA concentration was recorded in ng μl^{-1} from the water, sediment and faecal samples. The ratio of pure DNA should be ranged between 1.8 and 2.05 (Figure 2, Appendix 1) (Brown, 2010). Duplicate measurements were taken for each sample. In addition, the purified DNA samples were electrophoresed using agarose gel electrophoresis as described in section 2.5.4.

2.5.3 Conventional polymerase chain reaction (PCR)

The principle of PCR is to amplify a target DNA template to produce large numbers of a specific DNA product *in vitro*. It involves the use of a thermostable DNA polymerase, target DNA, two oligonucleotide primers, and

deoxynucleotide triphosphate (dNTPs). PCR cycles include three stages: (i) template denaturation at 94 ºC for 15 seconds to two minutes; (ii) primer annealing at 40-60 ºC for 15-60 seconds and (iii) primer extension at 70-74 ºC for one minutes followed by a final variable length extension step at 72 ºC (Saiki *et al.*, 1988).

PCR was used to detect *Bacteroides* genetic markers in the water, sediment and faecal samples by using host-specific primer sets previously designed by Bernhard and Field (2000a, b) and Dick *et al.* (2005a). Lyophilised primers were dissolved with DNase-RNase free water and mixed well to a final concentration of 50 pmol (Table 2.1).

PCR reactions were conducted in a total volume of 25 µl. Each reaction mixture contained 2 µl of template DNA, 1 µl (50 pmol) each of forward and reverse specific primers (Eurofins MWG, Germany), 8.5 µl molecular biology grade water (Fisher Scientific, UK) and 12.5 µl of ReadyMix™ Taq PCR reaction mix (Sigma Aldrich, UK). The mastercycler gradient programme (Eppendorf, Hamburg, Germany) was used to perform and optimize the PCR method. For *Bacteroides* 16S rRNA genetic marker from human (HF183F), generic (Bac32F), horse (HoF795F), and pig (PF163F) primers were used. The cycling parameters were as follows: 15 minutes at 95 ºC for initial denaturation, followed by 35 cycles of 94 ºC for 30 seconds, annealing temperatures (Table 2.1), 1.5 minutes at 72 ºC with a final 7 minutes extension at 72 ºC (Bernhard and Field, 2000b; Dick *et al.*, 2005a). For the detection of *Bacteroides* genetic marker from cow, CF128F primer was used. PCR conditions for this primer were as follows: 94 ºC for 3 minutes, followed by 35 cycles of 94 ºC for one minute, annealing temperature at 54.8 ºC for

one minute, and 72 ºC for 2 minutes. Finally, a final 10 minute extension was carried out at 72 ºC (Seurinck *et al.*, 2005; Gawler *et al.*, 2007; Ahmed *et al.*, 2009b). All the forward primers were coupled with reverse primer Bac708R. DNA template was used as a positive control, whereas reactions without DNA template were included in each assay as negative control.

2.5.4 Agarose gel electrophoresis

Agarose gel electrophoresis is a method aimed to separate DNA fragments based on their size, the strength of electrical charges and the concentration of agarose. Due to the overall negative charge of the phosphate backbone of DNA, movement forwards the positive electrode away from negative electrode is observed under an electrical current. Gel electrophoresis was conducted using a 1.5 % (w/ v) agarose gel (Bio-Rad, UK), prepared by mixing and boiling agarose with 1× Tris-acetate EDTA (TAE) buffer. Molten agarose was placed in a water-bath (50 ºC) to cool and 8 µl SYBR® safe nucleic acid gel stain (Invitrogen, UK) added. The molten agarose was poured into a gel casting tray with a comb inserted in order to create wells. Once set, the gel was placed in an electrophoresis tank (Fisher Scientific, UK) containing an appropriate volume of 1× TAE buffer. Loading buffer (2 µl, 6×- see Appendix 2) was mixed with 8 µl of the extracted DNA/ PCR product and loaded onto the gel. Eight µl of 50-1000 bp ladder was loaded (Bioline Biotech, UK) to help estimate the size of the DNA product. The gel was run at 90 volts for 45-60 minutes, followed by imaging under ultraviolet (UV) light (Universal Hood11, Italy). The gel image was visualized using Bio-Rad software (Bio-Rad, UK).

Table 2.1 The host-specific *Bacteroides* primers used in this study

Primer	Primer sequence (5'…….3')	Annealing temp. ºC	*Bacteroides* host	Amplicon size (bp)	References
Bac32F	AACGCTAGCTACAGGCTT	53.7	General	670	(Bernhard and Field, 2000b)
HF183F	ATCATGAGTTCACATGTCCG	55.3	Human	520	(Bernhard and Field, 2000b)
CF128F	CCAACYTTCCCGWTACTC*	54.8	Cow	580	(Bernhard and Field, 2000b)
Bac708R	CAATCGGAGTTCTTCGTG	-	-	-	(Bernhard and Field, 2000b)
HoF795F	GCGGATTAATACCGTATGA	56.7	Horse	129	(Dick *et al.*, 2005a)
PF163F	CCAGCCGTAAAATAGTCGG	52.4	Pig	563	(Dick *et al.*, 2005a)

*W: A or T; Y: C or T; temp: temperature; bp: base pair

2.5.5 Clean-up of PCR products

The PCR products were purified by using a SureClean purification kit as described by the manufacturer's protocol (Bioline Biotech, UK). In brief:

i. An equal volume (25 µl) of SureClean solution was added to the PCR product in Eppendorf tube (1.5 ml) and mixed thoroughly, then incubated at room temperature for 10 minutes. The mixture was centrifuged at 13000 ×*g* for 15 minutes.

ii. The supernatant was aspirated, and then a volume of 70 % molecular biological grade ethanol equal to 2× the original sample volume added. The mixture was vortexed for 10 seconds, before centrifugation at 13000 x *g* (Appleton Woods, UK) for 10 minutes.

iii. The supernatant was removed. The DNA pellet was dried by leaving the tube upside down at room temperature to ensure complete removal of ethanol. The eluted DNA was re-suspended in appropriate volume of water molecular biology grade (Fisher Scientific, UK). Both the purity and quantity of DNA products were measured using a UV spectrophotometer (NanoVue™, Fisher Scientific, UK).

2.5.6 Sequencing of PCR products

PCR products were cleaned using a SureClean purification kit (Bioline Biotech, UK) according to the manufacturer's instructions as described in section 2.5.5. DNA concentration was determined using a NanoDrop® UV spectrophotometer (NanoDrop®, ND-1000, UK). Purified PCR products (20 µl) at a concentration between 20-30 ng μl^{-1} were placed in separate 1.5 ml Eppendorf tubes and sent to the genome analysis and technology core

(GATC Biotech, UK) centre in London for sequencing. The partial sequencing results were obtained via the GATC website (www.gatc-biotechcom/en/index.html). Sequences were matched to those in available databases used the basic local alignment search tool (BLAST, GeneBank) services (www.blast.ncbinlm.nih.gov/Blast.cgi) to determine the phylogenetic relationships.

2.5.7 Quantification assay and absolute standard curve

Quantitative PCR was used to measure the quantity of target nucleic acid during amplification. The absolute standard curve was performed by purification of PCR product, ligation into the target plasmid, cell transformation and plasmid DNA preparation pGEM®-T easy vector as described by Nisr (2012) and Ruijter *et al.* (2013).

2.5.7.1 Molecular media and chemical solutions

Preparation and ingredients of the molecular media and the biochemical solutions are listed in Appendix 2. Luria-Bertani (LB) broth was prepared by dissolving tryptone (10 g), sodium chloride (10 g, NaCl), and yeast extract (5 g) in one litre of distilled water and autoclaved. Ampicillin (100 µg ml^{-1}) was then aseptically added. Bacteriological agar (15 g) was added onto one litre LB broth to make LB agar, autoclaved then cooled down at room temperature to approximately 50 ºC. Thereafter, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, 40 µg ml^{-1}), ampicillin (100 µg ml^{-1}) and isopropyl-β-thiogalactopyranoside (IPTG, 0.1 mM) were added to the LB agar as recommended by the manufacturer's instructions (Promega, UK). The media was then poured into Petri dishes, left to set, then stored at 4 ºC for future use.

2.5.7.2 Plasmid pGEM®-T easy vector

The pGEM®-T easy is a suitable vector for cloning PCR products. The pGEM®-T easy vector 3015 bp (Promega, UK) was provided as a linearized plasmid vector, included multi cloning sites for easy excision of insert, T7 and SP6 long sequencing primer for transcription and sequencing. Plasmid pGEM®-T easy vector contains an ampicillin resistance gene (Amp^r) to use as a selectable genetic marker and a β-galactosidase gene (lacZ) that glued the plasmid on both ends to prevent re-annealing of plasmid and provided compatible overhangs for PCR product (Figure 2.3).

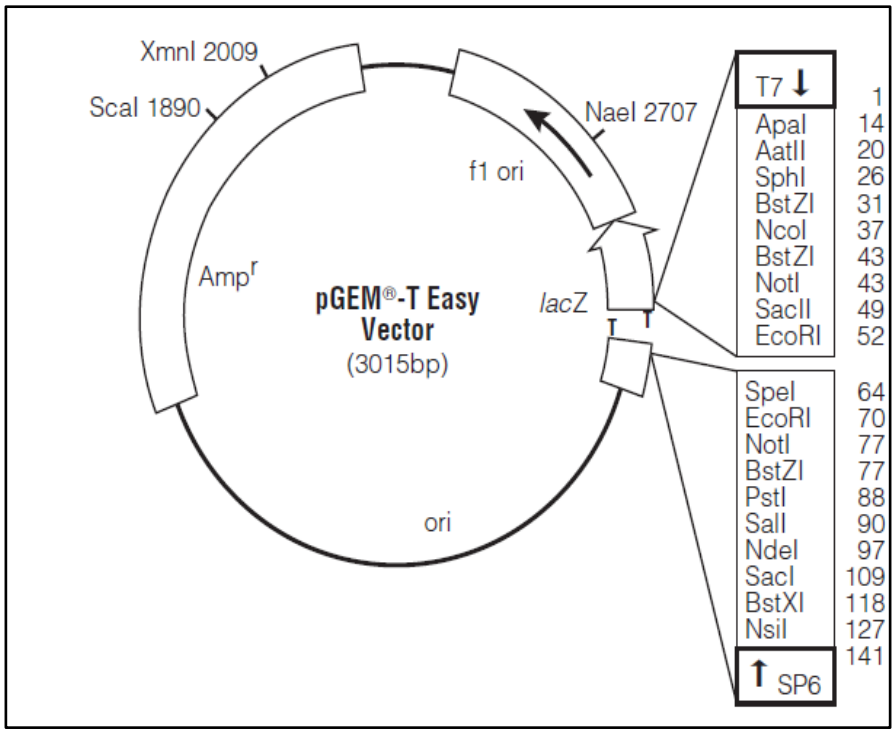


Figure 2.3 Schematic map of the pGEM®-T easy cloning plasmid vector provides convenient promoters (T7 and SP6) that serve as sequencing binding sites. It also contains the lacZ gene which acts as coding region to allow easy blue/ white screening of recombinant cells, (reproduced with permission from Promega, UK).

2.5.7.3 Ligation of PCR products into pGEM®-T easy plasmid

The purified PCR products were cloned into plasmid pGEM®-T easy using a Promega cloning system (Promega, UK). The PCR products were ligated within the pGEM®-T easy plasmid using a T_4 DNA ligase enzyme and their buffer provided with the cloning system. The ligation mixture (10 µl) was prepared in 1.5 ml Eppendorf tubes as below:

- T_4 DNA ligation buffer 5 µl
- pGEM®-T easy plasmid (50 ng μl^{-1}) 1 µl
- T_4 DNA ligase enzyme (3 U) 1 µl
- PCR amplicon DNA (20 ng μl^{-1}) 2 µl
- Molecular biology grade water up to 10 µl

The contents were mixed gently and the tubes were incubated at room temperature for 2 hours. Two types of control reactions were set for ligation:

i. the plasmid with inserted DNA was used as a positive control and

ii. the plasmid without inserted DNA was used as a negative control.

2.5.7.4 Competent cells transformation

Competent *E. coli* JM109 cells (Promega, UK) with high transformation efficiency (10^8 CFU μg^{-1}) provided with pGEM®-T easy cloning system were used according to the manufacturer's instructions. These cells were stored at - 80 ºC. In brief:

i. The ligation mixture (4 µl) was placed in 1.5 ml DNase/ RNase-free polypropylene tubes.

ii. Competent cells (50 µl) were added after thawing in an ice bath for 5 minutes and mixed gently, before being left on ice for 20 minutes. The

samples were then heat-shocked by placing at 42 ºC for 50 seconds. The sample was then immediately returned into ice for 2 minutes.

iii. Luria Bertani (LB) broth (200 µl) was added into the tubes containing the ligation mixture, and then incubated at 37 ºC with shaking (120 rpm, Thermo Scientific, UK) for 90 minutes. The mixture was plated (100 µl) onto LB agar plates containing ampicillin (100 µg ml^{-1}), IPTG (0.1 mM), and X-gal (40 µg ml^{-1}). The plates were incubated at 37 ºC overnight.

iv. White colonies were considered recombinant transformants, while blue colonies lacked inserts or non-recombinant (Brown, 2010). White and blue pure colonies were streaked again separately on LB agar plates containing X-gal/ IPTG/ ampicillin (Appendix 2). A single colony was inoculated in LB broth (10 ml) containing ampicillin (100 µg ml^{-1}), and then incubated overnight at 37 ºC with shaking (120 rpm, Thermo Scientific, UK). The inoculated broth then was used for the plasmid/ DNA extraction.

2.5.7.5 Plasmid/ DNA extraction

Plasmid/ DNA product was extracted from the culture of recombinant *E. coli.* The GenElute™ plasmid miniprep kit (Sigma Aldrich, UK) was used as described by the manufacturer's guidelines. In brief:

i. Aliquots (1 ml) of recombinant *E. coli* were harvested by centrifugation at 13000 × *g* for 2 minutes at room temperature, and then the supernatant discarded.

ii. The cell pellet was re-suspended by adding 200 µl of the re-suspension solution, mixed gently by pipetting.

iii. Lysis solution (200 µl) was added to lyse the re-suspended cells and release plasmid. The contents were mixed by gentle inversions then left for five minutes at room temperature.

iv. Neutralization binding solution (350 µl) was added to the pellet and mixed gently by inverting the tube for 5 minutes. Then, the mixture was centrifuged at 12000 × *g* for 10 minutes.

v. GenElute columns were prepared by adding 500 µl of the column preparation solution and centrifuged at 12000 × *g* for one minute.

vi. The clear supernatant from step 4 was transferred into spin columns, centrifuged at 12000 × *g* for one minute and the flow-through liquid discarded.

vii. Wash solution (750 µl) was added into spin columns, and then centrifuged at 12000 × *g* for one minute.

viii. The spin columns were transferred to fresh collection tubes and 100 µl of molecular biology grade water was added. Next, the columns were centrifuged at 12000 × *g* for one minute. Finally, the eluted plasmid DNA was stored at - 20 ºC for future use.

2.5.7.6 Restriction enzyme digestion

Double stranded DNA was cleaved by restriction enzymes at specific nucleotide sequences called restriction sites. One unit (U) of restriction enzyme is defined as the amount of enzyme required to cut the plasmid at 37 ºC. EcoR1 restriction enzyme (Promega, UK) was used to check the presence of the inserted target gene in the vector. Mixture reaction 25 µl was prepared in a 1.5 ml Eppendorf tube as the following:

- Plasmid/ DNA (20 ng μl^{-1}) 5 µl
- EcoR1 restriction enzyme (4 U) 2 µl
- EcoR1 restriction enzyme buffer 2 µl
- Bovine serum albumin (10 µg μl^{-1}) 1 µl
- Deionized water up to 25 µl

Digestion was performed at 37 ºC overnight and then electrophoresed into a 1.5 % agarose gel prepared as described in section 2.5.4 to check for the presence of the target insert at the correct size.

2.5.7.7 Target sequencing

The BigDye® terminator version 3.1 cycle sequencing kit (Applied Biosystems, USA) protocol was used to achieve fluorescence-based cycle sequencing reactions on PCR fragments. This method was used as per the manufacturer's instructions. For sequencing the target plasmid/ DNA, a master mix (20 µl) was prepared as below:

- BigDye® terminator ready mix 4 µl
- Forward or reverse primer (5 pmol) 1 µl
- BigDye® terminator buffer 3.5 µl
- Plasmid/ DNA (20 ng μl^{-1}) 1.5 µl
- Deionized water 10 µl

PCR was run on a GeneAmp® PCR system 9700 thermal cycle (Applied Biosystems, USA). The PCR conditions were: initial denaturation at 96 ºC for one minute, followed by 25 cycles of 96 ºC for 10 second, 50 ºC for 5 second, 60 ºC for 4 minutes, then final extension at 60 ºC for 5 minutes.

Purification and precipitation process was performed using the Ethanol/ EDTA precipitation protocol (BigDye® Applied Biosystems). In brief:

i. EDTA 125 mM (5 µl, pH 8) was added to each well of a MicroAmp® 96 well plate (Applied Biosystems, USA) containing sample reaction (20 µl).

ii. Absolute ethanol (60 µl) was added to each sample well.

iii. The MicroAmp® 96 well plate was sealed by aluminium tape and mixed by inverting 4 times, and then incubated at room temperature for 20 minutes.

iv. The MicroAmp® 96 well plate containing samples was centrifuged at 21000 × *g* (Appleton Woods, UK) for 20 minutes at 4 ºC. The supernatant was removed, and then the pellet washed using 110 µl of 70 % ethanol. The MicroAmp® 96 well plate was protected from light and left to air dry for 10 minutes.

v. The pellet was re-suspended using 15 µl HiDi® formamide (Sigma Aldrich, UK), mixed by pipetting several times, and transferred into a 96 well sequencing plate for running on the Applied Biosystems ABI 3130 genetic analyser (Applied Biosystems, USA) with the optimal programme using T7 primer (5´- TAATACGACTCACTATAGGG- 3´).

Partial sequences were obtained and then analysed using the basic local alignment search tool (BLAST) software from the National Centre for Biotechnology Information (NCBI). The BLAST-GeneBank software was used to compare the target DNA sequence identity with the closest accession numbers approximate phylogenetic relationships.

2.5.7.8 Quantitative PCR (qPCR) amplification

Quantitative PCR was performed (all samples prepared in triplicate) in a total volume of 25 µl using the MicroAmp® optical 96 well reaction plate (Applied

Biosystems, USA). The plate was tightly covered with adhesive film (Applied Biosystems, USA), and then run in the StepOne™ Plus real-time PCR system (ABI Applied Biosystems, USA). The thermal PCR conditions were included 40 cycles started at 94 ºC for denaturing for 2 minutes, primer-specific annealing temperature at 62.5 ºC or 63.5 ºC for one minutes, and extension stage at 72 ºC for 1 minute. Melting curves for PCR products were set between 60-90 ºC. The amplicons of the 16S rRNA target gene were amplified and reaction components added respectively, to the each reaction well as below:

- SYBR® Green1 JumpStart Taq ReadyMix (Sigma, UK) 12.5 µl
- Forward primer (20 pmol, MWG, Germany) 1 µl
- Reverse primer (20 pmol, MWG, Germany) 1 µl
- Reference dye (Rox, Invitrogen, UK) 0.25 µl
- DNA template (20 ng μl^{-1}) 2 µl
- Molecular biology grade water 8.25 µl

Total 25 µl

Tenfold dilutions of the target insert were prepared and used to make a dilution series to create absolute standard curves (Ahmed *et al.*, 2010; Nisr *et al.*, 2011).

To detect the sensitivities and PCR amplification among the experiments, the slopes of the standard curves were determined by performing a linear regression test with StepOne™ software version 2.2.2 (Applied Biosystems, USA). For the quantitative PCR standards, the concentration was plotted versus the cycle number at which the fluorescence signal exceeds the

threshold cycle (Ct) value. A reaction with 100 % efficiency will produce a slope - 3.32 (Okabe *et al.*, 2007; Ahmed *et al.*, 2010). The efficiency of amplification (Eff.) was determined using the slope of the standard curve and calculated using the following equation:

$$\text{Eff. \%} = (10^{-1/\text{slope}}) - 1 \quad (1)$$

The efficiency was then converted to percentage by multiplying by a factor of 100.

2.5.7.9 Creating copy number standard curves with plasmid DNA

To determine the copy numbers of the target *Bacteroides* genetic markers, tenfold dilutions of the pGEM®-T easy recombinant plasmid were subjected to qPCR in triplicate as described in section 2.5.7.8, in order to create absolute standard curves for each marker. The mass of the plasmid plus the insert was calculated using the following equation:

$$m = [n]\left[\frac{\text{MW of plasmid}}{\text{Avogadro's number}}\right] \quad (2)$$

Where m is a mass of single plasmid (g), n is a size of plasmid and insert (bp), Avogadro's number = 6.023×10^{23} molecules per mole, MW: molecular weight of plasmid = 660 g per mole (Applied Biosystems, 2003).

The mass of plasmid DNA containing 5×10^6 to 5×10^0 copies was calculated as per the following equation:

Mass needed = [mass of single plasmid × copy number of interest] (3)

A series of tenfold serial dilutions of the plasmid DNA was carried out using molecular grade water as the diluent to achieve a working stock of plasmid which was used in qPCR quantifications. The following formula was used to

calculate the volume which was needed to prepare serial dilutions for standard curves:

$$C_1 \times V_1 = C_2 \times V_2 \quad (4)$$

Where C is a concentration of plasmid (ng) and V is a volume (µl) of dilution (Applied Biosystems, 2003).

2.6 Statistical analysis

The results were statistically analysed using the SPSS programme version 21 and Minitab version 16. Analysis of variance (ANOVA) (one and two ways) test was used including Fisher's least significant differences (Fisher's LSD) *post hoc* analyses. Pearson's correlation (r) coefficient was also performed to estimate the correlation between the sites and sampling times. Data were presented as mean ± standard error (SE). A p value of 0.05 or less was considered significant. All Figures and Tables were prepared using SigmaPlot version 12.5 and Microsoft excel 2010 software.

Chapter Three
An evaluation of bacterial source tracking of faecal bathing water pollution in the Kingsbridge estuary

3.1 Introduction

Vinten *et al.* (2008) stated that certain areas of the United Kingdom such as South Wales, North Yorkshire and south west Scotland have problems with bathing water compliance. Areas of intensive dairy farming with a high cattle concentration, cool and humid summers are prone to bathing water pollution (Ray, 1989). Farm areas and cattle walkways are highly susceptible to direct runoff of microbially contaminated water into streams, and a widespread direct use of streams to supply drinking water to livestock in summer. Heavy rainfall can exacerbate these inputs from agricultural land and can also cause problems with sewage treatment systems which, if overloaded, may resort to the use of storm sewer overflows, discharging sewage and rainfall largely untreated (Kay *et al.*, 2000). A higher risk to bathers occurs from contaminated bathing water, as well as economic losses as a result of closed beaches and shellfish harvesting areas (Scott *et al.*, 2002). Faecal indicator bacteria (FIB) are commonly used to determine water pollution of public health significance (Kay *et al.*, 2008a). The identification and enumeration of indicator bacteria have several advantages. However, these methods fail to detect the source of faecal pollution (Scott *et al.*, 2002). Detection of the source is a pre-requisite for the effective and efficient management of these aquatic environments. It also reduces the time and cost of implementing remedial measures (Okabe *et al.*, 2007). The intestines of warm-blooded animals contain abundant indicator bacteria, and their presence in environmental waters indicates faecal pollution, including the potential presence of pathogenic microorganisms (Anderson *et al.*, 2005). Van Asperen *et al.* (1998) identified the quality of water which is necessary to

protect bathers from illness and the key feature of quality is the concentration of faecal pollution in the water. The EU bathing water directives 2006/ 7/ EC are mainly focused on the protection of the health of bathers and those involved in coastal recreation (EU, 2006; Kay *et al.*, 2007a). The bathing and recreation season in the UK starts from beginning of May and continues to the end of September (Rees *et al.*, 1998; Howarth and McGillivary, 2001).

This study aimed to investigate the possible source of faecal bacterial pollution on South Sands beach. To monitor FIB in and out of the bathing season at a site of known problems in this regard, in order to note any trends and evaluate *Bacteroides* PCR-based tracking to source human and non-human faecal pollution. Sediments were also monitored as a possible reservoir of faecal water pollution.

3.2 Materials and methods

A detailed description of the methods used is given in chapter two. This section highlights features specific to this chapter only.

3.2.1 Description of study area

This study was carried out to assess the possible sources of microbial pollution in bathing water of South Sands, Salcombe, UK. South Sands is situated in the South Hams district of the Devon country and in the lower reaches of the Kingsbridge estuary in the South West of England (latitude 50.13 ºN longitude - 3.47 ºW). This study mainly concentrates on the three locations of the South Sands waters and sediments, these sites consist of the South Sands beach (A), the pond near to the hotel (B) and the stream near to the caravan park (C, Figure 3.1).

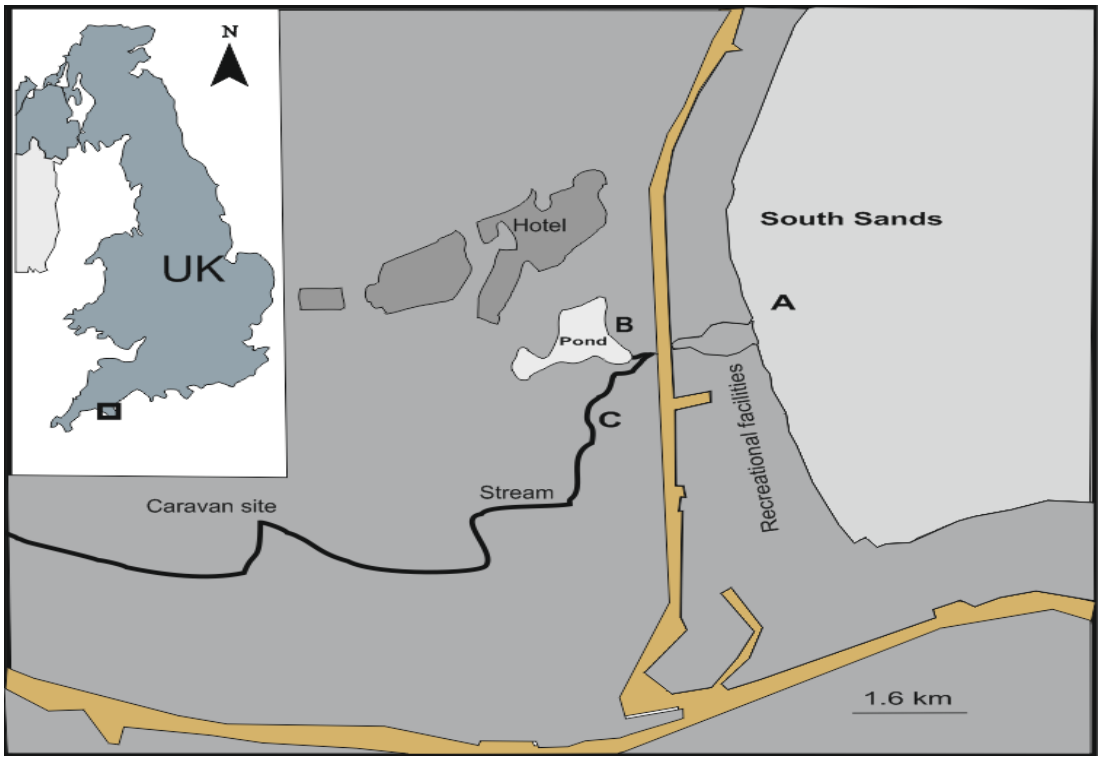


Figure 3.1 The study area, South Sands beach, Salcombe, Devon, UK. A: South Sands beach, B: pond near to the hotel, C: stream just below the caravan park.

3.2.2 Sample collection

Triplicate, separate samples of water and sediment were obtained from each site on four occasions in June, August, October 2010 and February 2011 as described in section 2.3 for the detection and enumeration of the FIB according to the EU bathing water directive 2006/ 7/ EC (EU, 2006). In addition, human faeces were collected from four adult volunteers, while animal faeces (cow, horse, and pig) were obtained from farms close to the study area.

4.2.3 Sample filtration

The membrane filtration method was used to process the water and the sediment samples, and subsequently culture-dependent analysis used to detect and enumerate Enterococci, *E. coli* and *Bacteroides* spp. One hundred millilitre of water was passed through filter membranes (0.45 µm, Whatman, UK) with suction by a negative pressure vacuum pump (section 2.4.1). Two grams of sediment sample were taken and added to sterile sea water up to a final volume of 20 ml as described in section 2.4.2. After filtration, the membranes were placed onto Slanetz and Bartley agar to detect Enterococci and membrane lauryl sulphate (MLS) broth to detect *E. coli.* Plates were incubated aerobically at 35 ºC for 4 hours for resuscitation of cells, then Enterococci cultures incubated at 44 ºC for 44 hours, and *E. coli* cultures were incubated overnight at 44 ºC. To detect *Bacteroides* spp., the membranes were placed onto *Bacteroides* bile esculin agar (BBE), and then incubated at 37 ºC for 72 hours at anaerobic condition (Don Whitley, UK). Results were expressed as CFU 100 ml^{-1} for the water samples and CFU g^{-1} for the sediment samples (Livingston *et al.*, 1978; Fricker and Fricker, 1996; Craig *et al.*, 2002).

3.2.4 DNA extraction and quantification

DNA was extracted from the water and faeces samples by using QIAamp stool DNA mini kit (Qiagen, UK) following the manufacturer's protocol (see sections 2.5.1.1 and 2.5.1.3). The SoilMaster^{TM} DNA extraction kit (Cambio, UK) was used for DNA extraction from the sediment samples following the manufacturer's protocol (see section 2.5.1.2).

The quantity and quality of extracted DNA was measured for all samples using a NanoDrop® (ND-1000) UV spectrophotometer as described in section 2.5.2.

3.2.5 Conventional polymerase chain reaction (PCR)

Conventional PCR was used to detect faecal *Bacteroides* 16S rRNA genetic markers in the water, sediment and faecal samples by using primer sets previously designed by Bernhard and Field (2000a; 2000b) and Dick *et al.* (2005b) (Table 2.1). PCR was run in a total volume of 25 µl per reaction mixture as described in section 2.5.3.

3.2.6 Agarose gel electrophoresis

Gel electrophoresis was used to determine the presence and size of PCR products. It was prepared and run as described in section 2.5.4.

3.2.7 Statistical analysis

The results were statistically evaluated using SPSS programme version 21 as described in section 2.6.

3.3 Results

The water samples showed no visible turbidity, and the pH was 7.8 ± 0.3 for seawater and 7.5 ± 0.3 for stream and pond waters. The salinity was 34 and < 0.5 practical salinity units (psu) for seawater and inland water (stream and pond), respectively.

3.3.1 Enumeration of faecal indicator bacteria

Overall, the results from water samples collected in February and October showed that there was a probability of more faecal pollution at the pond near to hotel and in the stream behind Caravan Park than in the seawater ($p < 0.05$). The numbers of *E. coli* isolated from three South Sands sites showed significant differences between beach, stream and pond ($p < 0.05$). *E. coli* numbers showed variable values at the four sampling events, and there was the probability of more microbial pollution during February and October ($p < 0.05$), and lesser contamination during June and August sampling events ($p > 0.05$). Also, there were significant differences between August and October and between August and February sampling events ($p < 0.05$, Table 3.1). In addition, the numbers of Enterococci in water samples showed significant differences between beach and stream and between beach and pond ($p < 0.05$), but no significant differences were observed between pond and stream South Sands sites ($p > 0.05$). According to the times of water collection, Enterococci numbers showed significant differences between October and other sampling times (June, August and February, $p < 0.05$, Table 3.2). *Bacteroides* spp. numbers showed significant differences between the beach and other South Sands sites (stream and pond, $p < 0.05$), but no significant differences were observed between stream and pond ($p >$

0.05). *Bacteroides* spp. numbers during February and October sampling events showed that there was the probability of more faecal pollution ($p < 0.05$) at stream and pond, but the number during June and August showed lesser concentration and no significant differences between June and August sampling events ($p > 0.05$, Table 3.3).

All sediment samples showed a loading of both FIB and *Bacteroides* spp. with a significant increase out of the bathing season. The transport of water from pond to stream to the beach is also reflected in the FIB and *Bacteroides* sediment values, the beach being the least contaminated. The results of culture-dependent analysis from the South Sands sediments are shown in Tables 3.4, 3.5 and 3.6. The results from sediment samples conducted in February and October showed the probability of more faecal pollution at pond and stream ($p < 0.05$). There were significant differences between the numbers of *E. coli* which were isolated from the three sites of South Sands (beach, stream and pond, $p < 0.05$). According to the sampling events, there were significant differences between February and other sampling times (June, August and October, $p < 0.05$), but no significant differences were observed between June and August ($p > 0.05$, Table 3.4). In addition, Enterococci numbers showed significant differences between all South Sands sites ($p < 0.05$, Table 3.5). According to the sampling times, Enterococci numbers showed significant differences between October and other sampling events (June, August and February, $p < 0.05$), but no significant differences were observed between August and February ($p > 0.05$). *Bacteroides* spp. numbers showed significant differences between three South Sands sites ($p < 0.05$). Significant differences were observed

between the sampling times in February and October and between June and August ($p > 0.05$, Table 3.6). In addition, high and positive correlations $r = 0.98$ and $r = 0.91$ were observed between *E. coli* and *Bacteroides* spp. numbers isolated from water and sediment samples, respectively.

3.3.2 Description of sampling intervals

The concentrations of each FIB were assessed at the time of sampling, and are shown in Figures 3.2, 3.3 and 3.4. In the June sampling, *E. coli* (Figure 3.2) showed low numbers at all sites (32, 24 and 54 CFU 100 ml^{-1}) in the waters of the stream, beach and pond, respectively, as well as in the sediment of the same respective sample sites (41, 35 and 68 CFU g^{-1}). In the August sampling, *E. coli* showed a similar distribution as the June sampling, in the water of stream (26, 16 and 47 CFU 100 ml^{-1}), beach, and pond, as well as in sediment of the same sites (36, 32 and 62 CFU g^{-1}). The October sampling showed an increase in *E. coli* isolates, with the highest concentrations in pond sediment (212 CFU g^{-1}), stream sediment (199 CFU g^{-1}), pond water (198 CFU 100 ml^{-1}), and stream water (161 CFU 100 ml^{-1}). The lowest concentrations of *E. coli* were in the water and sediment of the beach (96 CFU 100 ml^{-1}, 104 CFU g^{-1}). Moreover, the February sampling showed the highest concentration of *E. coli* in all sites of the study area than the June, August and October samplings, from the sediment of both the pond and the stream (297 and 280 CFU g^{-1}, respectively), as well as the pond water and the stream water (261 and 219 CFU 100 ml^{-1}, respectively).

Table 3.1 *E. coli* numbers associated with water taken from South Sands beach on four occasions between June 2010 and February 2011.

Site/ month	June	August	October	February
Stream	32 ± 4 $^{c, 2}$	26± 10 $^{c, 2}$	161± 16 $^{b, 2}$	219± 27 $^{a, 2}$
Beach	24± 4 $^{b, 3}$	16± 7 $^{b, 2}$	96± 17 $^{a, 3}$	97± 17 $^{a, 3}$
Pond	54± 11 $^{c, 1}$	47± 5 $^{c, 1}$	198± 16 $^{b, 1}$	261± 18 $^{a, 1}$

Values are presented as mean ± SD. Values with different superscript letters within each row are significantly different, while mean values with different superscript numbers within each column are significantly different.

Table 3.2 Enterococci numbers associated with water taken from South Sands beach on four occasions between June 2010 and February 2011.

Site/ month	June	August	October	February
Stream	197± 35 $^{b, 1}$	167± 97 $^{b, 1}$	364± 35 $^{a, 1}$	133± 10 $^{b, 2}$
Beach	35± 11 $^{b, 2}$	30± 14 $^{b, 2}$	101± 19 $^{a, 3}$	75± 10 $^{a, 3}$
Pond	221± 42 $^{a, 1}$	199± 38 $^{a, 1}$	228± 10 $^{a, 2}$	198± 22 $^{a, 1}$

Values are presented as mean ± SD. Values with different superscript letters within each row are significantly different, while mean values with different superscript numbers within each column are significantly different.

Table 3.3 *Bacteroides spp*. numbers associated with water taken from South Sands beach on four occasions between June 2010 and February 2011.

Site/ month	June	August	October	February
Stream	142± 47 $^{c, 1}$	243± 28 $^{b, 1}$	341± 49 $^{a, 1}$	369± 20 $^{a, 1}$
Beach	42± 31 $^{c, 2}$	161± 58 $^{b, 2}$	167± 9 $^{b, 2}$	199± 19 $^{a, 2}$
Pond	176± 12$^{c, 1}$	281± 57 $^{b, 1}$	327± 7$^{b, 1}$	374± 26 $^{a, 1}$

Values are presented as mean ± SD. Values with different superscript letters within each row are significantly different, while mean values with different superscript numbers within each column are significantly different.

Table 3.4 *E. coli* numbers associated with sediment taken from South Sands beach on four occasions between June 2010 and February 2011.

Site/ month	June	August	October	February
Stream	41± 12 $^{c, 2}$	36± 4 $^{c, 2}$	199± 14 $^{b, 2}$	280± 36 $^{a, 1}$
Beach	35± 6 $^{c, 2}$	32± 4 $^{c, 2}$	104± 14 $^{b, 3}$	141± 5 $^{a, 2}$
Pond	69± 15 $^{c, 1}$	62± 7 $^{c, 1}$	212± 20 $^{b, 1}$	297± 15 $^{a, 1}$

Values are presented as mean ± SD. Values with different superscript letters within each row are significantly different, while mean values with different superscript numbers within each column are significantly different.

Table 3.5 Enterococci numbers associated with sediment taken from South Sands beach on four occasions between June 2010 and February 2011.

Site/ month	June	August	October	February
Stream	64± 9 $^{b, 2}$	192± 35 $^{a, 1}$	214± 29 $^{a, 2}$	202± 12 $^{a, 2}$
Beach	42± 8 $^{b, 3}$	93± 17 $^{a, 2}$	112± 12 $^{a, 3}$	113± 13 $^{a, 3}$
Pond	112± 14 $^{c, 1}$	228± 51 $^{b, 1}$	432± 41 $^{a, 1}$	266± 43 $^{b, 1}$

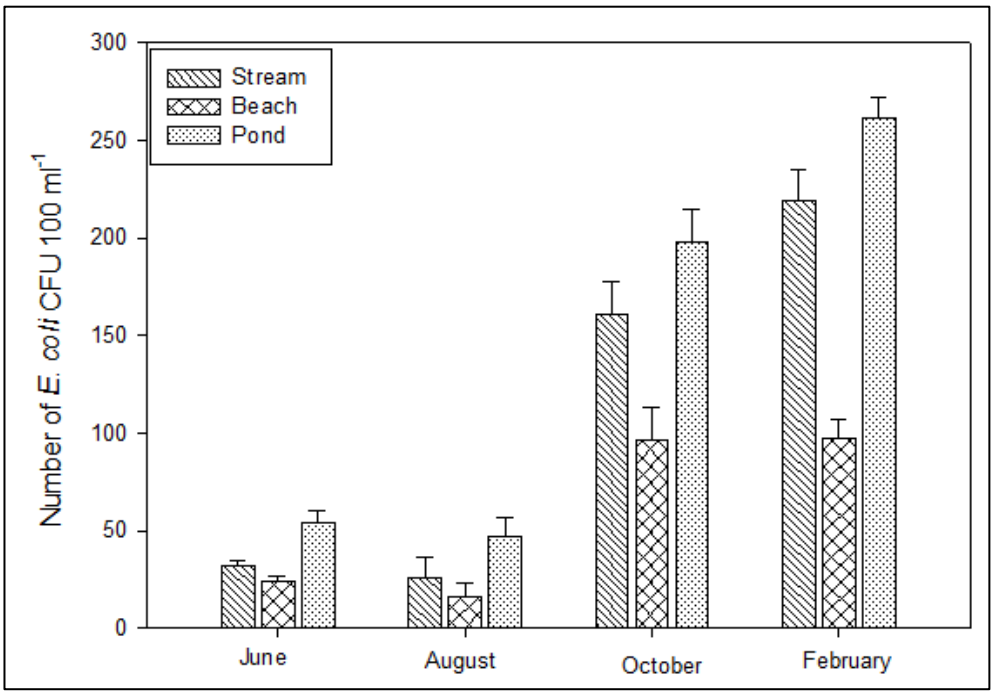
Values are presented as mean ± SD. Values with different superscript letters within each row are significantly different, while mean values with different superscript numbers within each column are significantly different.

Table 3.6 *Bacteroides* spp. numbers associated with sediment taken from South Sands beach on four occasions between June 2010 and February 2011.

Site/ month	June	August	October	February
Stream	67± 7 $^{c, 2}$	220± 21 $^{b, 1}$	398± 33 $^{a, 2}$	388± 34 $^{a, 2}$
Beach	83± 15 $^{b, 2}$	131± 42 $^{b, 2}$	137± 26 $^{b, 3}$	229± 20 $^{a, 3}$
Pond	198± 11 $^{b, 1}$	245± 51 $^{b, 1}$	416± 25 $^{a, 1}$	408± 13 $^{a, 1}$

Values are presented as mean ± SD. Values with different superscript letters within each row are significantly different, while mean values with different superscript numbers within each column are significantly different.

a



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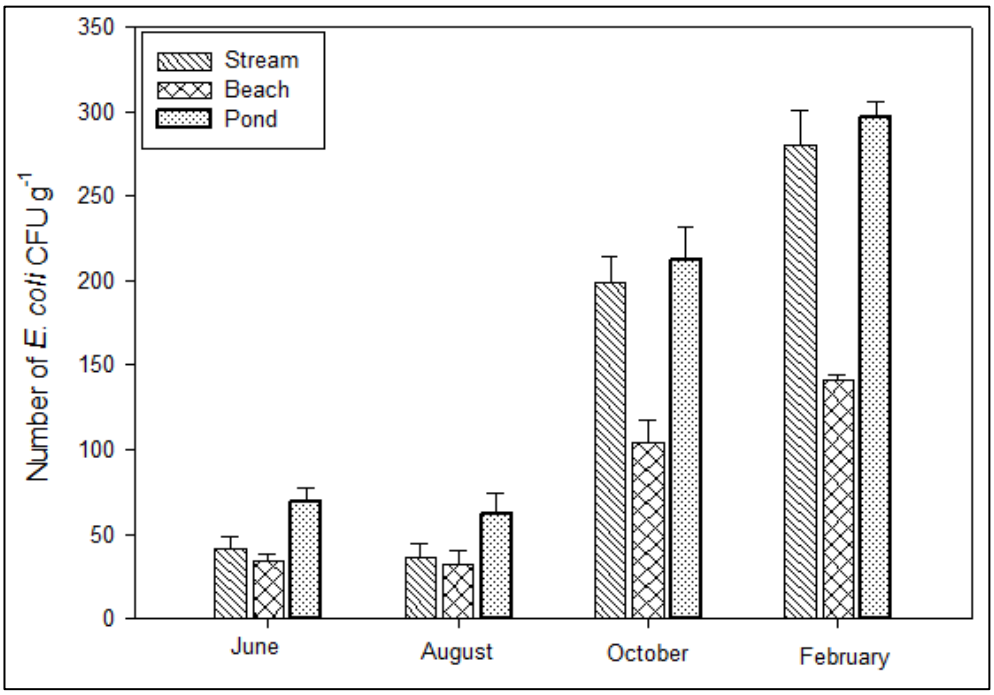
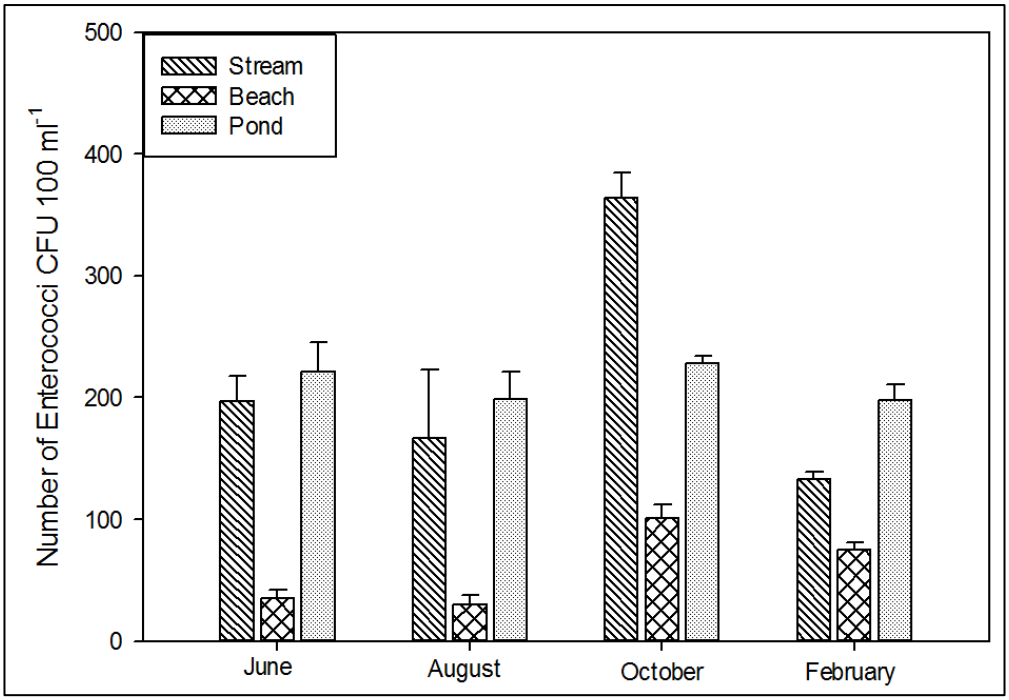


Figure 3.2 *E. coli* numbers (CFU) associated with water (a) and sediment (b) taken from South Sands beach on four occasions between June 2010 and February 2011. Data is presented as mean ± SE of triplicate samplings.

The June sampling results showed the highest concentration of Enterococci (Figure 3.3) in the pond water (221 CFU 100 ml^{-1}) and in the stream water (197 CFU 100 ml^{-1}), whereas, there was the lowest concentration in the beach water (35 CFU 100 ml^{-1}), stream sediment and the beach sediment (64 and 42 CFU g^{-1}). August samples also showed the highest concentration in pond sediment (228 CFU g^{-1}) and the stream (192 CFU g^{-1}), pond water (199 CFU 100 ml^{-1}), stream water (167 CFU 100 ml^{-1}) and the lowest level in beach water (30 CFU 100 ml^{-1}), and beach sediment (93 CFU g^{-1}). The October sampling showed the highest concentration of Enterococci in the pond sediment (432 CFU g^{-1}), stream water (364 CFU100 ml^{-1}) and the lowest in the pond water (228 CFU100 ml^{-1}), stream sediment (214 CFU g^{-1}), beach water and sediment (101 CFU 100 ml^{-1} and 112 CFU g^{-1}), respectively. Furthermore, the highest concentration of Enterococci in pond sediment (266 CFU g^{-1}) followed by stream sediment (202 CFU g^{-1}) and pond water (198 CFU 100 ml^{-1}).

a



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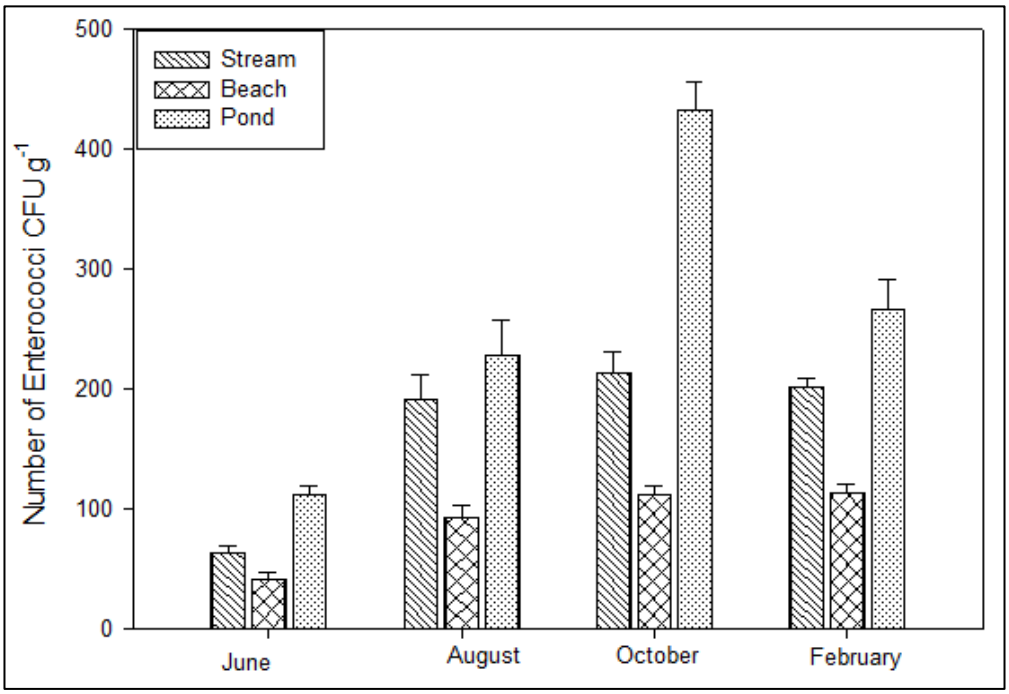
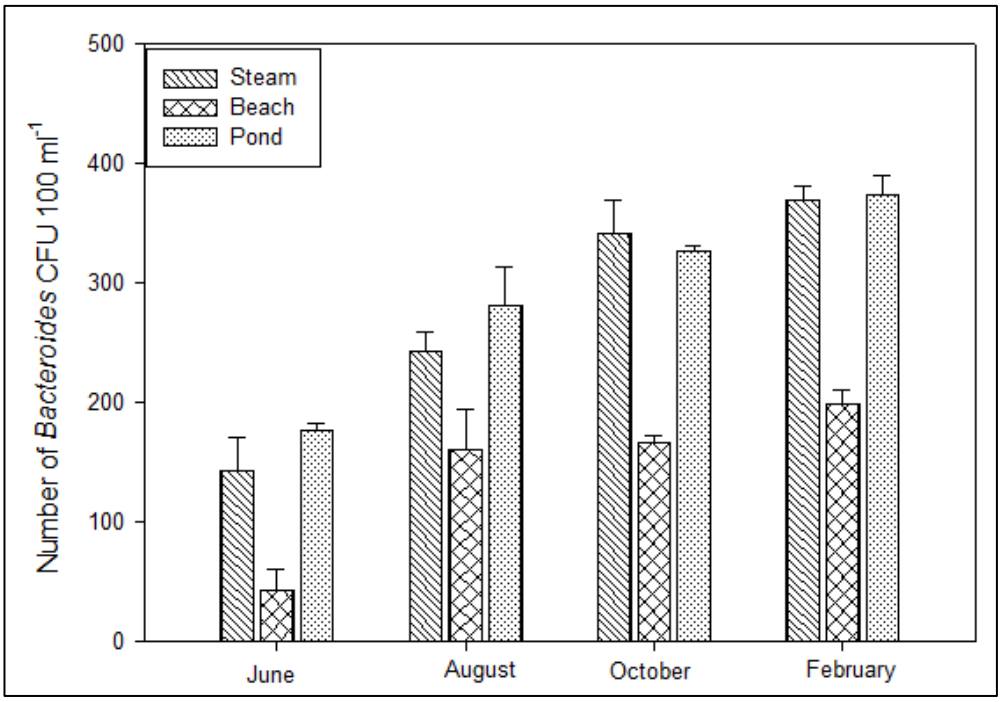


Figure 3.3 Enterococci (CFU) numbers associated with water (a) and sediment (b) taken from South Sands on four occasions between June 2010 and February 2011. Data is presented as mean ± SE of triplicate samplings.

From Figure 3.4, the June sampling appeared the highest number of *Bacteroides* spp. in pond sediment (198 CFU g^{-1}), pond water and stream (176, 143 CFU100 ml^{-1}) and the lowest concentration in water and beach sediment (43 CFU 100 ml^{-1}, 84 CFU g^{-1}) and stream sediment (67 CFU g^{-1}). The August sampling showed the highest number of *Bacteroides* spp. in water and sediment of the pond (281 CFU 100 ml^{-1}, 245 CFU g^{-1}), water and sediment of the stream (243 CFU 100 ml^{-1}, 220 CFU g^{-1}). In addition, the lowest number showed in water and sediment of the beach (161 CFU 100 ml^{-1}, 131 CFU g^{-1}, respectively).

The October sampling, the highest number of *Bacteroides* spp. showed in sediment and water of the pond (416 CFU g^{-1} and 327 CFU 100 ml^{-1}), sediment and water of the stream (398 CFU g^{-1} and 341 CFU 100 ml^{-1}), water and sediment of the beach (167 CFU 100 ml^{-1} and 137 CFU g^{-1}). In addition, The February samplings showed the highest concentration of *Bacteroides* spp. in all sites (408 and 388 CFU g^{-1}) from the sediment of the pond and the stream from the water of the pond and the stream (374, 369 CFU 100 ml^{-1}), respectively.

a



b

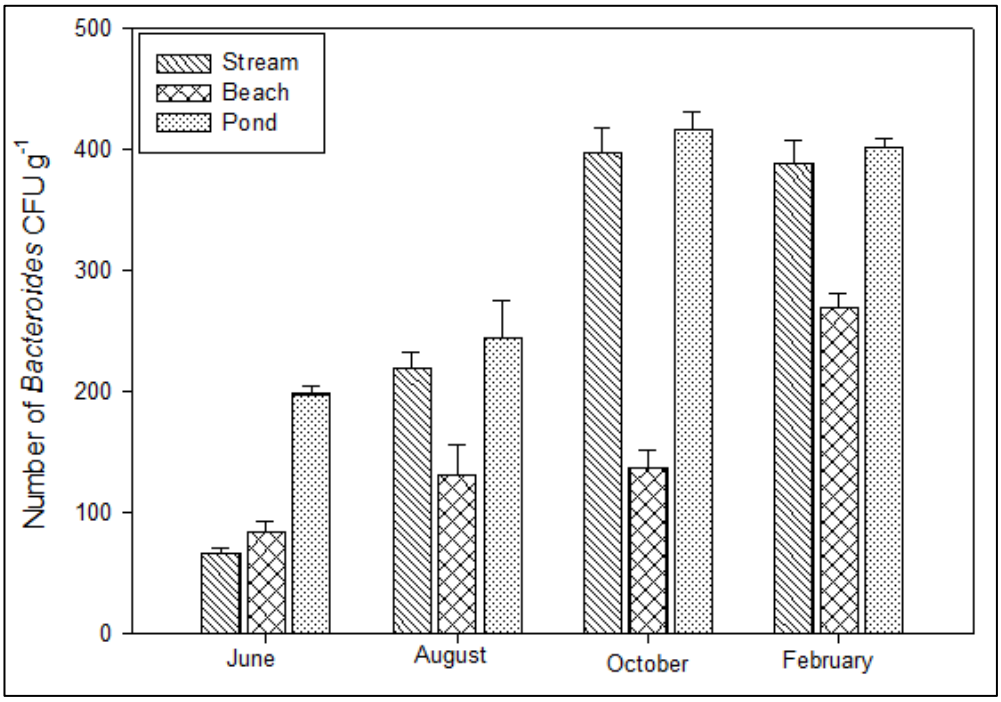


Figure 3.4 *Bacteroides* spp. numbers (CFU) associated with water (a) and sediment (b) samples taken from the South Sands beach on four occasions between June 2010 and February 2011. Data is presented as mean ± SE of triplicate samplings.

3.3.3 Microbial source tracking

The results of host-specific PCR of *Bacteroides* 16S rRNA genetic markers are shown in Figures 3.5, 3.6, and 3.7. The five forward host-specific primers were used to detect the source(s) of faecal water and sediment pollution (Table 3.7).

3.3.3.1 Detection of *Bacteroides* using host-specific primer sets

The generic *Bacteroides* primer set Bac32F-Bac708R was used to detect *Bacteroides* 16S rRNA genetic marker in the water and the sediment samples. All samples gave positive results with this primer set with a product size of 670 bp (Figure 3.5). This confirmed that all water and sediment samples from the site contained faecal *Bacteroides* spp. To determine the source of these faecal *Bacteroides* the samples were then investigated with human, cow, horse and pig host-specific primer sets.

Table 3.7 Combined presence (+)/ absence (-) data relating to host-specific *Bacteroides* genetic markers in water (W) and sediment (S) of South Sands beach on the four sampling occasions.

Specific markers	Stream		Pond		Beach	
	W	S	W	S	W	S
Human HF183F	-	+	-	-	-	-
Generic Bac32F	+	+	+	+	+	+
Cow CF128F	-	-	-	-	-	+
Horse HoF597F	-	-	-	-	-	-
Pig PF163F	-	-	-	-	-	-

W: water, S: sediment, positive and negative results represent for four sampling events.

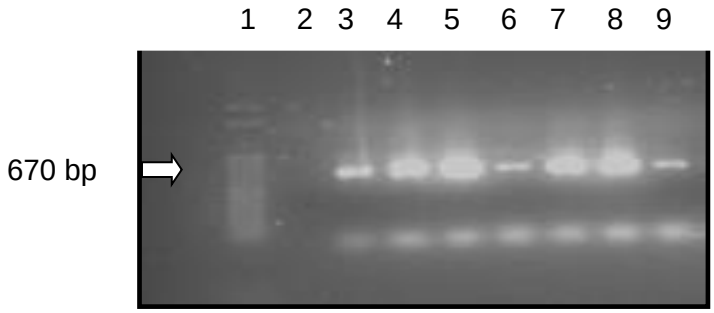


Figure 3.5 Host-specific *Bacteroides* 16S rRNA genetic marker amplified with generic *Bacteroides* (Bac32F-Bac708R) primer set. Presence of this marker was indicated by a 670 bp PCR product, as indicated on fragment. Lane 1: 50-1000 bp ladder, lane 2: negative control, lane 3: positive control (*B. fragilis* NCTC 9343 DNA template), lanes 4 and 5: stream water and sediment, lanes 6 and 7: pond water and sediment and lanes 8 and 9: water and sediment of beach.

The human-specific primer set (HF183F-Bac708R) was used to detect human origin *Bacteroides* 16S rRNA genetic marker in the water and sediment samples. The sediment of the stream showed a positive result, yielding a 520 bp product. However, all samples from other sites gave negative result, i.e. no product (Figure 3.6).

A cow-specific primer set (CF128F-Bac708R) was used to detect *Bacteroides* 16S rRNA genetic marker from cow faeces. Cow-specific *Bacteroides* genetic marker showed positive reactions with water and sediment of the stream and sediment of the beach at product size 580 bp (Figure 3.7). Moreover, CF128F-Bac708R primer gave positive findings with cow and horse faeces at 580 bp.

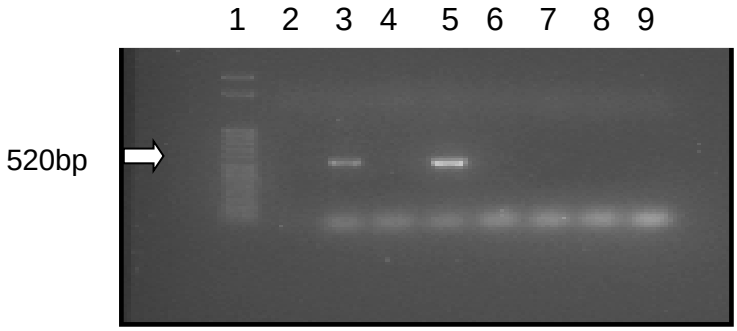


Figure 3.6 Host-specific *Bacteroides* 16S rRNA genetic marker amplified with the human-specific primer at 520 bp. Lane 1: ladder 50-1000 bp, lane 2: negative control, lane 3: positive control (Human faeces DNA template), lane 4: stream water, lane 5: stream sediment, lane 6: pond water, lane 7: pond sediment , lane 8: beach water, lane 9: beach sediment. The only positive reaction was in the lane 5.

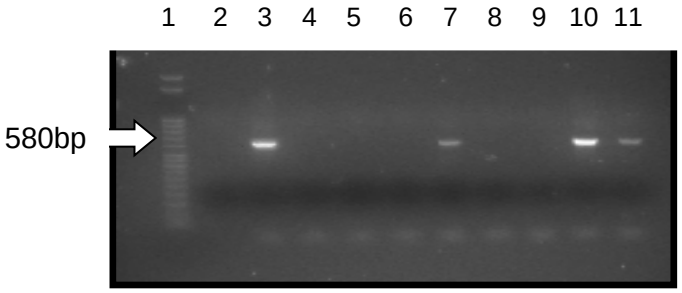


Figure 3.7 Host-specific *Bacteroides* 16S rRNA genetic marker amplified with cow primer set (CF128F-Bac708R) at 580 bp. Lane 1: ladder 50-1000 bp, lane 2: negative control, lane 3: positive control (cow faeces DNA template), lane 4: pond water, lane 5: pond sediment, lane 6: beach water, lane 7: beach sediment, lane 8: pond water, lane 9: pond sediment, lane 10: stream water, lane 11: stream sediment.

A horse-specific primer set (HoF597F-Bac708R) was used to detect *Bacteroides* 16S rRNA genetic marker from horse faeces in the water and the sediment samples. The results of the PCR with this marker gave negative results with all the water and the sediment samples. In addition, a pig-specific primer set (PF163F-Bac708R) was used to detect the *Bacteroides* 16S rRNA genetic marker from pig faeces in the water and sediment samples. The negative results were observed in the case of all water and sediment samples (data not shown).

3.4 Discussion

The results of this study have revealed that the quality of the water was better than or close to quality standards set by the EU bathing water directive 2006 for Enterococci and *E. coli*. There is no current EU bathing water directive for *Bacteroides* spp. The probability of greater bacterial pollution in inland water and sediment samples was shown in the results which were obtained from a bacteria count of water and sediment samples from the study area. The highest concentrations of the FIB appeared in the stream behind the caravan park and the pond near the hotel than on the South Sands beach. This is possibly because these sites (stream and pond) were exposed to greater levels of faecal pollution from a variety of sources such as agricultural activities and farm animal faeces. From the results obtained, it appeared that culturable *Bacteroides* spp. and Enterococci were at a higher concentration than *E. coli*. Rees *et al.* (1998) found that the concentration of FIB (Enterococci and *E. coli*) on beaches in Spain, Greece and Italy was less than on selected six UK beaches, and that these UK beaches were more variable in quality, with most of the beaches meeting with the standards set by the EU bathing water directive.

The results from this study are in disagreement with a study carried out by Joseph (2009) found the *Bacteroides* spp. at lower concentrations but the *E. coli* and Enterococci at higher concentrations compared with this study. This may be due to surface runoff from the nearby areas and during different weather conditions. Runoff from agricultural land and pastureland can be a significant source of FIB (Rees *et al.*, 1998; An *et al.*, 2002; Craig *et al.*, 2002; Crowther *et al.*, 2002).

From the results obtained from the sediments it also appeared that there was a probability of higher pollution by bacteria in the stream sediment behind the caravan park and the pond sediment near to the hotel than on South Sands beach.

The EU bathing water directive guidelines 2006 do not include or require monitoring of sediment samples and there are many complicating issues related to their sampling and analysis. Montagna (1982), Bradley *et al.* (1999) have stated that the sampling problems included differences in sediment types (for example; sediment, sand and shingle) and also the methods used to obtain the sediment sample which can be taken directly or by the use of a remote grab. Sediment presents difficulties with direct bacterial count because bacteria may adhere to the surface of sediment particles. In addition, Goyal *et al.* (1977) and Ferguson *et al.* (2005) have demonstrated that re-suspension of sediments occurs due to runoff, animal traffic and storms, which can increase the concentration of FIB in the sediment samples. The number of microorganisms in both water and sediment increases with heavy rainfall and the sediment acts as a reservoir of different types of the bacteria. Several reports refer to the fact that there are many potential sources of both FIB and pathogenic microorganisms which are found in aquatic environments (Ellis, 2000; Ferguson *et al.*, 2005).

Moreover, there were significant differences between samples taken throughout the year in this study. October and February samplings yielded higher concentrations of the FIB than June and August samplings. This difference may have occurred due to the heavy rainfall during October and

February samplings, whereas when the June and August samples were collected there was good weather.

The high abundance of FIB found during the October and February samplings in the water and sediment seen at all sites in this study may be partly due to local rainfall, the pattern of which has been shown previously to affect bacterial pollution levels (Kay *et al.*, 2007b; Martinez *et al.*, 2014). In addition, Kay *et al.* (2005; 2008a; 2008b) have demonstrated that the rainfall will facilitate the release of bacteria from streambed sediments. Furthermore, a study has carried out in the Wales and England by Kay *et al.* (2010), ≥40 % of the FIB determined as coming from agricultural sources such as livestock faeces and spread manure, farmyard and manure heaps, and adjacent water.

Bacteroides 16S rRNA genetic markers are preferred to the genetic markers from other FIB because they are restricted to warm-blooded animals including humans, and they make up a large number of faecal bacteria. In addition, they cannot survive long in natural aerobic waters (Meays *et al.*, 2004). Bernhard and Field (2000a) have stated that the use of molecular methods such as PCR may be preferable to cultured-based methods in water quality detection. From the PCR results here, all water and sediment samples tested positive for the generic *Bacteroides* genetic marker (Bac32F-Bac708R), i.e. all sites included faecal pollution with *Bacteroides* spp. from a variety of sources. The difficulty of *Bacteroides* spp. to survive under aerobic conditions suggests that all sampling sites were regularly subjected to recent pollution by faecal material (Fremaux *et al.*, 2009). Furthermore, the human-specific *Bacteroides* 16S rRNA genetic marker (HF183F-Bac708R) was used

to detect pollution of human origin in the water and the sediment samples. The result showed that this pollution was present in the stream sediment behind the caravan park. The reason for this faecal human pollution is thought to be because of the presence of caravan users. This is in agreement with a study carried out by Joseph (2009) who found that pollution of human origin in the water of the stream near to the same caravan park was detected. It has been demonstrated that the HF183F genetic marker showed 100 % sensitivity in all four Atlantic Rim countries of the European Union such as France, Ireland, Portugal and UK, (Gawler *et al.*, 2007), 100 % sensitivity in the USA (Bernhard and Field, 2000b; Layton *et al.*, 2013), 94 and 100 % in Canada (Fremaux *et al.*, 2009) and Australia, respectively (Ahmed *et al.*, 2008c; 2009b).

The results obtained with the CF128F *Bacteroides* cow-specific genetic marker showed a positive finding in the water and sediment of the stream and the sediment of the beach. This is presumably because the stream potentially carries of microbial pollution to the South Sands beach. The cow genetic marker appeared in water and sediment of the stream and indicates pollution of the stream by cow faeces. This is possibly because the genetic markers come from the large farms with cattle and other ruminant animals close to the stream. Gawler *et al.* (2007) have stated that the cow genetic marker displayed a high sensitivity (100 %) for cow *Bacteroides*, but low specificity (62.5 %) because of cross detection with other *Bacteroides* genetic marker from horse faeces. As well as being a genetic marker for cow and other ruminant faeces; it has also been shown to be present in pig faeces in France, Portugal and UK. It was also present in human and

chicken faeces in Portugal, which suggests a limitation of this genetic marker (Gawler *et al.*, 2007). The horse genetic marker HoF597F showed negative with all water and sediment samples, but it showed high sensitivity with horse faeces (100 %). On the other hand, Dick *et al.* (2005a) have demonstrated that the horse and pig *Bacteroides* genetic markers showed a high sensitivity with horse (100 %) and pig (90 %) faeces, respectively.

In conclusion, the culture-dependent results of this study showed that whilst FIB (Enterococci, *E. coli* and *Bacteroides*) were present in all water and sediment samples from South Sands, the bathing water still met the EU bathing water directive 2006 standards. However, the water quality deteriorated out of the bathing season; this is possibly because of higher rainfall. Sediment samples, especially those of the stream and pond, could potentially be a reservoir for 'spikes' which occur in the FIB under certain conditions. The *Bacteroides* 16S rRNA host-specific PCR was successful in showing that although human faecal genetic markers were not present on the beach they were present in the stream, indicating a possible hazard, whilst the positive detection of the cow genetic marker in water and sediment of the stream indicated that the presence of FIB were primarily due to cow faecal pollution at this site.

The results from this chapter have been presented as poster presentations at the following conferences in the UK: Faecal Indicators: Problem or Solution (FIPS) an international conference in Edinburgh. 6th-8th June 2011, Environmental Pollution: Chemical and Biological Approaches for Protecting Organisms" 1st Annual Conference 2011, and Ecotoxicology Research and Innovation Centre (ERIC), Plymouth University, 4th April 2011 and Centre for

Research in Translational Biomedicine (CRTB), Annual Research Day, Plymouth University. 5th April 2011. Also the results have published as a full paper in the proceeding of the FIPS conference by the Royal Society of Chemistry (RSC, ISBN: 9781849731690, Appendix 1).

Chapter Four

Development and use of *Bacteroides* 16S rRNA PCR assays for source tracking dog faecal pollution in bathing waters

4.1 Introduction

Faecal indicator bacteria (FIB) such as *Escherichia coli* (*E. coli*) and faecal Enterococci currently are used to determine faecal bathing water pollution are found in a variety of warm-blooded animals and are not unique to the intestinal flora of humans (Kreader, 1995). Thus, the need to distinguish between faecal pollution source(s) has stimulated the search for species-specific indicators. To understand microbial community structure and function in specific ecosystems, several researchers have utilized the 16S rRNA gene and associated phylogenetic analysis markers as an essential and powerful tool for bacteriological studies (Hongoh *et al.*, 2003; Zhou and Hernandez-Sanabria, 2009; Perumbakkam and Craig, 2011; Rastogi and Sani, 2011). Various strategies have now been followed to track faecal bacteria contaminating bathing water. MST is an increasingly popular method for determining host-specific contributions of faecal pollution to water systems, thus helping to identify unknown sources. The main MST method is the detection of host-specific 16S rRNA genetic markers of *Bacteroides* spp*.*, which are found exclusively in the faeces of humans and animals, usually in greater abundance than FIB (Paster *et al.*, 1994; Kildare *et al.*, 2007). MST aims to determine the relative amounts of host-specific faecal pollution in bathing water samples. The main objective being to directly quantify host-specific *Bacteroides,* depending on the relationship between the total number of *Bacteroides* sequences and host-specific genetic markers detected (Kildare *et al.*, 2007). The analysis of the 16S rRNA genes investigate complex microbial communities in environmental studies and they have some limitations, namely the heterogeneity in the copy number of this gene

among the same bacterial species (Case *et al.*, 2007), an inability to always differentiate closely related species and strains (Khamis *et al.*, 2004) and a high similarity of 16S rRNA sequences among some organisms, which differ at whole genome level (Glazer and Nikaido, 2007). As a result of these limitations, researchers have looked at alternative universally present genes that occur as a single copy and can be used in conjunction with the 16S rRNA genetic marker (Perumbakkam and Craig, 2011). The following factors should be considered in designing specific primers (Dieffenbach *et al.*, 1993; Heilig *et al.*, 2002; Chen *et al.*, 2003; Grunenwald, 2003; Promega UK, 2009; Brown, 2010; Shanks *et al.*, 2012):

i. the degree of mismatching between the nucleotides with related species,
ii. a need for less than 50 % G+C to enable a suitable annealing temperature to be used,
iii. avoidance of sequence complementarities in a primer set to minimize dimer phenomena in PCR product,
iv. the size of the primer should be between 18-22 nucleotides to increase the sensitivity and avoid the formation of secondary structures.

In urban areas there are many sources that may lead to the contamination of water supplies, such as urban runoff and negligent waste management, as well as discharge from domestic pets; these represent important potential sources of faecal pollution in aquatic systems (Crowther *et al.*, 2001; Schriewer *et al.*, 2013). In developed countries the populations of domestic dogs (*Canis lupus familiaris*) have grown over the last two decades (Murray

et al., 2010). For example, in the UK the number of dogs is about 9.4 million according to a public survey carried out by Asher *et al.* (2011). A variety of microbial pathogens can inhabit apparently healthy domestic dogs with the possibility of transmission of zoonotic risks between humans and dogs (Damborg *et al.*, 2009; Atwill *et al.*, 2012). Dogs are now banned from various UK bathing beaches during the bathing season (Figure 4.1), but there is currently no simple and/ or inexpensive method for estimating dog faecal pollution on these beaches and thus the actual effectiveness of such bans. TaqMan® labelled assays have been designed to quantify dog-specific *Bacteroides* (Kildare *et al.*, 2007). However, this study aimed to develop and use specific and sensitive conventional PCR primer sets and qPCR assays based on SYBR® green fluorescent binding dye, and to amplify a section of the 16S rRNA gene unique to *Bacteroides* spp. from dog faeces. This will enable the monitoring of bathing water and sediment pollution at surfing and bathing beaches in areas where dogs are either allowed or banned during the bathing season.

4.2 Materials and methods

The methods applied for this study are described in detail in chapter two. In this section only changes will be discussed in detail.

4.2.1 Description of the study area

Bigbury-on-Sea is situated in the South Hams district of the country of Devon, in south west England (latitude 50.28 ºN longitude - 3.89 ºW) approximately 250 meters from Burgh Island (tidal island). Bigbury-on-Sea is popular for human recreation events; it is a small beach with sand and some shingle. This study concentrates on two beaches one of which allows dogs (A) another site (B) which imposes a dog ban (Figure 4.2). Water samples were also collected from the Plymouth offshore station L4 (7 miles off the Plymouth coast 50.15 ºN, - 4.13 ºW) to use as a dog contamination free control.

4.2.2 Sample collection

Triplicate samples of water and sediment were collected from the study area on three occasions at irregular intervals, the first on 20th July and the second on 15th August and the third on 30th August 2012 (see sections 2.3.1 and 2.3.2). In addition, 58 faecal samples (10 dogs, 12 cows, eight horses, four pigs, eight sheep, four deer, two cats and six ducks) were collected from local sources in Devon, UK and four human faecal samples were obtained from adult volunteers (see section 2.3.3).

Figure 4.1 The banned dog symbol on the left site of the beach on Bigbury-on-Sea. In the UK, dog bans on specific beaches in the summer months start from the 1st of May and last until to the 30th of September (Rees *et al.*, 1998).

4.2.3 Sample filtration and culture-dependent analysis

Water samples were filtered using a vacuum pump onto 0.45 µm pore size membrane filters (Whatman, UK) and placed on BBE agar as described in section 2.4.1.3 (Livingston *et al.*, 1978). Two grams of sediment was taken and added to sterile sea water to 20 ml to make an initial tenfold dilution as described in section 2.4.2. Faecal *Bacteroides* spp. colonies were counted and expressed as colony forming units (CFU) 100 ml^{-1} and CFU g^{-1} for water

and wet sediment, respectively (Craig *et al.*, 2002; Ferguson *et al.*, 2005). Each set of samples was prepared in triplicate.

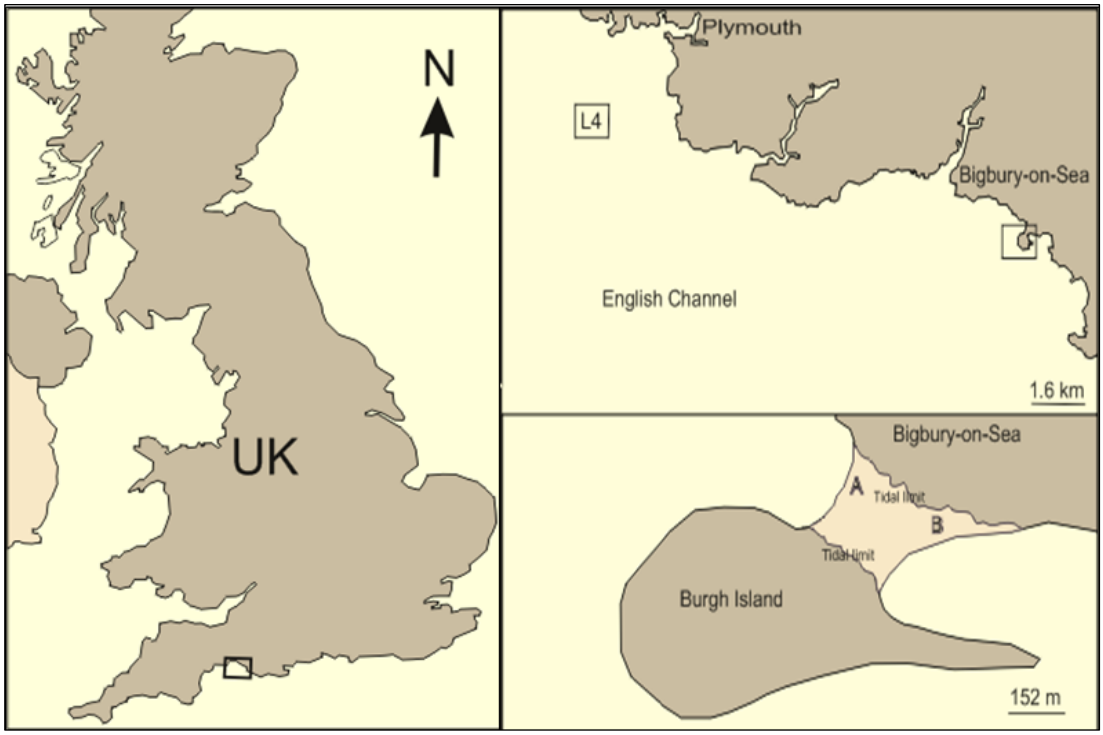


Figure 4.2 The beach on Bigbury-on-Sea, Devon, UK. A: area where dogs are allowed access, B: area where dogs are banned in summer months, L4: L4 Plymouth off-shore station (dog contamination-free control).

4.2.4 DNA extraction

DNA was extracted from the water and faecal samples by using the QIAamp DNA mini kit (Qiagen, UK) following the manufacturer's protocol as described in section 2.5.1.1 and section 2.5.1.3. The SoilMaster^{TM} DNA extraction kit (Cambio, UK) was used for DNA extraction from the sediment samples following the manufacturer's protocol (see section 2.5.1.2). DNA was stored at - 80 ºC for future use.

4.2.5 Quantification of extracted DNA

The quantity and quality of extracted DNA were measured in all samples using a NanoDrop® (ND-1000) UV spectrophotometer as described in section 2.5.2.

4.2.6 Conventional PCR for detecting *Bacteroides* genetic markers

Conventional PCR was used to detect *Bacteroides* 16S rRNA gene in water, sediment and faecal samples using generic *Bacteroides* primer pairs (Bernhard and Field, 2000a). PCR reactions were conducted in a total volume of 25 µl as described in section 2.5.3. A human-specific *Bacteroides* primer (HF183F-Bac708R) was used (Bernhard and Field, 2000b). For horse-, pig- and cow-specific faecal *Bacteroides* genetic markers; HoF795F, PF163F and CF128F specific primers were used as explained in section 2.5.3 (Table 2.1). The PCR products were purified using SureClean purification kit (Bioline Biotech, UK) as described in the manufacturer's protocol (see section 2.5.5).

4.2.7 DNA sequencing and analysis

The purified PCR amplicons of dog faecal *Bacteroides* 16S rRNA genetic markers generated using the primer set Bac32F-Bac708R were commercially sequenced using the value read service from genome analysis and technology core (GATC Biotech, London, http://www.gatc-biotech.com/en/index.html). Identification of *Bacteroides* spp. was performed by using the basic local alignment search tool (BLAST) software from the national centre for biotechnology information (NCBI, http://www.ncbi.nlm.nih.gov/) as described in section 2.5.6. The NCBI-BLAST database was used to identify the DNA sequence, and the evolutionary relationship

(phylogenetic tree) between the 16S rRNA gene from dog *Bacteroides* spp. and other (human, cow, horse, pig, cat and duck) was created using molecular evolutionary genetics analysis (MEGA) version 5.2.2 (Hall, 2011; Tamura *et al.*, 2011). The evolutionary history was deduced by using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). All positions containing gaps and missing data were deleted. Clustalw2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was also used to produce a multiple sequence alignment pattern between the amplified sections of the *Bacteroides* 16S rRNA gene from dog faeces and the other animal faeces and to assign sequences to operational taxonomic units (OTUs). OTUs were defined by assigning 16S rRNA gene sequences with a> 98 % similarity to each other the same species (Morales and Holben, 2009; Wooley *et al.*, 2010). The mismatching sequence regions of the 16S rRNA gene were then utilized to design specific primers for dog faecal *Bacteroides* species (Figure 4.3).

4.2.8 Primer design and PCR amplification

The mismatching sequence regions of the 16S rRNA gene were utilized to design specific primers for the dog *Bacteroides* spp. Three sets of dog-specific primer were designed (DF53F-DF606R, DF113F-DF472R, and DF418F-DF609R, Table 4.1) with aid of the Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and purchased from Eurofins, MWG, Germany (http://www.eurofinsgenomics.eu/). These primer sets were then used to amplify the 16S rRNA genetic marker of the dog *Bacteroides*.

```
human    AGCTTGCTAAGGCTGATGGCGACCGGCGCACGGGTGAGTAACACGTATCCAACCTGCCGT 120
pig      AGCTTGCTAAATTTGATGGCGACCGGCGCACGGGTGAGTAACGCGTATCCAACCTTCC-C 119
dog      G--TTGCGTGGTCTGATGGCGACCGGCGCACGGGTGAGTAACGCGTATCCAACCTCCCGC 67
cow      AGC--AATGCATGGGC-GGCGACCGGCGCACGGGTGCGTAACGCGTATCCAACCTTCCCG 116
sheep    AGCTTGCTAAATCCGCTGGCGACCGGCGCACGGGTGCGTAACGCGTATCCAACTTTCCCG 120
duck     ----------------------CCGGAG----------TAACACGTATCCAACCTACCAT 55
                              **** *         **** ********** * **

human    CTACTC-TTGGCCAGCCTTCTGAAAGGAAGATTAATCCAGGATGGGATCATGAGTTCACA 179
pig      CTGTCCACGGGATAGCCCGTCGAAAGGCGGATTAATACCGTATGAGGTCACAAGCGGGCA 179
dog      ATACTC-GGGGATAGCCTTCTGAAAGGAAGATTAATACCCGATGGTATCTCAAGAGCACA 126
cow      TTACTC-ATGGATAGCCTTCCGAAAGGGAGATTAATACATGATGGTGTTGAAATTCCGCA 175
sheep    TTACTC-AGGGATAGCCTTCCGAAAGGGAGATTAATACCTGATGGTGTTTGAAGTTCGCA 179
duck     TCTTTC-GGGGATAGCCTTTCGAAAGAAAGATTAATACCCGATAGTCTAGGAATAAAGCA 114
              *   **  ****    *****   ******* *   **    *    *     **

human    TGTCCGCATGATTAAAGGTATTTTCCGGTAGACGATGGGGATGCGTTCCATTAGATAGTA 239
pig      TCTAATTGTGACGAAAGGT-TTTGCGGACAGA-GATGGGGATGCGTCCGATTAGGTAGTC 237
dog      TGCAATTAAGATTAAAGAA-TTT--CGGTATGCGATGGGGATGCGTTCCATTAGGTAGTA 183
cow      TGTTATTTCAACTAAAGAT--TTATCGGTAACGGATGGGGATGCGTTCCATTAGCTTGTT 233
sheep    TGTTCTTCAAACTAAAGAT--TTATCGGTAACGGATAGGGATGCGTGACATTAGATAGTA 237
duck     TTTTATTTTTAGTAAAGAA--TT-TCGGTGTTTGATGGGGATGCGTTCCATTAGATTGTT 171
          *         *  ****    **   *      *** *********   ***** * **

human    GGCGGGGTAACGGCCCACCTAGTCAACGATGGATAGGGGTTCTGAGAGGAAGGTCCCCCA 299
pig      GGCGGGGTAACGGCCCACCGAGCCGACGATCGGTAGGGGTTCTGAGAGGAAGGTCCCCCA 297
dog      GGCGGGGTAACGGCCCACCTAGCCATCGATGGATAGGGGTTCTGAGAGGAAGGTCCCCCA 243
cow      GGCGGGGTAACGGCCCACCAAGGCTTCGATGGATAGGGGTTCTGAGAGGAAGGTCCCCCA 293
sheep    GGCGGGGTAACGGCCCACCTAGTCTACGATGTCTAGGGGTTCTGAGAGGAAGGTCCCCCA 297
duck     GGTGAGGTAACGGCTCACCAAGTCTTCGATGGATAGGGGTTCTGGGAGGAAGGTCCCCCA 231
         ** * ********* **** ** *  ****   ********** ***************

human    CATTGGAACTGAGACACGGTCCAAATTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCA 359
pig      CATTGGAACTGAGACACGGTCCAAACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCA 357
dog      CATTGGAACTGAGACACGGTCCAAACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCA 303
cow      CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCA 353
sheep    CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCA 357
duck     CATTGGAACTGAGACACGGTCCAAACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCA 291
         ** ********************* * ********************************

human    ATGGGCGATGGCCTGAACCAGCCAAGTAGCGTGAAGGATGACTGCCCTATGGGTTGTAAA 419
pig      ATGGGCGAGAGCCTGAACCAGCCAAGTAGCGTGCAGGATGACGGCCCTATGGGTTGTAAA 417
dog      ATGGGCGCGAGCCTGAACCAGCCAAGTAGCGTGAAGGATGACTGCCCTATGGGTTGTAAA 363
cow      ATGGCCGGAAGGCTGAACCAGCCAAGTAGCGTGAAGGATGAAGGTTCTATGGATTGTAAA 413
sheep    ATGGTCGGAAGACTGAACCAGCCAAGTAGCGTGAAGGATGAAGGTTCTATGGATTGTAAA 417
duck     ATGGACGAGAGTCTGAACCAGCCAAGTAGCGTGAAGGACGAAGGCCCTACGGGTCGTAAA 351
         **** **   * ********************* **** **  *  *** ** * *****

human    CTTCTTTTATAAAGGAATAAAGTCGGGTATGCATACCCGTTTGCATGTACTT-TATGAAT 478
pig      CTGCTTTTATGCGGGGATAAAGTGAGGGATGCGTCCCTTTTTGCAGGTACCG-CATGAAT 476
dog      CTTCTTTTGTCCGGGAATAAAACCGCCTACGTGTAGGCGCTTGTATGTACCGGTACGAAT 423
cow      CTTCTTTTATGAGGGAATAAAACCTCCCACGTGTGGGAGCTTGTATGTACCT-TATGAAT 472
sheep    CTTCTTTTATATAGGAATAAAACCTCCCACGTGTGGGAGCTTGTATGTACTA-TATGAAT 476
duck     CTTCTTTTATAGGGGAATAAAGTGGAGAACGTGTTCTCCTTTGTATGTACCC-TACGAAT 410
         ** ***** *   ** *****       * *  *      *** * ****    * ****

human    AAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCGAGCGTTATCCG 538
pig      AAGGACCGGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGAAGGTCCGGGCGTTATCCG 536
dog      AAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCGAGCGTTATCCG 483
cow      AAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCGAGCGTTATCCG 532
sheep    AAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCGAGCGTTATCCG 536
duck     AAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCG 470
         *** * ******* ***************************** * * *  **********
```

Figure 4.3 Multiple sequence alignments of *Bacteroides* spp. from different sources using mismatching regions in order to design dog-specific primer sets. Data was generated using the GenBank database (NCBI, www.ncbi.nlm.nih.gov).

The PCR amplification was carried out using the programme mentioned above and the annealing temperature was optimized using different temperatures (55, 57, 60, 62.5, 63.5 and 65 ºC) for each primer set. Each primer set was tested in amplification reactions for 16S rRNA genetic markers in total DNA isolated from human, cow, pig, horse, sheep, deer, cat, and duck faecal samples. The amplified products were electrophoresed in a 1.5 % agarose gel, and the images were captured (section 2.5.4). PCR products were cleaned as described previously (section 2.5.5).

4.2.9 Nucleotide sequence accession numbers

The sequences of dog-specific *Bacteroides* 16S rRNA gene determined in this study have been deposited in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers JX431865, JX431866 and JX431867 (Appendix 1).

4.2.10 Quantification assays

PCR products were purified and ligated into the pGEM®-T easy plasmid vector 3015 bp (Promega, UK). The ligated products were transformed into high efficiency *E. coli* JM109 competent cells (Promega, UK) and plated onto Luria-Bertani (LB) agar plates containing 40 µg ml^{-1} X-gal, 0.1 mM IPTG (Appendix 2) and 100 µg ml^{-1} ampicillin as recommended by the manufacturer. Plasmid DNA was extracted from the culture of recombinant *E. coli* using a GenElute™ plasmid miniprep kit (Sigma Aldrich, UK) as described in section 2.5.7.

qPCR was then performed in total reaction volumes of 25 µl containing 12.5 µl SYBR® Green 1 JumpStart™ Taq ReadyMix™ (Sigma Aldrich, UK), as described in section 2.5.7.8. The mixture was applied into MicroAmp optical

96-well reaction plate, which was covered tightly with adhesive film; the plate was then run in the StepOnePlus Real-Time PCR system (Applied Biosystems, USA). The reactions were carried out as described in section 2.5.7.8. Melting curves for PCR products were established between 60-90 °C with a resolution of 0.3 ºC after cycling to determine amplification specificity. Triplicate positive control samples (*Bacteroides*-plasmid) and triplicate 'no-template' negative control samples containing sterile seawater samples from Plymouth L4 (dog-free) station were used for quality control purposes. Efficiency of amplification (Eff.%) was determined (section 2.5.7.8) by the slope of the standard curve and calculated using the following equation: Eff.%= ($10^{-1/slope}$) -1 (Okabe *et al.*, 2007; Ahmed *et al.*, 2010).

Table 4.1 The new dog-specific *Bacteroides* primer sets designed and used in this study

Primer	Primer sequence (5'----3')	Length (bp)	Annealing temp. ºC	Amplicon size (bp)
DF53F	TATCCAACCTCCCGCATAC	19	62.5	570
DF606R	CATTTCACCGCTACACCAC	19		
DF113F	ATCTCAAGAGCACATGCAA	19	62.5	380
DF472R	AATAAATCCGGATAACGCTC	20		
DF418F	ACGAATAAGCATCGGCTAAC	20	63.5	210
DF609R	AAGCATTTCACCGCTACA	18		

bp: base pair

4.2.11 Limit of detection of qPCR

The limit of detection (LOD) is defined as the lowest amount of measurable target in a single reaction (Nutz *et al.*, 2011). Serial ten-fold dilutions of the sample (10^{-1} to 10^{-8}) were prepared and DNA was extracted from each dilution and analysed using conventional PCR and qPCR. The LOD was determined as the quantity of DNA matching to the threshold cycle (Ct). *Bacteroides* spp. cell number was enumerated using the membrane filtration method for each dilution. PCR products were cloned into the pGEM®-T easy vector plasmids and then used to calibrate unknown samples. To create absolute standard curves, a known copy number plasmid pGEM®-T easy vector was used as described in section 2.5.7.9. *B. fragilis* has six 16S rRNA operons per cell as stated by the ribosomal DNA operon copy number database (Klappenbach *et al.*, 2001).

4.2.12 Statistical analysis

Results were statistically analysed using the SPSS statistical programme version 21 as described in section 2.6. One Way Analysis of Variance (ANOVA) was carried out to determine the significance of differences between the Ct values obtained (n= 3 for each run) from DNA isolated from water and sediment samples.

4.2.13 Sensitivity and specificity

To calculate the effectiveness of the MST method on faecal samples, sensitivity (%) and specificity (%) were calculated as:

$$\text{Sensitivity} = \frac{\text{True positive}}{(\text{True positive}+\text{False negative})} \qquad (1)$$

$$\text{Specificity} = \frac{\text{True negative}}{(\text{True negative}+\text{False positive})} \qquad (2)$$

Where 'true positive' is the total number of positive reactions for the dog-specific genetic markers in dog faecal samples, 'false negative' is the total number of negative reactions for the dog-specific genetic markers in dog faecal samples, 'false positive' is the total number of positive reactions for the dog-specific genetic markers in 'non-dog' faecal samples, 'true negative' is the total number of negative reactions for the dog-specific genetic markers in 'non-dog' faecal samples (Mieszkin *et al.*, 2009; Schriewer *et al.*, 2013). Values of 1 correspond to 100 %. A p value equal to or less than 0.05 was considered to indicate a significant difference.

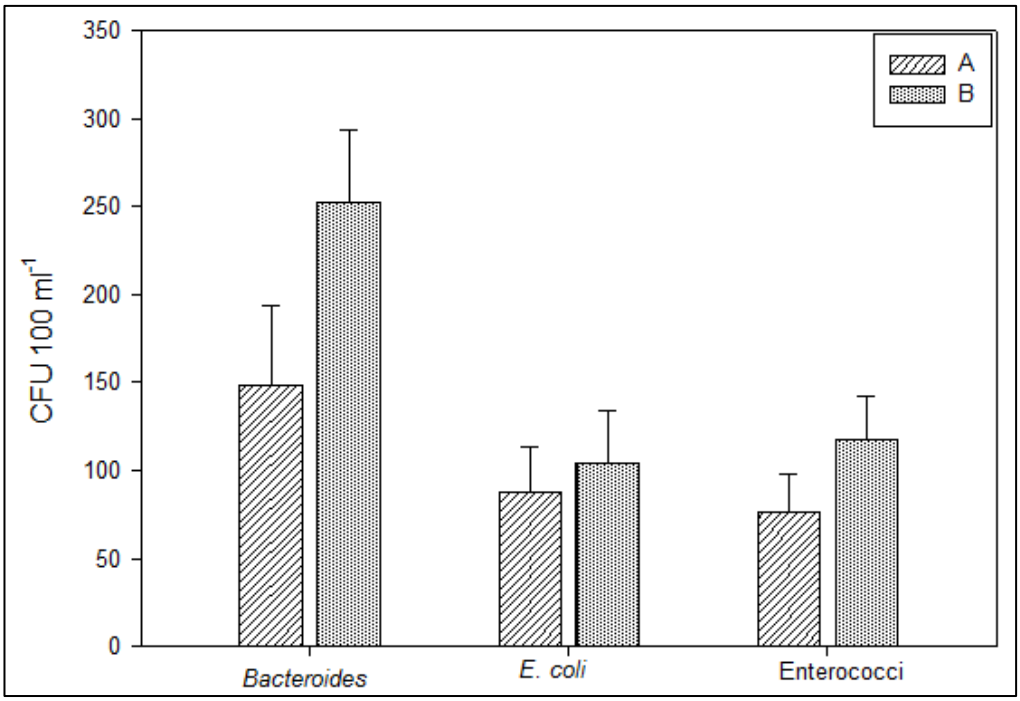
4.3 Results

The pH and salinity across the three sampling times was 7.8 ± 0.3, and 34 practical salinity units (psu), respectively. The water samples in the second sampling (15th August) showed slight visible turbidity.

4.3.1 FIB counts from water and sediment samples

Figures 4.4, 4.5 and 4.6 show the results of culture-dependent analysis of the water and sediment samples for *E. coli*, Enterococci, and *Bacteroides* spp. over the course of three field visits at the two sites. No significant differences were observed between the numbers of FIB which were isolated from water (Figure 4.4a) and sediment (Figure 4.4b) samples on 20th July 2012 ($p > 0.05$). The results of the second sampling (15th August) showed a significant difference between water samples ($p = 0.03$, Figure 4.5a), but no significant difference was observed between sediment samples ($p > 0.05$, Figure 4.5 b). In addition, no significant differences were observed between bacteria numbers associated with water (Figure 4.6a) and sediment (Figure 4.6b) samples on 30th August 2012 ($p > 0.05$). Overall, these results indicated a good or sufficient quality of beach waters, based on the EU bathing water directive 2006 (< 200 and < 500 CFU 100 ml^{-1} for Enterococci and *E. coli,* respectively). *E. coli*, Enterococci and *Bacteroides* spp. numbers were relatively high in the beach water and sediment at the second sampling. The only breach of EU bathing water directive 2006 standards was observed in the case of Enterococci in beach water site B on the second sampling event (Figure 4.5).

a



b

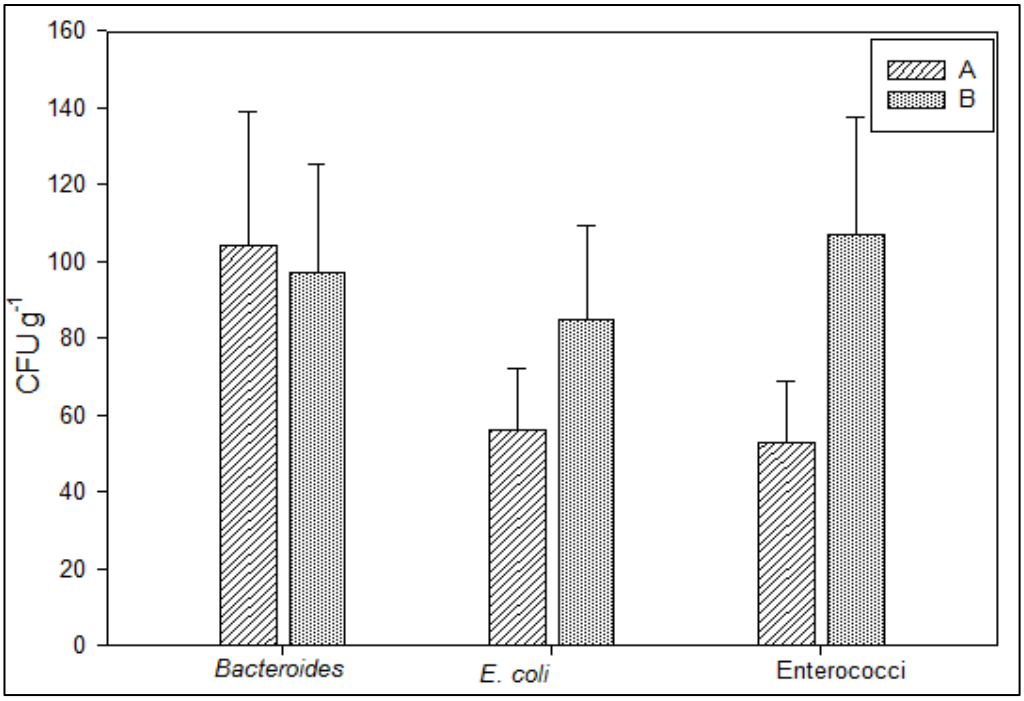
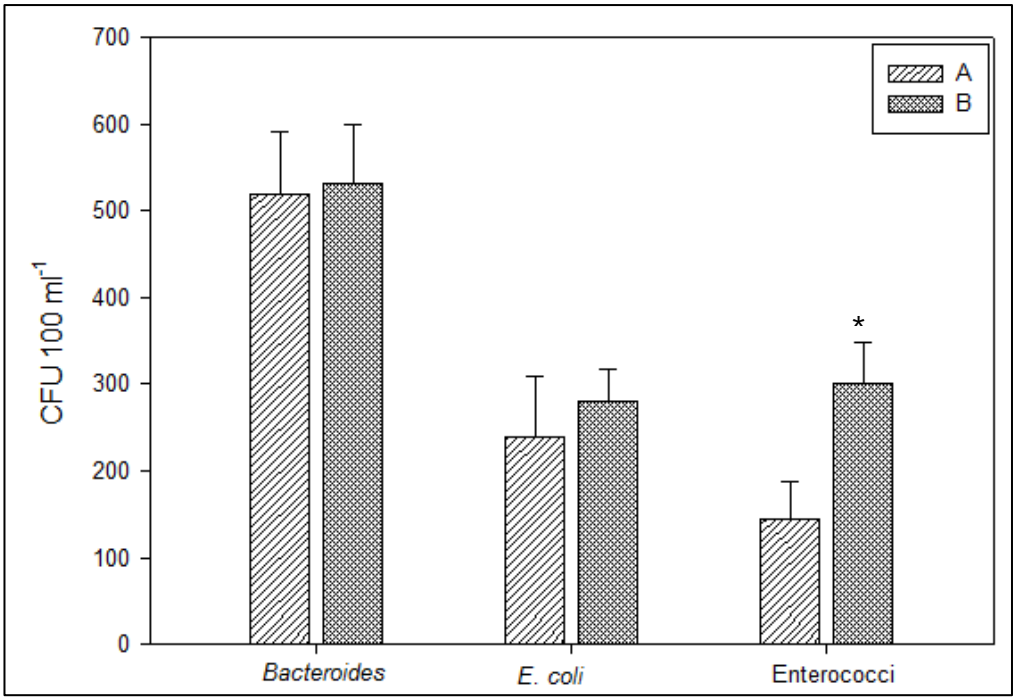


Figure 4.4 The distribution and frequency of culturable *Bacteroides* spp., *E. coli* and Enterococci in Bigbury-on-Sea water (a) and sediment (b) samples from two sites on 20th July 2012. Data is presented as mean ± SE of triplicate samplings. A: an area where dogs are permitted access, B: an area where dogs are banned during the summer months.

a



b

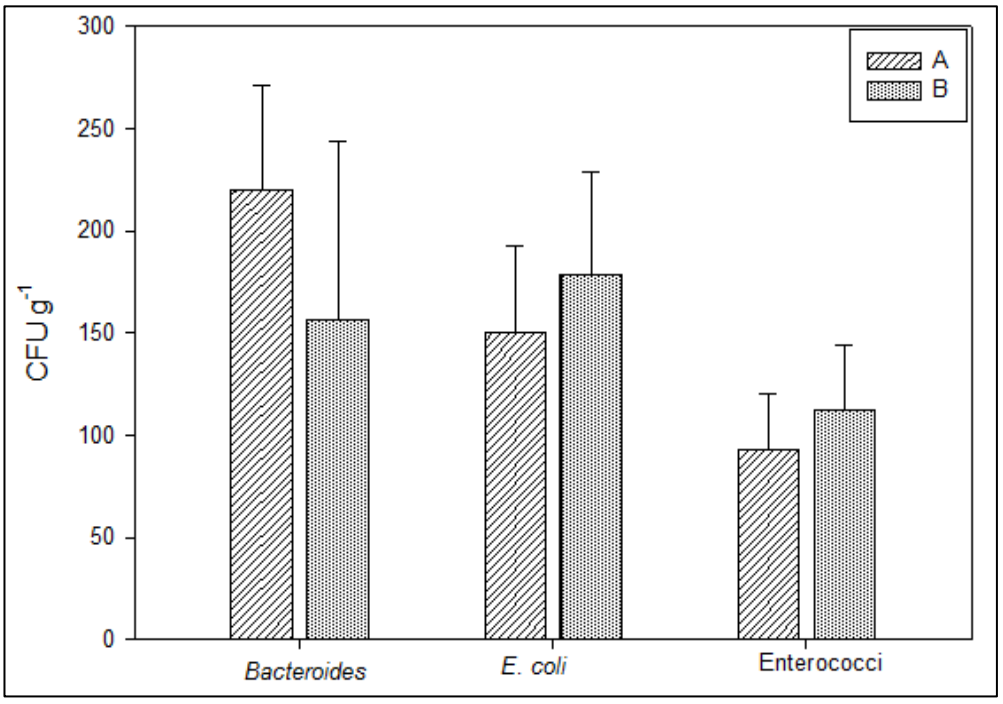
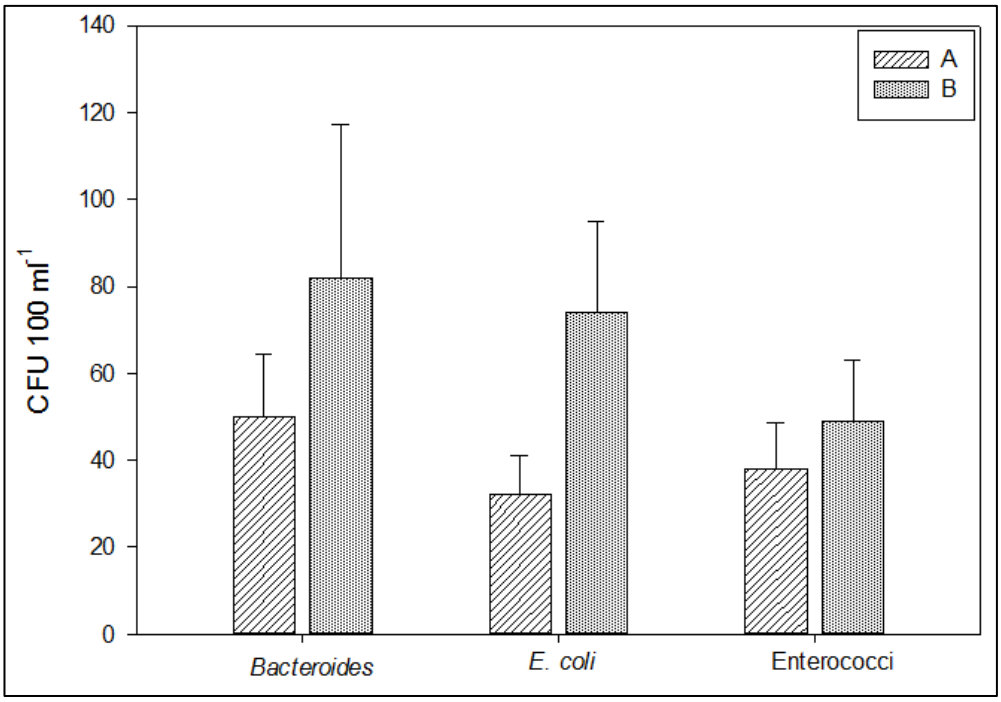


Figure 4.5 *Bacteroides* spp., *E. coli* and Enterococci CFU distribution in Bigbury-on-Sea water (a) and sediment (b) samples from two sites on 15th August 2012. Data is presented as mean ± SE of triplicate samplings. A: an area where dogs are permitted access, B: an area where dogs are banned during the summer months, *the only breach of the EU bathing water directive 2006.

a



b

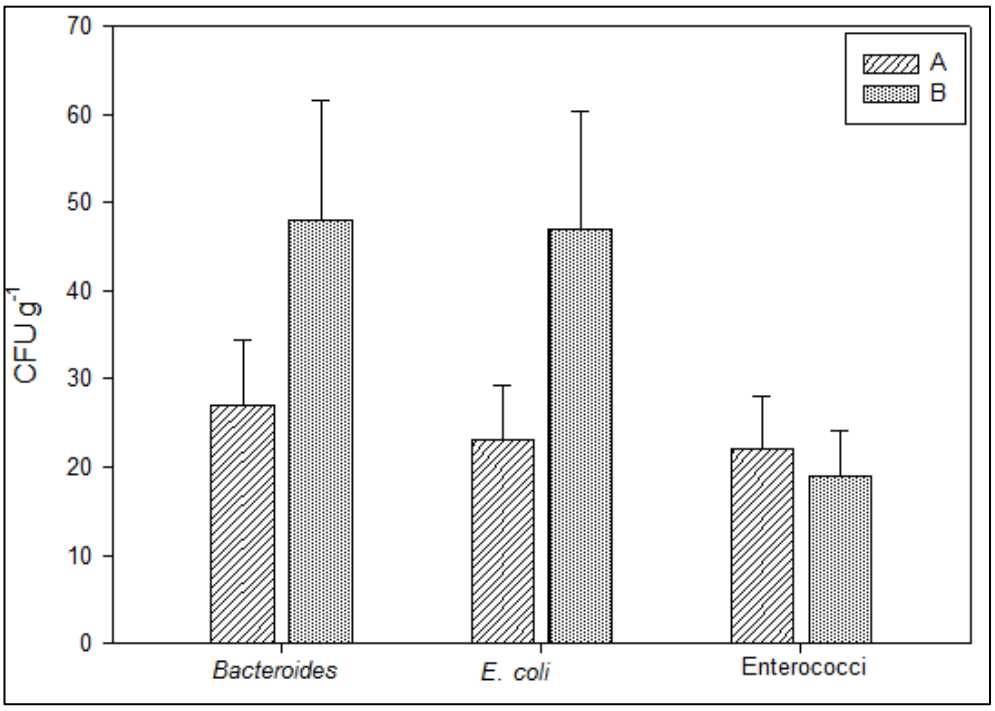


Figure 4.6 The distribution of *Bacteroides* spp., *E. coli* and Enterococci in the Bigbury-on-Sea water (a) and sediment (b) samples from two sites on 30th August 2012. Data is presented as mean ± SE of triplicate samplings. A: an area where dogs are permitted access, B: an area where dogs are banned during the summer months.

4.3.2 Detection of *Bacteroides* genetic markers

The 16S rRNA genetic marker of *Bacteroides* was successfully amplified from samples of water, sediment and faeces using generic *Bacteroides* primer set (Bac32F-Bac708R), producing a 670 bp amplicon (Figure 4.7). No successful amplification of human-, horse- and pig-specific faecal *Bacteroides* genetic markers was detected any of the water or sediment samples. In the case of cow-specific genetic markers, a positive reaction was only observed in sea water from site B (Table 4.2).

Table 4.2 The identification of host-specific genetic markers in the water and sediment samples from Bigbury-on-Sea beach on three occasions during July and August 2012. Site A: an area where dogs are permitted access, site B: an area where dogs are banned.

Host of *Bacteroides*	Primer sets	Site A		Site B	
		Water/	Sed.	Water/	Sed.
Generic	Bac32F-Bac708R	+	+	+	+
Human	HF183F-Bac708R	-	-	-	-
Cow	CF128F-Bac708R	-	-	+	-
Horse	HoF795F-Bac708R	-	-	-	-
Pig	PF163F-Bac708R	-	-	-	-

Sed.: Sediment, positive and negative results represent combined data from three sampling events.

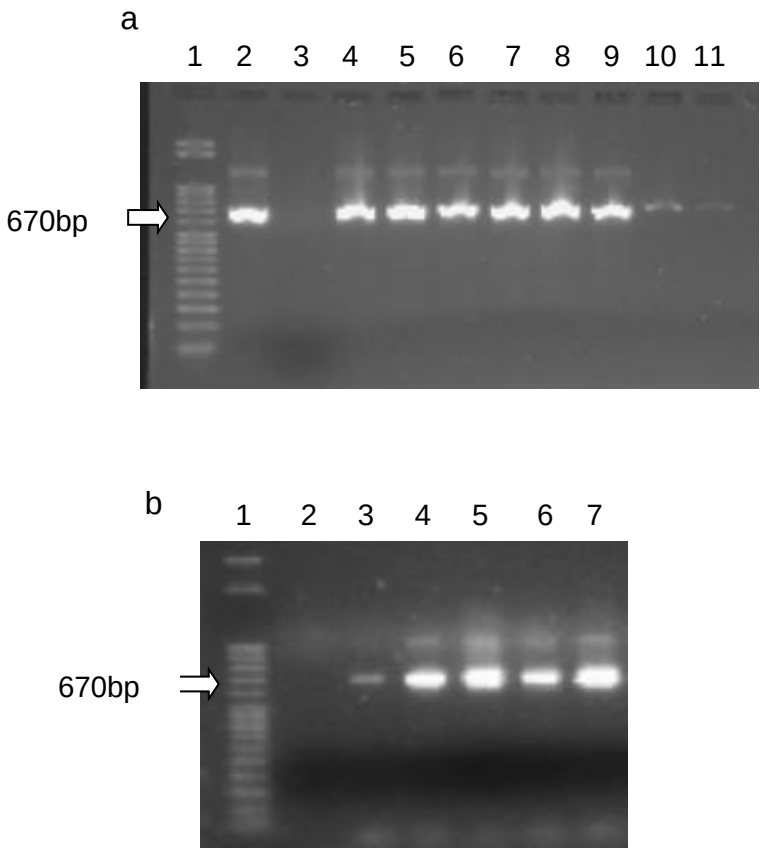


Figure 4.7 Confirmation of the presence of *Bacteroides* spp. genetic markers in extracted DNA from water, sediment and faecal samples collected from various animals, using the generic *Bacteroides* primer set Bac32F-Bac708R. (a) Confirmation of the presence of *Bacteroides* spp. genetic markers in faecal samples of human (lane 4), cow (5), horse (6), pig (7), sheep (8), deer (9), cat (10) and duck (11). (b) Confirmation of the presence of *Bacteroides* spp. genetic markers in in water and sediment samples. Lanes 4 and 5: water and sediment beach A, lanes 6 and 7: water and sediment beach B. In each image, lane 1: ladder 50-1000 bp, lane 2 a and 3 b: positive controls (DNA template) at product size 670 bp, lane 3 a and 2 b: negative controls.

4.3.3 Specific primer design for 16S rRNA gene of dog *Bacteroides*

The 16S rRNA gene sequences from faecal *Bacteroides* amplified from dog faeces or pure cultures were used to design three sets of specific primers differentiating 16S rRNA gene amplicons specific to dog *Bacteroides* spp. from faecal *Bacteroides* spp. originating from other animals. The annealing temperature of each primer set was optimized and it was found that 62.5 ºC and 63.5 ºC (Table 4.1) were the optimal annealing temperatures, producing a single band at product sizes 570, 380 and 210 bp for DF53F-DF606R, DF113F-DF472R and DF418F-DF609R, respectively (Figure 4.8).

The PCR amplifications of the first set of dog-specific *Bacteroides* primer (DF53F-DF606R) successfully showed a single band with extracted DNA from dog faeces, whereas no products were detected using extracted DNA from human and other animal faeces (Figure 4.8a). The second set (DF113F-DF472R) showed no products with all animal faecal DNA samples, a positive result was seen with dog faecal DNA samples (Figure 4.8b). However, PCR amplifications using the third set (DF418F-DF609R) showed a strong positive band with extracted DNA from dog faeces as well as weak bands with a human faecal DNA sample, indicating a slightly lower specificity for this primer set; negative results were found with all the other animal faecal DNA samples tested (Figure 4.8 c).

4.3.4 Sensitivity and specificity

The specificity and sensitivity of the first and second dog-specific primer sets (DF53F-DF606R and DF113F-DF472R) was 100 % due to the true positive results obtained in 10 out of 10 dog faecal samples tested, coupled with the fact that products were not detected in any of the other 48 animal faecal

samples. The values were then converted to percentages by multiplying by a factor of 100:

Sensitivity of the three dog-specific primer sets $= \frac{10}{10}$ = 100 %

Specificity of the first and second dog-specific primer sets $= \frac{48}{48+0}$= 100 %

Specificity of the third dog-specific primer sets $= \frac{44}{44+4}$= 92 %

Whilst the third primer set (DF418F-DF609R) gave a true positive reaction in 10 out of 10 (100 %) dog faecal samples, it produced a true negative reaction in 44 out of 48 other faecal samples (92 %).

4.3.5 Phylogenetic analysis

When designing dog-specific faecal *Bacteroides* primer sets, faecal *Bacteroides* spp. sequences were tested using:

i. the partial *Bacteroides* 16S rRNA gene sequences obtained from dog faeces (accession numbers JX431865, JX431866 and JX431867) and
ii. partial *Bacteroides* 16S rRNA gene sequences of other faecal animal provided by the GenBank database (*in silico*).

Seventy two operational taxonomic units (OTUs) were closely related (similarity 98 % or greater) to the partial dog uncultured *Bacteroides* sequences (GenBank accession number JX431865) from dog faecal samples (data not shown). This sequencing was used to produce the phylogenetic analysis. In addition, the sequence investigation of faecal *Bacteroides* showed a similarity of identity up to 89 % to the 16S rRNA gene of known dog-specific *Bacteroides* spp. (Table 4.3).

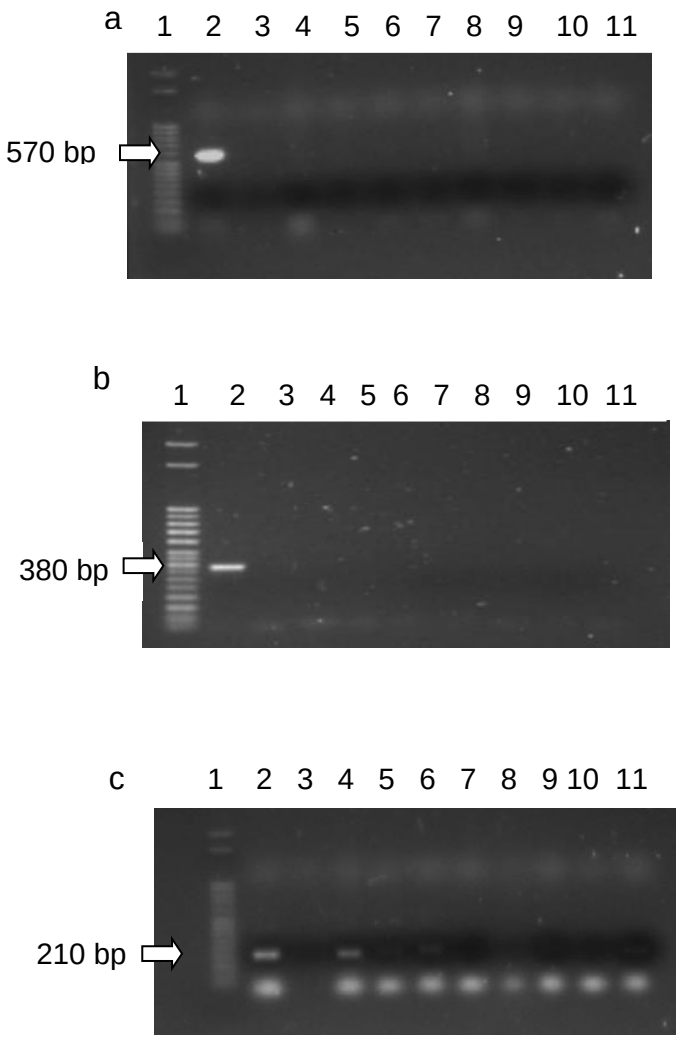


Figure 4.8 Use of dog-specific faecal *Bacteroides* primer sets DF53F-DF606R (a), DF113F-DF472R (b) and DF418F-DF609R (c) to amplify portions of the 16S rRNA gene of *Bacteroides* DNA extracted from human and animal faeces. Lane 1: ladder 50-1000 bp, lane 2: DNA positive controls (dog faeces DNA template), lane 3: no-template negative controls, lanes 4-11: human, cow, horse, pig, sheep, deer, cat and duck, respectively.

Table 4.3 Comparison of sequence similarities between dog-specific *Bacteroides* (from PCR amplicons using dog-specific primer sets described in this study) using the accession number JX431867 and other host-specific *Bacteroides* sequence information obtained from the GenBank database (NCBI, www.ncbi.nlm.nih.gov).

Bacteroides host	Length (bp)	Similarity (%)
Dog	639	100
Human	706	89
Cow	700	87
Pig	704	84
Duck	638	82
Sheep	707	87
Cat	706	82

The sequencing of PCR products produced from DNA samples isolated from dogs and other animals showed significant similarity to the 16S rRNA gene of *Bacteroides* spp. Phylogenetic analysis of *Bacteroides* based upon the neighbour-joining of partial 16S rRNA gene sequences showed that these sequences were derived from *Bacteroides* spp. of different animals. Phylogenetic analysis involved 29 sequences. The similarity percentage between individual clusters is indicated in Figure 4.9. For example, there was an 86 % similarity between the four *Bacteroides* dog sequences (pre-fixed by JX and including FJ221360) and the two human-sourced *Bacteroides* sequences (pre-fixed by HQ/ EF). In addition, the sequences investigation of faecal *Bacteroides* spp. showed a similarity of identity up to 89 % to the 16S rRNA gene of known dog-specific *Bacteroides* (Table 4.3). The nucleotide

sequence of 16S rRNA gene amplicons of dog *Bacteroides* amplified by the generic primers was aligned with 16S rRNA *Bacteroides* from other animals in order to detect region(s) with strong mismatch sequences, which can be used to design specific primers for dog *Bacteroides* spp. The mismatch effects of primer sequences of dog-specific *Bacteroides* spp. were investigated with other *Bacteroides* sequences from different sources. DF53F-DF606R, DF113F-DF472R and DF418F-DF609R showed 3 to 11 oligonucleotide mismatches with all tested sequences. To determine the effect of the primer mismatch, 16S rRNA genetic markers were amplified by PCR using dog-specific primer sets, and the products were sequenced again by GATC biotech laboratories (GATC, UK).

4.3.6 Application of the new primer sets

The new dog-specific *Bacteroides* 16S rRNA primer sets were used to detect dog genetic markers in the beach water and sediment of two sites of the Bigbury-on-Sea. The PCR amplification showed positive results for dog *Bacteroides* genetic markers in the beach water of site A where dog are permitted and site B where dog banned. Negative PCR results were observed using dog-specific *Bacteroides* primer sets in the beach sediments (site A and B) on three occasions of the sampling collections (see section 4.2.2 and Figure 4.10).

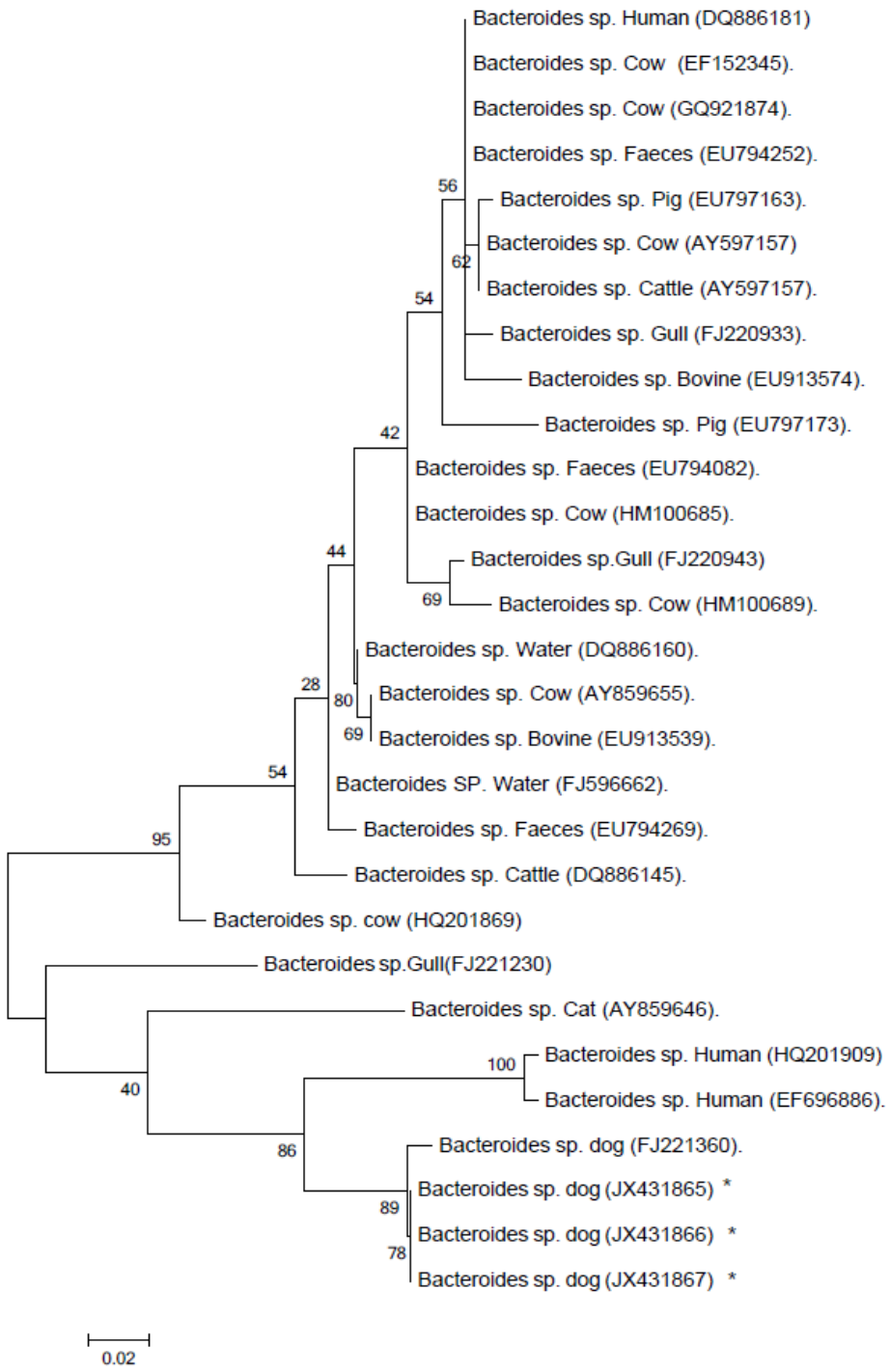


Figure 4.9 The evolutionary relationships of host-specific *Bacteroides* associated with related animals, water and unknown faecal sources; phylogeny of the *Bacteroides* 16S rRNA genes was inferred by distance based analysis using Tamura-Nei distance estimates of aligned nucleotide sequences derived from the PCR sequence data. *Accession numbers of *Bacteroides* genetic markers from dog faeces created in this study.

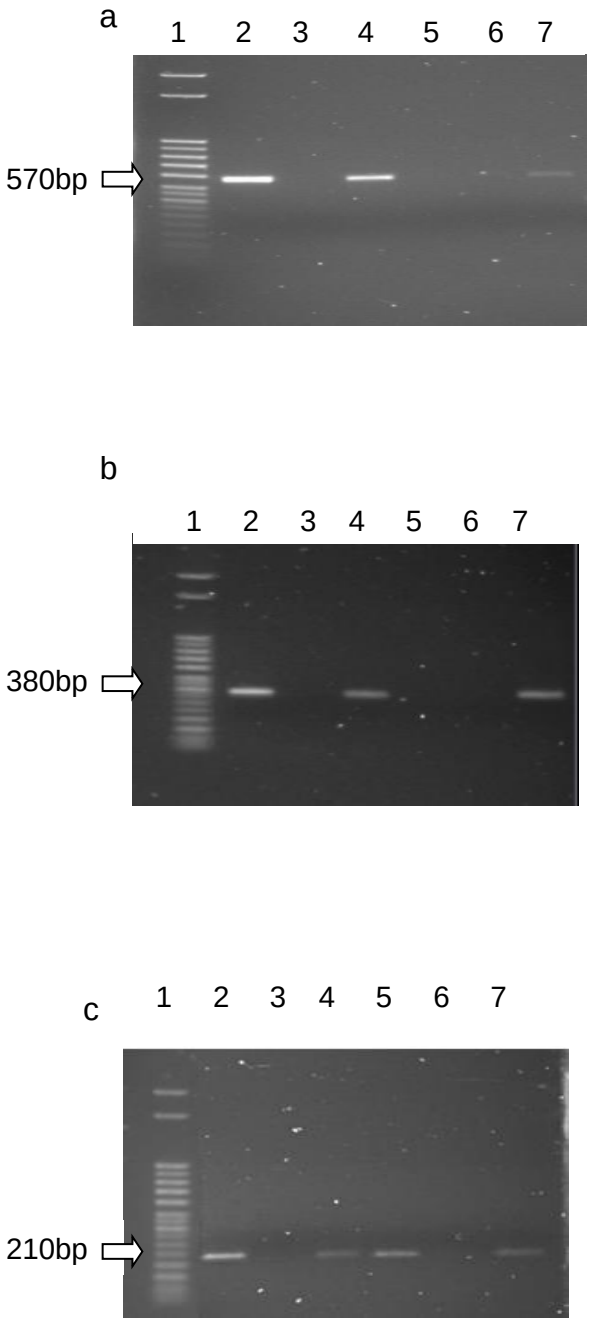


Figure 4.10 Conventional PCR amplified with dog-specific *Bacteroides* primer sets DF53F-DF606R (a), DF113F-DF472R (b) and DF418F-DF609R (c). Lane 1: ladder 50-1000 bp, lane 2: positive controls (dog faeces DNA template), lane 3: no-template negative controls, lane 4: water of beach A, lane 5: sediment of beach A, lane 6: sediment of beach B, and lane 7: water of beach B.

4.3.7 Standard curve and qPCR amplification of dog-specific primers

The standard curves (Figure 4.11) showed a linear slope and the quantity of the genetic marker was from 0 to 6 $\log_{10}$ copy number of dog-specific *Bacteroides* genetic markers per microliter of plasmid DNA extracted. The amplification efficiencies (Eff. %) of each qPCR run were ranged between 91 and 115 % for both genetic markers (DF113F-DF472R and DF418F-DF609R). The correlation coefficient (R^2 value) was between 0.960 and 0.998 (Table 4.4). In addition, the amplification plots of the qPCR products were confirmed by verifying the melting curve points which ranged between 83 and 87 ºC. Two dog-specific *Bacteroides* genetic marker assays (DF113F-DF472R and DF418F-DF609R) were used in this study and plasmid DNA containing partial 16S rRNA gene insert was run as standard.

4.3.8 Limit of detection of qPCR amplifications

Quantitative PCR assays were used to determine the copy number and limit of detection of dog-specific *Bacteroides* genetic markers in water and sediment samples. Quantitative PCR performance characteristics based on absolute standard curves (range of slopes, efficiencies, coefficient of correlation and quantification) are shown in Table 4.4. Amplification was observed in all water and sediment samples. The copy number ranged from 2.9×10^1 to 3.68×10^2 copies in the water samples, and ranged from 4×10^0 to 1.5×10^1 copies in the sediment samples. There was a marginally significant difference ($p= 0.04$) between the copy numbers of dog *Bacteroides* genetic markers in the beach water and sediment at both sites on all three sampling occasions.

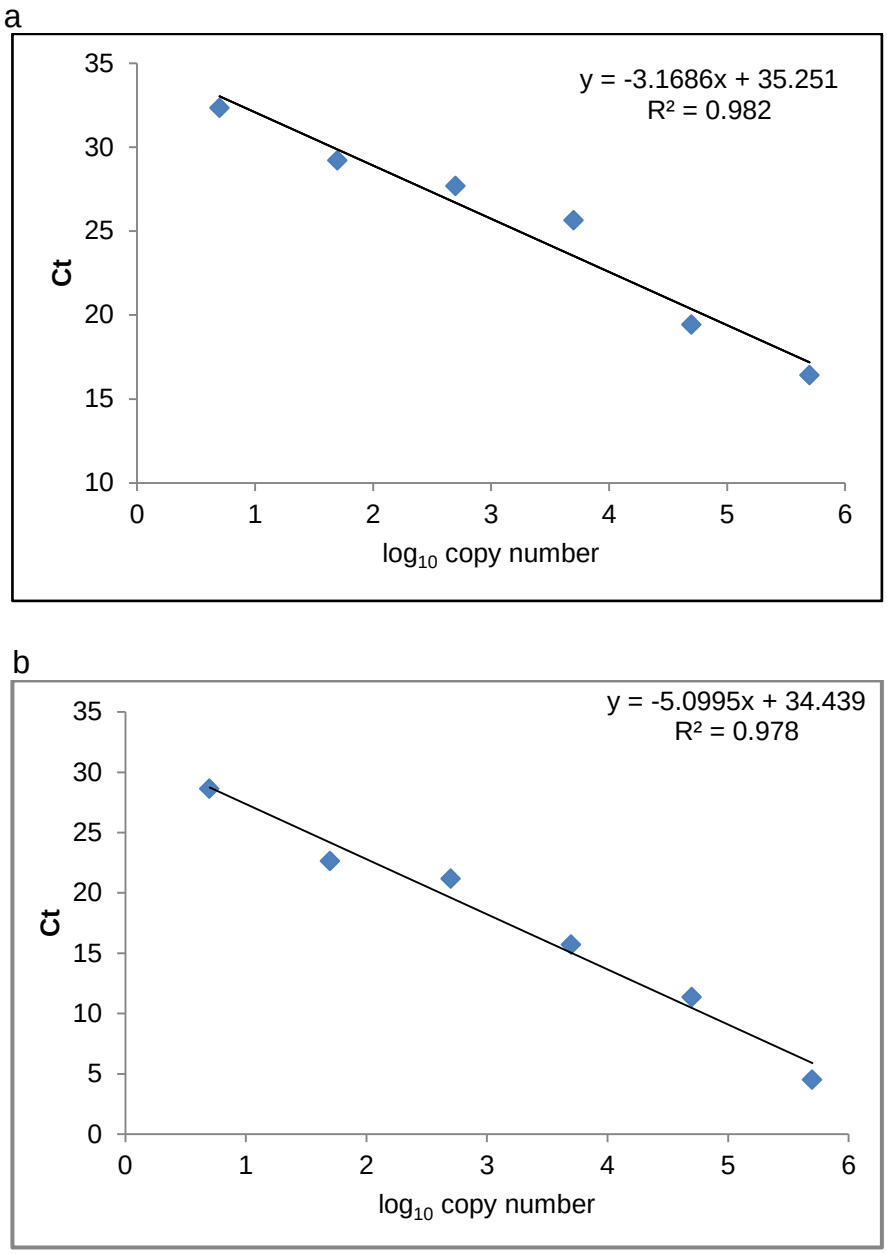


Figure 4.11 Standard curves created from tenfold serial dilution series of recombinant pGEM®-T easy plasmid containing the target sequence of the genetic marker illustrating the threshold cycle (Ct) and $\log_{10}$ copy number measurements using dog-specific *Bacteroides* primer sets DF418F-DF609R (a) and DF113F-DF472R (b).

Table 4.4 Quantitative PCR dog-specific *Bacteroides* primer set performances based on absolute standard curves run separately over this study

Primer sets	Samples	Slope range	R^{2*} range	Eff.% range	Quantification range (copies)
DF113F-DF472R	Water	-3.594, -3.012	0.979 - 0.998	91 - 114	29 - 368
DF418F-DF609R	Water	-3.563, -3.069	0.961 - 0.997	91 - 111	29 - 368
DF113F-DF472R	Sediment	-3.594, -3.012	0.979 - 0.998	91 - 115	4 - 15
DF418F-DF609R	Sediment	-3.563, -3.069	0.960 - 0.997	91 - 112	4 - 15

R^2: Coefficient of correlation, Eff.: Efficiency= $10^{(-1/slope)}$-1, quantification range: copy number per reaction.

4.4 Discussion

In this study, culture-dependent results showed good/ sufficient quality of beach waters based on the EU bathing water directive 2006 for *E. coli* and Enterococci at the both sites on each occasion. The highest level of FIB was in the second sampling (15th August 2012) which was possibly because of associated bad weather and heavy rainfall. Kay *et al.* (2010) have demonstrated that ≥40 % of the FIB come from agricultural sources. Moreover, concentrations of FIB have often been shown to increase with rainfall resulting from runoff from urban and agricultural land (Rajal *et al.*, 2007; Green *et al.*, 2012). This study has shown that even whilst the water quality met the requirements of the directive, pollution from dog faeces may still be present. In the past, the principle management measures in the event of the directive standards being breached focused on sewage treatment facilities but, increasingly, it has been recognised that other diffuse sources of contamination may also be important. Therefore, knowledge of the source and longevity of bacteria found in bathing waters, as has been demonstrated for dogs, is critical in order to manage the risks to human health. Thus, even whilst this water quality was found to be good/ sufficient quality the dog-specific primers designed here found dog faecal *Bacteroides* spp. in water samples from both sites.

Host-specific conventional and qPCR primer sets were designed to target 16S rRNA genetic marker of faecal *Bacteroides* to source track dog faecal pollution and to distinguish this from other animal sources. The resulting amplicons were aligned with 16S rRNA genetic markers of *Bacteroides* from other animals in order to detect region(s) with strongly mismatching

sequences, which were then used to design specific primer sets for *Bacteroides* dogs. The amplicon size and sequence of the conserved 16S rRNA genes are informative parameters that have been used in phylogenetic studies of these species (Wang *et al.*, 2002). Specific primers can be used for many purposes such as phylogenetic analysis and gene expression (Shoemaker *et al.*, 2001).

In the current study, distinguishing (source tracking) *Bacteroides* spp. from dog faeces and other *Bacteroides* spp. colonizing the same environment but from different source, was the major aim. This can be complicated as the 16S rRNA genetic marker of *Bacteroides* species has strong homology (84 to 89 %), and primers for a particular species can cross-react with other *Bacteroides* spp. (Shelburne *et al.*, 2002). However, all three primer sets (DF53F-DF606R, DF113F-DF472R and DF418F-DF609R) appeared to have high sensitivity and high specificity (100, 100 and 92 %, respectively) *in vitro* and *in silico* and thus successfully detected the *Bacteroides* spp. from dog faeces amongst other types of *Bacteroides* spp. This compared favourably with BacCan-UCD assay (Kildare *et al*., 2007) which was showed 62.5 % sensitivity. Some cross-reactivity was shown with human faecal *Bacteroides* PCR. This was because the similarity of nucleotide amplicons between the sequences of *Bacteroides* from dog and human faeces was a quite high (89 %), and the specificity of the third primer set (DF418F-DF609R) was slightly below 100 % (92 %). Other techniques such as denaturing gradient gel electrophoresis (DGGE), and next generation sequencing (NGS) have been used recently to distinguish sequences in closely related species (Kuboniwa *et al.*, 2010; Denecke *et al.*, 2012). However, these techniques are relatively

expensive and time consuming compared with PCR using the host-specific primers.

When utilised on bathing waters at a designated beach on sites at which dogs are banned and permitted, all the samples showed positive results with the generic *Bacteroides* genetic markers indicating some degree of animal faecal pollution. However, no *Bacteroides* genetic markers from human, horse and pig origins were detected in any sample but cow and the newly designed dog *Bacteroides* genetic markers were detected in the beach water indicating that cow and dog faecal pollution may have come from areas close by.

The results of qPCR showed that the dog *Bacteroides* genetic markers were present and could be detected in the beach waters and even in the beach sediments at both sites, although in low numbers. Low copy numbers, as well as variations in sensitivity and specificity associated with different genetic markers have also been reported in other studies using human and other animal *Bacteroides* genetic markers (Layton *et al.*, 2013), or other animal *Bacteroides* genetic primers (Okabe *et al.*, 2007). In addition, Schriewer *et al.* (2013) found 5.7×10^1 and 3.81×10^2 copy numbers per reaction as a lower limit of quantification for the primers DogBact and BacCan-UCD, respectively. On the other hand, several researchers have calculated the limit of detection of *Bacteroides* genetic markers, and they found different values ranging from 1 to 800 copy numbers per reaction (Table 1, Appendix 1).

In this study, SYBR® Green 1 fluorescent binding dye protocol was used in qPCR analysis to detect dog-specific *Bacteroides* genetic marker instead of the more expensive TaqMan® protocol. SYBR® Green 1 dye and TaqMan® probe protocols have about the same limit of detection, reproducibility, and thermodynamic range, but the accumulation of primer dimers and the amplification of non-specific PCR products can be detected only in SYBR® Green 1 protocol (Lee *et al.*, 2006a). Unlike TaqMan®-based assays, SYBR® Green also allows determination of the specificity of qPCR using melting curves (Quellhorst *et al.*, 2005).

In conclusion, dog-specific faecal *Bacteroides* 16S rRNA genetic PCR assays were designed and appeared to be both specific and sensitive. These primer sets were successfully used to detect the presence of faecal *Bacteroides* genetic marker from dogs in water from both areas of a bathing beach on which dogs were banned and dogs were permitted. Quantitative PCR detected low copy numbers (near the limit of detection) of the genetic markers in water and sediment samples. In this case, traditional FIB analysis methods found that water quality was ‘good’, whereas source tracking was used to demonstrate that *Bacteroides* spp. can even reach areas where access for dogs is restricted. Quantitative PCR assays with newly designed host-specific 16S rRNA primer sets were successfully developed and used for identification and quantification of dog-specific faecal pollution, which cannot be done by culture-dependent methods. The current study results revealed that a qPCR assay could sufficiently discriminate and quantify dog-specific faecal pollution. The use of such genetic markers to identify the source of bacteria in a case of a breach of the bathing water quality

standards or an outbreak of disease may prove invaluable in future public health studies relating to faecal contamination of bathing water.

The results from this chapter have been presented as oral or poster presentations at the following conferences: the Society of Applied Microbiology (SfAM) Conference, Edinburgh, UK, 2nd-5th July 2012, the Centre for Agricultural and Rural Sustainability (CARS), Duchy college, UK, 19th June 2013 and the BioMicroWorld, V International Conference on Environmental, Industrial and Applied Microbiology, Madrid, Spain, 2nd-4th October 2013. Also, the results of this chapter have published in the Journal of Hydrology: Current Research 2014 (doi:10.4172/2157-7587.1000163).

Chapter Five

Survival and persistence of *Bacteroides* species as faecal indicators and the recovery of 16S rRNA markers under controlled conditions

5.1 Introduction

Faecal *Bacteroides* species have been suggested as an alternative indicator of recent faecal pollution, because of their higher abundance in populations of faecal bacteria compared to traditional FIB (Kreader, 1995; Eckburg *et al.*, 2005; Layton *et al.*, 2006; Liang *et al.*, 2012). Source-specific indicators using the PCR of host-specific genetic markers from *Bacteroides* spp. have been suggested as a rapid diagnostic tool to identify faecal pollution and faecal source discrimination (Balleste and Blanch, 2010). Green *et al.* (2011) have suggested that a measure of genetic marker persistence is provided by traditional PCR and the absolute quantification of their genetic markers provided by quantitative PCR (qPCR). Anderson *et al.* (2005) demonstrated that the intestines of warm-blooded animals contain abundant FIB, and their presence in environmental waters indicates faecal pollution and the accompanying presence of potential pathogens. Whilst, it has been reported that FIB such as *E. coli* may replicate in the environment (Ferguson and Signoretto, 2011), little information is known about the survival and persistence of faecal *Bacteroides* spp. and their genetic markers in aquatic environments after being released into bathing water (Okabe and Shimazu, 2007). Enumeration of non-cultivable host-specific faecal *Bacteroides* spp. in faecal samples has been attempted with 16S rRNA gene labelling and immune-capture followed by PCR (Bae and Wuertz, 2009b). Consequently, proper predictive MST models must be developed in order to examine the survival of *Bacteroides* spp. Additionally, the relationship between *Bacteroides* spp. survival and persistence of other usual microbial indicators should be considered (Balleste and Blanch, 2010). However, the persistence

of *Bacteroides* spp. after release into different environmental waters and sediments is not well understood (Scott *et al.*, 2002). To this end, Green *et al.* (2011) have stated that microcosms are frequently used to study environmental methods under highly controlled circumstances.

Furthermore, bacteria show an affinity for inorganic sediment particles, and chemical factors play a role in increasing their adhesion (Hipsey *et al.*, 2006). Microbial ecologists are interested in microbial morphology, localization, adhesion, abundance and activity to determine the phylogeny of environmental microorganisms. Fluorescence *in situ* hybridization (FISH) with nucleic acid probes that target regions of 16S rRNA gene molecules have been used to investigate the adhesion of the *Bacteroides* spp. with sediment particles (Daims *et al.*, 2005; Hipsey *et al.*, 2006). FISH and comparative 16S rRNA gene sequences are key methods for identifying the diversity and composition of complex microbial communities (Amann *et al.*, 2001). 16S rRNA gene is commonly used as a target molecule for FISH because it has genetic stability (Woese, 1987). This study aimed to determine the culture-dependent survival of *Bacteroides* spp. and PCR-based recovery of human- and dog-sourced *Bacteroides* genetic markers from river water and seawater laboratory microcosms containing sediment.

5.2 Materials and methods

Some methods used in this chapter are described in detail in chapter two. In this section only changes will be discussed in detail.

5.2.1 Water and sediment samples

Samples of river water and sediment were collected from the Plym River, (50.38 ºN and - 4.08 ºW), England. Water samples were collected at a depth of approximately 30 cm below the surface of water using five litre buckets. Sediment samples were collected in the small bags using a spade. Sea water samples were obtained from environmental laboratories, Davy building, Plymouth University. All samples were sterilized by autoclaving at 121 ºC and 15 psi for 15 minutes.

5.2.2 Faecal sample collection

Four human faecal samples were donated from adults in the sterile utensils and placed in the sterile 50 ml tubes. Dog faecal samples (n = 10) were collected from domestic dogs in Plymouth, Devon using sterile containers with the help of owners. Faecal samples were immediately transported to the laboratory. Two grams of faecal samples were mixed with up to 20 ml sterile PBS. The sample was blended for two minutes in a Stomacher® Lab-Blender (Seward, UK). Tenfold serial dilutions (10^{-2} to 10^{-6}) were prepared, the samples left to settle for 10 minutes, and 100 µl of each dilution was spread onto *Bacteroides* bile esculin agar (BBE) plates and incubated at 37 ºC for 72 hours under anaerobic condition (Don Whitley, UK). Pure colonies of *Bacteroides* spp. were randomly selected and inoculated into 50 ml of BPRM broth, then incubated at 37 ºC for 72 hours anaerobically. Optical density (OD) was measured at 590 nm to determine total bacterial numbers in each

sample as described in section 2.4.3. The cells were centrifuged at 8000 × *g* for 10 minutes and washed twice with 5 ml sterile PBS. The cell pellets were re-suspended in 50 ml sterile PBS in the final cell concentrations of 10^3 - 10^5 cells ml^{-1}.

5.2.3 Microcosm setup

Microcosms were designed to simulate environmental conditions by allowing exposure to controlled temperature and salinity. The study was conducted in the Davy building laboratories, Plymouth University. The four microcosms consisted of four sterile 9 litre buckets, two of which were filled to capacity with sterile river water, while the other two were filled to capacity with sterile sea water (Figure 5.1). Sterile sediment was added to the buckets to a thickness of about one to two cm from the bottom. Clear plastic film covers were put on the microcosms to minimize the evaporation of water. Treatments included two salinities; river water (< 0.5 practical salinity units, psu) and sea water (34 psu). In addition, salinities (< 0.5 and 34 psu) of microcosms were performed as the two types of river water and seawater in the previous study areas. Ambient temperatures of 17 ± 0.5 ºC (two buckets) and 10 ± 0.5 ºC (two buckets) were used to replicate local surface temperature during cold and warm seasons. These temperatures were chosen as they reflect those of bathing water around the Devon coastline during cold and warm seasons (METNET,www4.plymouth.ac.uk/metnet/). The average water temperature in the cold months is 10 ± 2 ºC and the average of water temperature in the warm months is 17 ± 1.5 ºC (Webb and Walling, 1992; McDowell and Trudgill, 2000). Ninety millilitres of isolated human- and dog-sourced *Bacteroides* suspension was added into each 9

litre bucket. All microcosms were kept in the dark. The temperature was checked daily in each microcosm before and after opening of incubators and did not vary by more than ± 0.5 ºC. River water and seawater samples without inoculation were incubated at 10 ºC and 17 ºC as control.

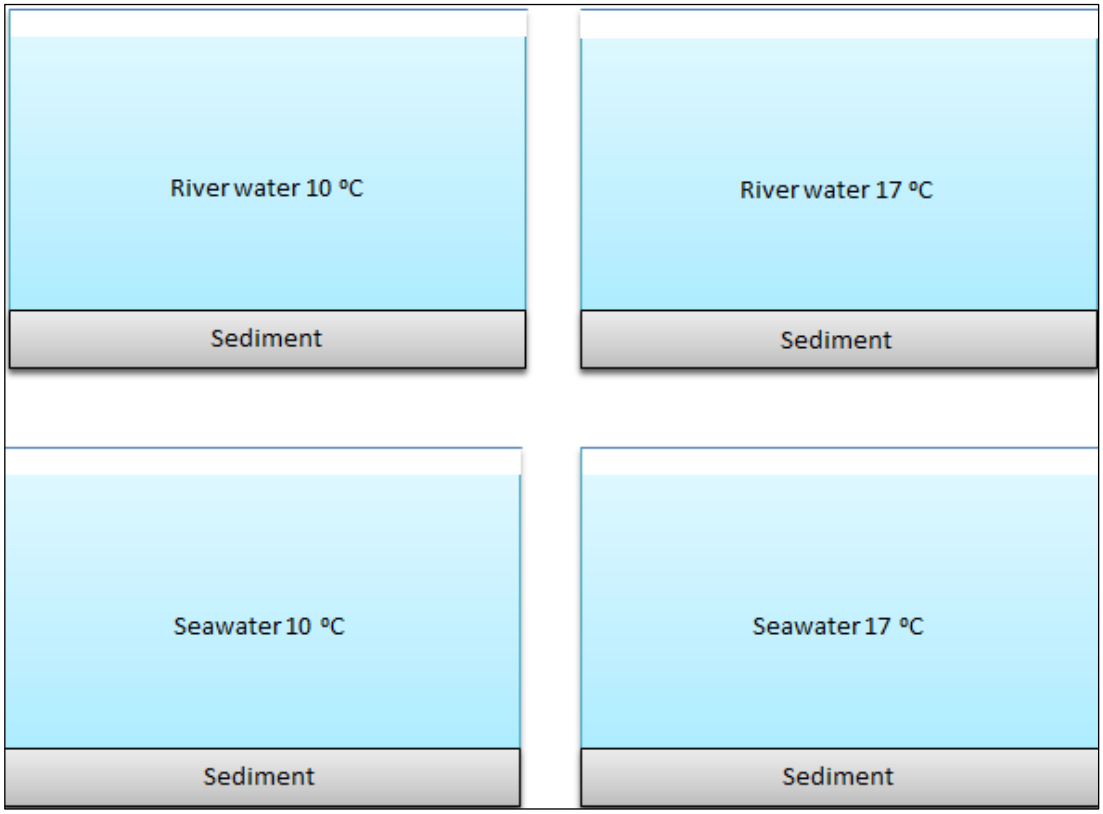


Figure 5.1 Schematic diagram illustrating conditions in controlled microcosms used in this study.

5.2.4 Sample collection

Water samples had no visible turbidity. Triplicate 100 ml water and 2 g sediment samples were taken from each microcosm every two days for 14 days, and then daily until day 18. Water samples were filtered by using a vacuum pump through 0.45 µm pore size cellulose nitrate membrane filters (Whatman, UK) and placed on to BBE agar as described in section 2.4.1.3 (Livingston *et al.*, 1978). Faecal *Bacteroides* colonies were counted as CFU 100 ml^{-1}. In addition, sediment samples (2 g) were taken and sterile seawater or sterile river water added up to 20 ml final volume to make the initial 10^{-1} dilution as explained in section 2.4.2. The results were expressed as CFU g^{-1} (Craig *et al.*, 2002; Ferguson *et al.*, 2005). Each set of the samples was processed in triplicate, and the means were reported.

5.2.5 DNA extraction

From each microcosm, other 100 ml of water samples were collected and filtered through 0.45 µm pore size cellulose nitrate membrane filters using vacuum filtration. The membrane filters were placed into separate sterile polypropylene tubes (15 ml) with 700 µl of guanidine isothiocyanate lysis buffer, mixed several times to ensure complete wetting of the membrane filters, then stored at - 80 ºC until further DNA extraction (Dick and Field, 2004; Shanks *et al.*, 2006b; Schulz and Childers, 2011). DNA was extracted from water samples by QIAamp DNA mini kit (Qiagen, UK) as described in the manufacturer's instructions (section 2.5.1.1). A SoilMaster^{TM} DNA extraction kit (Cambio, UK) was used for DNA extraction from the sediment samples as per the manufacturer's protocol, as described in section 2.5.1.2.

5.2.6 DNA quantification

Extracted DNA was quantified by using a NanoDrop UV spectrophotometer (ND-1000, Labtech, UK). Two µl of each sample was used for the quantification of DNA from the water and sediment samples as described in section 2.5.2.

5.2.7 Conventional polymerase chain reaction (PCR)

The PCR was carried out in a total volume of 25 µl as described in section 2.5.3. The generic *Bacteroides* primer set Bac32F-Bac708R, and human-specific *Bacteroides* primer set HF183F-Bac708R was used (Bernhard and Field, 2000b) as described in 2.5.3. For *Bacteroides* spp. from dog faeces, dog-specific primer sets were used, which were designed previously (Table 4.1). The cycling parameters of PCR were as listed in section 2.5.3. Finally, to detect the amplified products, PCR product was visualized on a 1.5 % (w/ v) agarose gel with SYBR® safe DNA gel stain (Invitrogen, UK) as described in section 2.5.4.

5.2.8 Quantitative polymerase chain reaction

Faecal *Bacteroides* qPCR host-specific primer sets were used. For generic *Bacteroides,* qBac560F-qBac725R primer set was used (Okabe *et al.*, 2007). Human-specific *Bacteroides* HF183F-HFR primer set was used (developed previously by Bernhard and Field 2000b and Seurinck *et al.* 2005) . Seurinck *et al.* (2005) developed a new reverse primer (HFR) for using with human-specific *Bacteroides* forward primer to decrease the amplicon length to a suitable size (82 bp) for qPCR detection (Table 5.1). DF113F-DF472R and DF418F-DF609R dog-specific primer sets were used which were designed in a previous study (chapter 4) to detect dog specific *Bacteroides* 16S rRNA

genetic markers. Target genetic markers were cloned into the pGEM®-T easy vector plasmid (Promega, UK). The ligated products were transformed into high efficiency *E. coli* JM109 competent cells (Promega, UK) as described in section 2.5.7.3. Recombinant *E. coli* colonies were inoculated in LB broth as mentioned in section 2.5.7.4. LB agar containing 100 µl ampicillin, 40 µg ml^{-1} X-gal and 0.1 mM IPTG were used for screening the white-blue colonies as described in section 2.5.7.1. Plasmid DNA was extracted (see section 2.5.7.5). Enzyme digestion was performed (section 2.5.7.6). Partial sequencing was performed using BigDye® kit (Applied Biosystems, USA) as described in section 2.5.7.7. The qPCR thermal programmes were carried out as described in section 2.5.7.8. However, for qBac560F-qBac725R and HF183F-HFR primer sets the thermal conditions were applied as follows, incubation at 50 ºC for 2 minutes and at 95 ºC for 10 minutes, followed by 40 cycles started with denaturing at 95 ºC for 15 second (qBac560F-qBac725R) or 30 second (HF183F-HFR), annealing temperature (Table 5.1) and extension at 62 ºC (qBac560F-qBac725R) or 60 ºC (HF183F-HFR) for 1 minute (Seurinck *et al.*, 2005; Okabe *et al.*, 2007). For determination the target copy numbers in water and sediment samples, absolute standard curves were created as the method described in section 2.5.7.9.

Table 5.1 Additional *Bacteroides* host-specific qPCR primer sets used in this study

Primer	Sequence (5'----------3')	Host of *Bacteroides*	Annealing temp. ºC	References
qBac560F	TTTATTGGGTTTAAAGGGAGCGTA	Generic	62	(Okabe *et al.*, 2007)
qBac725R	CAATCGGAGTTCTTCGTGATATCTA	Generic		(Okabe *et al.*, 2007)
HF183F	ATCATGAGTTCACATGTCCG	Human	53	(Bernhard and Field, 2000b)
HFR	TACCCCGCCTACTATCTAATG	Human		(Seurinck *et al.*, 2005)

5.2.9 Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) was used to detect bacteria by using a fluorescently labelled oligonucleotide probe that hybridizes specifically to its complementary target sequence within the intact cells (Moter and Göbel, 2000). Samplings from sediment were performed every two days for seven days and then daily until day 10. FISH consists of four steps. The more details in subsequent subdivisions (Daims *et al.*, 2005; Daims, 2009).

i. **Fixation**

Sediment samples (200 mg) were re-suspended in 500 µl sterile PBS and fixed with 500 µl 4 % (w/ v in PBS) ice-cold fresh paraformaldehyde solution (Sigma Aldrich, UK) for three hours at 4 ºC. The sample was then centrifuged at 13000 *x g* for 5 minutes, and washed twice with 200 µl sterile PBS to remove residual paraformaldehyde. The sample was then re-suspended in 1:1 (v/ v) 200 µl ice-cold PBS and ice-cold 96 % molecular grade ethanol (Sigma Aldrich, UK).

ii. **Dehydration**

After fixation and washing, 35 µl of re-suspended sample was spotted onto Polylysine® glass slides (Thermo Scientific, UK). Dehydration of the samples was achieved by passing the slides through a series of increasing concentrations of ethanol (50, 80 and 96 % v/ v in sterile ddH_2O three minutes in each). The slides then were dried at 46 ºC for 15 minutes or longer at room temperature.

iii. **Hybridization**

Fixed slides were hybridized with 50 pmol bacterial-specific probe EUB338 [5'- GCTGCCTCCCGTAGGAGT -3'] or *Bacteroides* specific probe Bac303 [5'- CCAATGTGGGGGACCTT -3'] (Manz *et al.*, 1996). Both probes were labelled with fluorescein isothiocyanate (FITC) at the 5' end (Eurofins MWG, Germany). One microliter from each probe was mixed with 10 µl of fresh hybridization buffer onto Polylysine® slide, and then these slides placed into 50 ml plastic tubes horizontally to prevent evaporation. The plastic tube was closed and placed in a horizontal position in an incubator at 46 ºC for 90 minutes. After hybridization, washing was performed using pre-warmed washing buffer (Appendix 2) at 48 ºC for 20 minutes. The slides then were washed briefly in ice-cold sterile water then dried with compressed air.

iv. **Mounting and examination**

Citifluor antifadent (Fluoroshield™, Sigma, UK) solution was applied to the sample and cover slip added, and then examined using either a Nikon 80i epifluorescence microscope or a Zeiss LSM 510 confocal laser scanning microscope (Zeiss, Germany).

5.2.10 Decay rate calculation

Decay rates of cultured *Bacteroides* spp. were calculated using the following equation (Okabe and Shimazu, 2007):

$$\text{Decay rate} = \frac{[\ln(N_t) - \ln(N_0)]}{t} \qquad (1)$$

Where N_t: $\log_{10}$ (CFU or copies 100 ml^{-1}) at time t, N_0: $\log_{10}$ (CFU or copies 100 ml^{-1}) at time zero, ln: natural $\log_{10}$ and t: time (in days).

5.3 Results

Four microcosms were set up and maintained for 18 days including 9 litres of water and 1-2 cm depth sediment. The water samples showed no visible turbidity, and the pH was 7.8 ± 0.3 for seawater and 7.5 ± 0.3 for river water. The salinity was 34 and < 0.5 psu for seawater and river water, respectively.

5.3.1 Bacteriological counts

5.3.1.1 Culture-dependent recovery

The mean initial populations of culturable *Bacteroides* spp. were 1.2×10^4 CFU 100 ml^{-1} in water and 8.0×10^3 CFU g^{-1} in sediment (Table 2, Appendix 1). The linear recovery in number of faecal *Bacteroides* spp. was calculated down to 1.4×10^1 CFU 100 ml^{-1} in the water microcosms at day 14 and 2.7×10^1 CFU g^{-1} in the sediment microcosm at day 16. The culturability of faecal *Bacteroides* spp. from water samples at both temperatures and both salinities decreased linearly. Results from the river water and seawater showed decreasing *Bacteroides* spp. count which declined sharply after 6 days. The count of faecal *Bacteroides* spp. in sediment slowly decreased before a sharply decline showed after 8 days. The percentage recovery of *Bacteroides* spp. were calculated in CFUs per 100 ml water or per g sediment as a number of CFU divided on initial number, there were no significant differences between the decline rates of water samples (p= 0.997) and between sediment samples (p= 0.845, Figure 5.2).

5.3.1.2 Decay rates of *Bacteroides* spp.

The decay rates of *Bacteroides* spp. were slightly different according to temperature and salinity and are presented in Table 5.2. Decay rates of

Bacteroides spp. on day 14 in water microcosms ranged from - 3.4×10^{-2} to - 9.4×10^{-2} CFU 100 ml^{-1} per day. Decay rates in sediment microcosms (day 14) ranged from - 1.9×10^{-2} CFU g^{-1} to - 2.8×10^{-2} CFU g^{-1} per day (Table 5.2). There was no significant difference (p= 0.592) in the calculated decay rates of faecal *Bacteroides* in water and sediment microcosms.

Table 5.2 Decay rates of *Bacteroides* spp. at day 8 and 14 in all water and sediment microcosms.

Sample	Temp. °C	Salinity psu[a]	Decay rate[b] (log_{10} CFU 100 ml^{-1} or CFU g^{-1}) per day	
			day 8	day 14
Water	10	< 0.5	- 8.2×10^{-3}	- 4.1×10^{-2}
Sed.	10	< 0.5	- 1.6×10^{-3}	- 2.0×10^{-2}
Water	10	34	- 7.3×10^{-3}	- 3.4×10^{-2}
Sed.	10	34	- 1.1×10^{-3}	- 1.9×10^{-2}
Water	17	< 0.5	- 1.1×10^{-2}	- 9.0×10^{-2}
Sed.	17	< 0.5	- 1.1×10^{-3}	- 2.3×10^{-2}
Water	17	34	- 1.1×10^{-2}	- 9.4×10^{-2}
Sed.	17	34	- 1.2×10^{-3}	- 2.8×10^{-2}

[a]psu: practical salinity units, [b]negative value represents an actual decrease in bacterial numbers, sed.: sediment

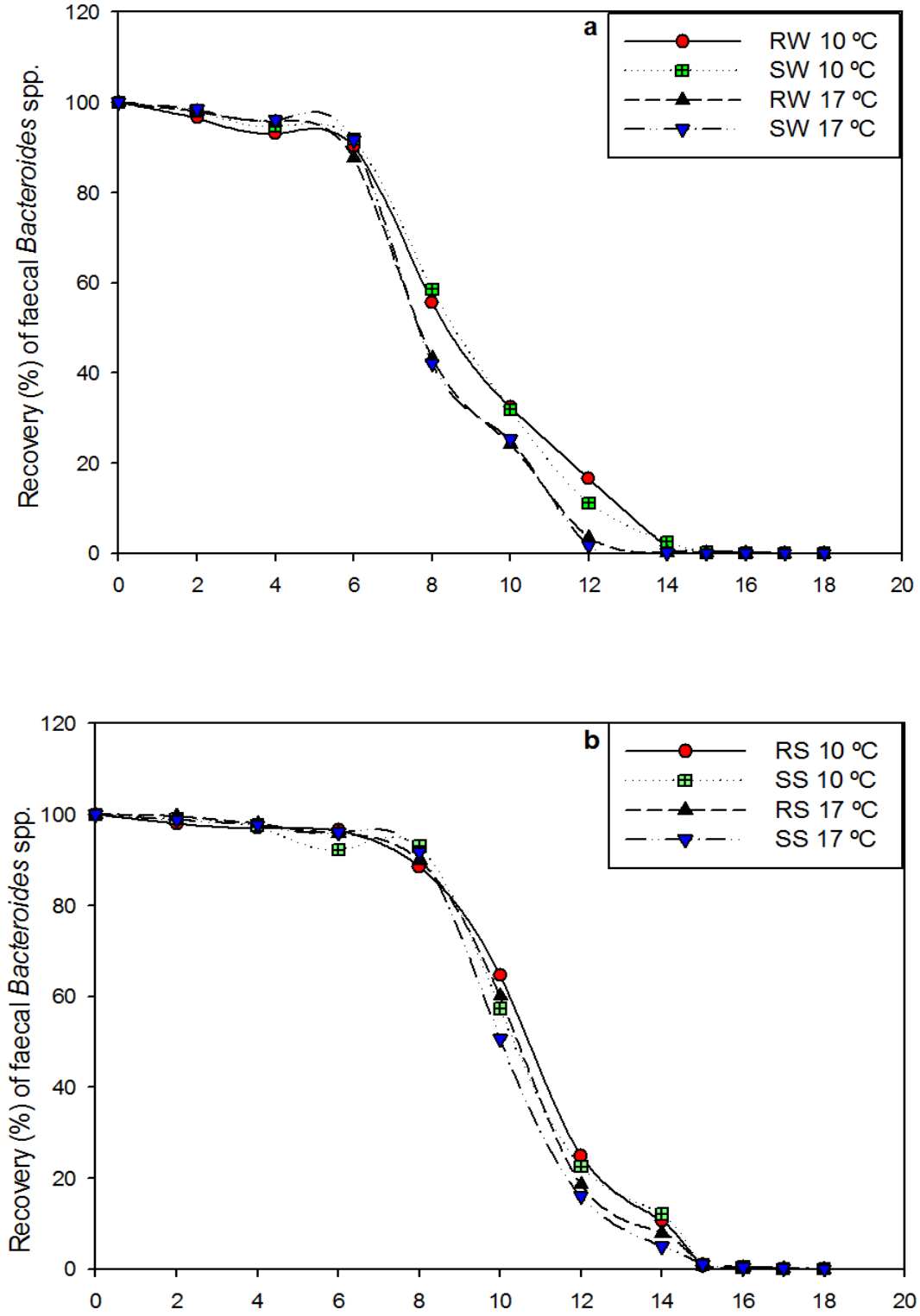


Figure 5.2 Percentage recovery of human- and dog-sourced faecal *Bacteroides* spp. in the water microcosms (a), and in the sediment microcosms (b). RW: river water; SW: seawater; RS: river sediment; SS: sea sediment.

5.3.2 Molecular methods for genetic marker analysis

Conventional PCR and qPCR were used for measuring the persistence of *Bacteroides* 16S rRNA genetic markers and to estimate the copy number of target DNA obtained from each microcosm. The survival of *Bacteroides* spp. and the persistence of their genetic markers were compared concurrently.

5.3.2.1 Conventional polymerase chain reaction (PCR)

The results for generic-, human- and dog- *Bacteroides* 16S rRNA genetic markers showed positive findings in the river water and sea water samples until day 12 in 17 ºC microcosms, whereas in 10 ºC microcosms these genetic markers were still detectable until day 14. These genetic markers showed positive results in sediment samples until day 14 in 17 ºC microcosms, whereas in 10 ºC microcosms they were detected until day 16 (Table 5.3). The patterns of recovery for each marker were identical.

5.3.2.2 Quantitative polymerase chain reaction

The DNA concentration for *Bacteroides* genetic markers was estimated on day 0 and did not significantly differ between river water and seawater microcosms ($p= 0.397$) and between river and sea sediment microcosms ($p= 0.624$). There were linearly decline rates of *Bacteroides* genetic markers in water and sediment samples (Figure 5.5). Human- and dog-sourced *Bacteroides* genetic markers had decreased in water samples to 1.2×10^1 and 6.0×10^0 copy number per 100 ml at day 16 of water samples, respectively. In addition, these genetic markers had decreased in sediment samples at day 16, and low copy numbers of host-specific *Bacteroides* genetic markers ranged from 1.0 × 101 to 4×10^0 copy numbers per reaction.

Table 5.3 Recovery of *Bacteroides* for generic-, human- and dog-specific 16S rRNA genetic markers on each sampling day as determined by conventional PCR in water and sediment microcosms. Recovery was identical for all three markers.

	Water samples*				Sediment samples			
Day	RW 10 °C	SW 10 °C	RW 17 °C	SW 17 °C	RS 10 °C	SS 10 °C	RS 17 °C	SS 17 °C
0	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+
14	+	+	-	-	+	+	+	+
15	-	-	-	-	+	+	-	-
16	-	-	-	-	+	+	-	-
17	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-

*RW: river water; SW: seawater; RS: river sediment; SS: sea sediment

Table 5.4 Quantitative PCR host-specific *Bacteroides* primer set performances based on absolute standard curves run separately in water and sediment samples over this study

Primer sets	Samples	Target *Bacteroides*	Slope range	R^2 range	Eff.% range	Quantification range (copies)
qBac560F-qBac725R	Water	Generic	-3.531, -3.110	0.953 - 0.991	91 - 110	20 - 1.2 × 10^4
HF183F-HFR	water	Human	-3.541, -3.011	0.960 - 0.996	92 - 114	12 - 5.7 × 10^3
DF113F-DF472R	water	Dog	-3.594, -3.012	0.979 - 0.998	91 - 114	8 - 6.4 × 10^3
DF418F-DF609R	Water	Dog	-3.522, -3.022	0.968 -0.988	92 - 114	6 - 6.6 × 10^3
qBac560F-qBac725R	Sediment	Generic	-3.563, -3.069	0.961 - 0.997	91 - 112	24 - 7.0 × 10^3
HF183F-HFR	Sediment	Human	-3.551, -3.131	0.965 – 0.999	91 - 113	10 - 3.3 × 10^3
DF113F-DF472R	Sediment	Dog	-3.535, -3.023	0.959 – 0.992	91 - 111	4 - 3.6 × 10^3
DF418F-DF609R	Sediment	Dog	-3.501, -3.098	0.969 -0.987	93 - 110	4 - 3.5 × 10^3

*R^2: Coefficient of correlation, Eff.: Efficiency= $10^{(-1/slope)}$-1, quantification range: copy number per reaction.

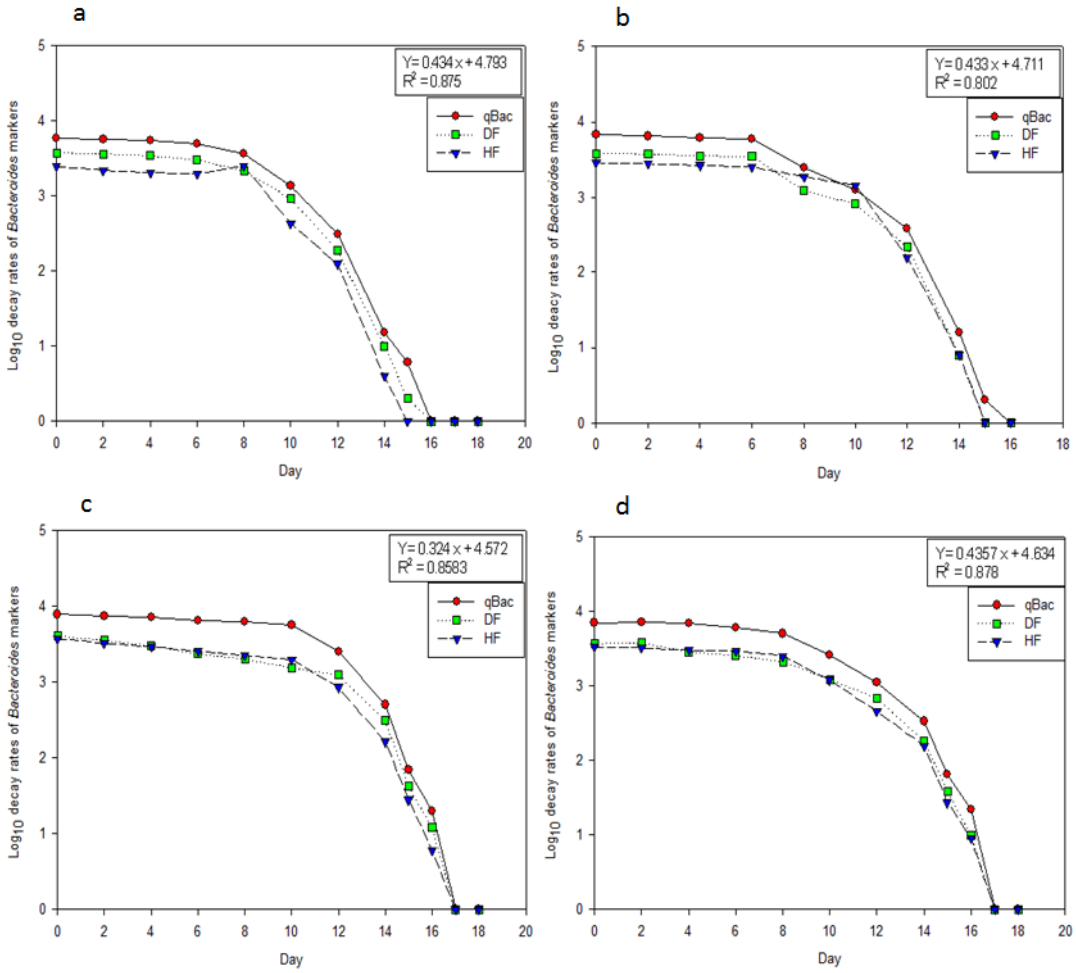


Figure 5.5 The persistence of *Bacteroides* spp. genetic markers in river water samples (a), seawater samples (b), river sediment samples (c) and sea sediment samples (d). qBac: generic *Bacteroides* genetic marker, DF: dog-specific genetic marker and HF: human-specific genetic marker.

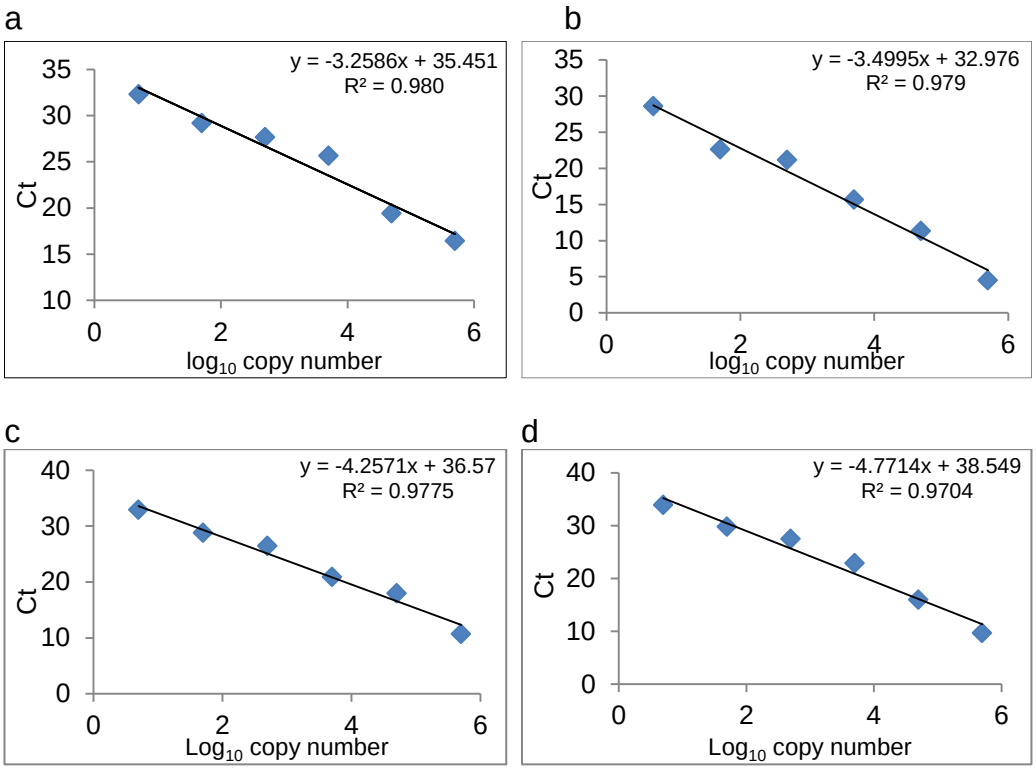


Figure 5.6 Standard curves created from tenfold serial dilution series of a recombinant pGEM-T plasmid containing the target sequence of the genetic marker, illustrating the threshold cycle (Ct) against $\log_{10}$ copy number measurements using dog-specific *Bacteroides* primer sets DF418F-DF609R (a), DF113F-DF472R (b), qBac560F-qBac725R (c) and HF183F-HFR (d).

A plasmid containing target sequences was used to create absolute standard curves for target *Bacteroides* primer sets, and the quantities were estimated based on these curves (Figure 5.6). The performance characteristics of qPCR primer sets based on absolute standard curves (range of slopes, efficiencies, coefficient of correlation and quantification) are shown in Table 5.4. Melting curve analysis of the specific PCR products was 85 ± 2 ºC. qPCR was used to determine persistence profiles for generic-, human- and dog-specific *Bacteroides* 16S rRNA genetic markers. The persistence profile of these genetic markers was similar in both water microcosms, which a

sharp decline in the number of genetic markers from day 6 onwards, whilst, in sediment microcosms the persistence of genetic markers was slight longer before a sharp decline from day 8 onwards.

5.3.3 Fluorescence *in situ* hybridization

The adherence of *Bacteroides* onto particles of sediment was investigated by FISH using two probes, EUB338 and Bac303, in river and sea sediment microcosms at 10 ºC and 17 ºC on days 1, 3, 5, 7 and 8 (Table 5.5). The adherence of *Bacteroides* species was observed in the sediment samples until day 8 (Figure 5.7). No *Bacteroides* were detectable after day 8.

Table 5.5 Detection of adherence of *Bacteroides* sing the general bacterial probe EUB338 and the *Bacteroides* specific probe Bac303 in sediment microcosms.

Day	EUB 338 probe				Bac303 probe			
	River sed.		Sea sed.		River sed.		Sea sed.	
	10 ºC	17 ºC	10 ºC	17 ºC	10 ºC	17 ºC	10 ºC	17 ºC
1	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+
9	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-

Sed.: sediment

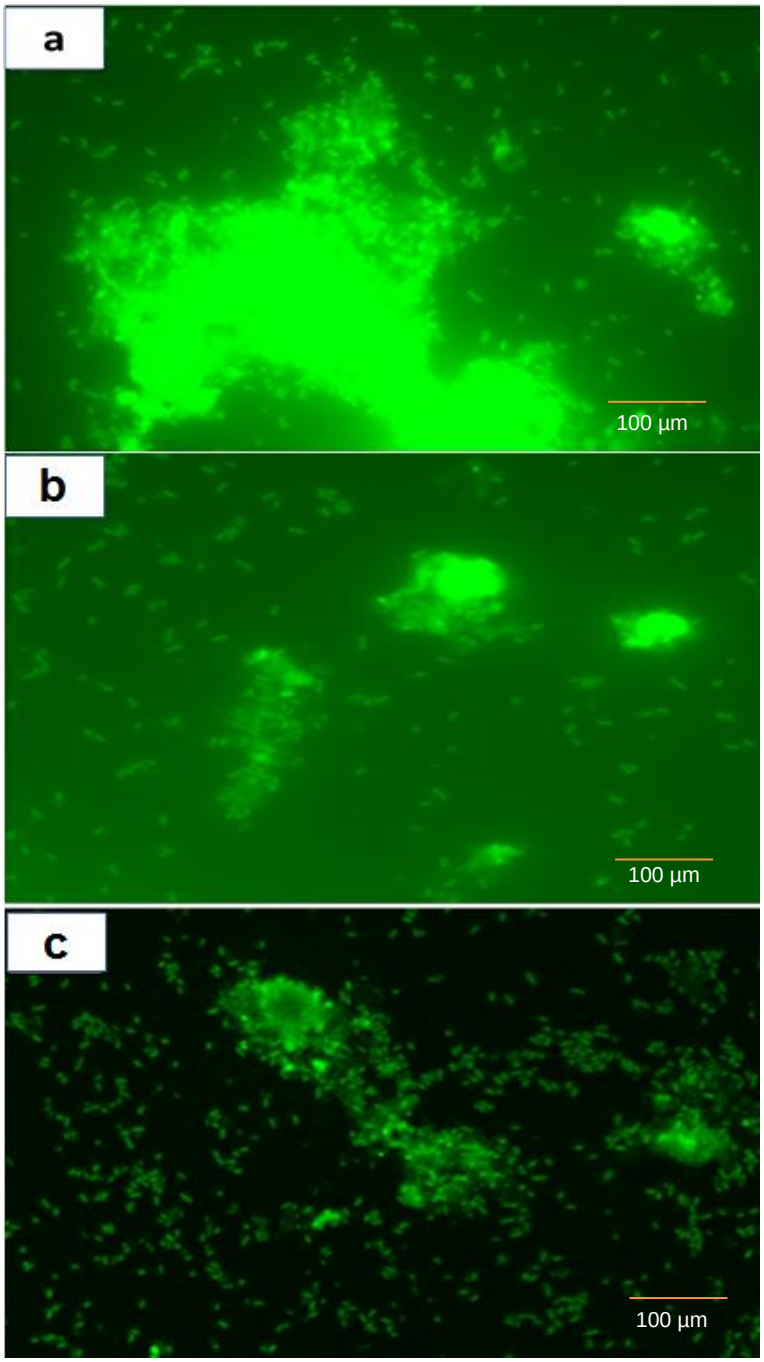


Figure 5.7 Adherence of *Bacteroides* spp. cells to particles of sediment detected using FISH. Cells were labelled with either FITC-labelled EUB338 (a), or FITC-labelled Bac303 oligonucleotide probes (b and c). Images were captured using a Nikon 80i epifluorescence microscope (a and b) or a Zeiss LSM 510 confocal laser scanning microscope (c) equipped with a ×60/ ×63 oil objectives, respectively.

5.4 Discussion

Environmental microcosms were used to trace the survival of *Bacteroides* spp. isolated from dog and human faeces in water and sediment, as well as to identify the persistence of their genetic markers in the same environmental conditions. Rates of decay of faecal *Bacteroides* spp. are useful in the interpretation of the results of environmental studies of faecal pollution of bathing water and the persistence of their genetic markers is considered to be an indicator of faecal pollution (Schulz and Childers, 2011). Many factors affect survival and persistence of FIB including temperature, solar irradiation, oxygen tolerance, predators, salinity, and availability of nutrient materials (Garcia-Lara *et al.*, 1991; Menon *et al.*, 2003; Walters and Field, 2009).

In both water and sediment microcosms, the concentration of *Bacteroides* spp. started to decline sharply from day 6 in water and from day 8 in sediment microcosms, while the host-specific *Bacteroides* genetic markers persisted longer namely up to day 14 in water microcosms and up to day 16 in sediment microcosms. The decay rates of human- and non-human (dog) faecal *Bacteroides* varied slightly according to the temperature and salinity used, but there was no statistical difference between them. This is in agreement with studies carried out by Schulz and Childers (2011), Okabe and Shimazu (2007) who had found the persistence of *Bacteroides* genetic markers persist up to day 14, but it is in disagreement with Green *et al.* (2011) who inoculated raw sewage effluent into river and marine water microcosms and detected faecal *Bacteroides* genetic markers only up to day 6 in river water microcosms and up to day 7 in seawater microcosms. Furthermore, Walters and Field (2009) observed the persistence of faecal *Bacteroides*

genetic markers for 6 days, but Seurinck *et al.* (2005) observed longer persistence until day 25 for the human *Bacteroides* genetic marker at 4 ºC and 12 ºC in fresh water. On the other hand, Bae and Wuertz (2009b) have detected that sunlight did not impact on the survival and persistence of *Bacteroides* spp. and their DNA, and they persisted for 24 days.

There were no noticeable differences in survival and persistence profiles of human and dog faecal *Bacteroides* genetic markers between the river and seawater incubations and between the 10 ºC and 17 ºC microcosms. Liang *et al.* (2012) have stated that the detection of DNA directly from environmental samples by molecular methods lacks the ability to discriminate between live (including VBNC) and dead bacteria. Also, Rudi *et al.* (2005) have demonstrated that researchers have often encountered a major issue regarding the distinction between viable and dead cells as the source of isolated DNA. Furthermore, Bae and Wuertz (2009a) have confirmed that the detection of *Bacteroides* DNA without distinguishing between live and dead cells may provide misleading information about the timing of recent faecal pollution events, especially in the case of sewage pollution which is derived from numerous sources. In contrast, applying qPCR can provide data on the fate of *Bacteroides* spp., and support management practices to maintain recreational water quality. The results suggest that *Bacteroides* spp. do not survive for long even in anaerobic sediment, whilst DNA persists for a little longer. Therefore, it may be useful to include sediment analysis in future MST data. As a result, variations in persistence are inevitable; it is possible that the longer persistence of these genetic markers in this case may be associated with dark incubation conditions or the presence of sediment.

Sediment may be considered an important source of bacteria in the natural water systems (Fries *et al.*, 2008). Although there were no obvious differences in the survival profiles of ruminant *Bacteroides* genetic markers (CF128) between light and dark microcosms in the work by Walters and Field (2009), there was no sediment in these microcosms or those of Green *et al.* (2011).

The adherence of *Bacteroides* spp. to particles of sediments was determined using FISH; there was clearly observed adhesion between faecal *Bacteroides* spp. and sediment grains until day 8. After 8 days the adherence was no longer detectable; largely because the number of the bacteria decreased sharply. Gillan and Pernet (2007) have stated that the adhesions of microorganisms tend to raise the rate of mineral dissolution and may lead to the release of adsorbed pollutants. Cooksey and Wigglesworth-Cooksey (1995) have stated that marine bacteria can exploit surface-bound substances of marine layers and some microorganisms attached to particles of sediment can help their adhesion. In this study, the adhesion of bacteria to particles of sediment indicates that the sediments act as reservoir for adhesive bacteria and may release them at some conditions such as tidal movement.

In conclusion, the short survival period of *Bacteroides* spp. outside of its natural host (gut of warm-blooded animals) suggests that this species can be used as faecal indicator for recent pollution, and this may help environmental agencies to track the source(s) of pollution. The short persistence of *Bacteroides* genetic markers indicates that this may be associated with the survival of the cells; this also suggests that *Bacteroides* spp. and their

markers can be used as faecal indicators. The use of qPCR for the detection of the persistence of *Bacteroides* spp. in environmental studies can provide data on the survival, decline and transport of these bacteria in water, and help environmental management agencies to predict recreational water quality. In addition, the similarity in persistence of human- and dog-specific *Bacteroides* 16S rRNA genetic markers to viable cells in environmental water and sediment samples suggests that *Bacteroides* spp. do not persist in the natural environment for long and that 16S rRNA genetic markers may be of value as alternative faecal indicators of bathing water pollution and source tracking in the future.

The results from this chapter have been presented as poster presentations at the following conferences: the American Society of Microbiology (ASM), San Francisco, USA. 16^{th}-19^{th} June 2012, the Centre for Research in Translation Biomedicine (CRTB), Annual Research Day, Plymouth University, 4^{th} July 2013 and in the BioMicroWorld, V International Conference on Environmental, Industrial and Applied Microbiology, Madrid, Spain, 2^{nd}-4^{th} October 2013. Also, the results of this chapter have accepted to publish as full paper in the proceeding book entitled 'Industrial, Medical and Environmental Applications of Microorganisms: Current Status and Trends' (2014).

Chapter Six

General discussion and recommended future work

This study focuses on the use, evaluation and development of microbial source tracking (MST) methods to determine faecal pollution source(s) in bathing water and sediment. Water quality issues have always been a concern to humans, particularly in areas where water can become polluted with pathogens from human sewage, animal waste and other waste waters. In recent years, concerns have increased both in developed and developing countries, where people have more leisure time which they may spend at the coast bathing or pursuing other water sports. In addition, increased urbanization can lead to overload of sewer systems. In spite of work which has been done to improve sewage treatment, researchers and environmental organisations still detect bacterial pollution in some beach waters, even in developed countries. However, faecal pollution may not only originate from sewage but from other sources such as animal waste. Crowther *et al.* (2001) have stated that in spite of a huge investment in sewerage and waste water treatment infrastructure coastal towns and cities in the UK, some bathing water is still failing to achieve sufficient standards. Good water quality is essential for the environmental ecosystem and to keep the environment safe for all uses and to improve public health (GCRWQ, 2012; UNEP, 2014).

Many waterborne pathogens, including bacteria, viruses and parasites, may impact negatively on a water system. However, it is difficult to monitor these pathogens for many reasons. Goodridge *et al.* (2009) have demonstrated that the processes which are used for monitoring, isolating and identifying individual waterborne pathogens are difficult, expensive and labour intensive. In order to address this problem with pathogen monitoring, FIB are monitored instead of pathogenic microorganisms.

To quantify the faecal pollution that threatens public health, normal gut flora bacteria (faecal coliforms, *E. coli*, and Enterococci) have been used worldwide as faecal indicators of the possible presence of dangerous pathogens that could exist in bathing water, drinking water and harvested seafood. The faecal indicator programme simplifies environmental management in terms of monitoring water quality and predicting faecal pollution source distribution, as well as then recommending effective strategies to improve the bathing water quality. The acceptable thresholds of these bacteria as faecal indicators for pathogenic microbes vary by continent and country (Mesquita and Noble, 2013).

While these indicators have been used reasonably effectively in many countries for years, basic water hygiene concerns have been raised about its effectiveness, particularly with regard to bathing water. Three key problems have been identified which, if addressed, could enable more effective and efficient water management:

i. Identification of the exact source(s) of pathogens is difficult. While many pathogens may originate from untreated human sewage waste, other mammals and birds have similar gut microflora and hence can contribute to the pollution (Kinzelman and McPhail, 2012). The management actions required to deal with each of these wastes individually varies significantly and so knowledge of the source is essential.

ii. The presence of pathogens and indicators in beach sediments is often not considered. However, this may be a significant source of exposure for young children or those involved in sports activities such as surfing,

when the active waves may agitate the sediments and re-suspend them, along with any associated bacteria (GCRWQ, 2012).

iii. The survival of pathogens and indicator bacteria, and their associated genetic markers has to be known and comparable if the indicator bacteria are to be a useful management tool (Rogers *et al.*, 2011).

6.1 The use of FIB to indicate faecal pollution

The first step for the current study was to determine the concentration of FIB in particular bathing water at a 'problematic' bathing area. This was measured using membrane filtration plate counts, as per conventional methods, and by seeing whether the use of *Bacteroides* spp. as an alternative FIB gave equivalent results. The results showed that at the site of the case study the conventional FIB (Enterococci, *E. coli*) and *Bacteroides* spp. were present in all water and sediment samples at similar concentrations and that these concentrations complied with the EU bathing water directive (EUBWD), indicating that the bathing water quality was 'good'. Thus, this suggests that *Bacteroides* spp. could be substituted or at least used alongside the current FIB to assess bathing water samples. The use of FIB methods seems suitable for the establishment of bathing water profiles required by the EU bathing water directive (BWD, 2006/ 7/ CE).

Traditional culture-dependent procedures such as membrane filtration are currently used to isolate and enumerate FIB, including faecal Enterococci and *E. coli* in bathing water samples. The membrane filtration method involves filtering the water samples using filters with a standard diameter and pore size, and incubating each filter on selective media. This method varies for different bacteria types, with different media and conditions favouring

particular species of bacteria. At present, the concentrations of FIB as detected by membrane filtration plates counts is an accepted method for regulatory determinations to measure the safety of bathing waters for recreational purposes (Shuval, 2003; Sinigalliano *et al.*, 2010). However, one major limitation of this method is that it is not really possible to use the presence or numbers of these faecal bacteria to determine the source of the pollution.

However, the correlation between FIB and digestive system diseases contracted after bathing water exposure shows that *E. coli* and Enterococci are the best indicators of health risk from fresh recreational water, whilst Enterococci are better indicators in marine water because of their salt tolerance (EPA, 2011; Yau, 2011). Faecal coliforms are known to be poor health indicators but are still used in many countries as primary indicators (EPA, 2011); this is possibly because the associated culturing/ enumeration method is inexpensive and does not need a high level of technical expertise. Limited epidemiological studies conducted in coastal water contaminated by nonpoint source(s) have not shown a significant association between human health and the presence of these indicator bacteria (Boehm *et al.*, 2009; Sinigalliano *et al.*, 2010). In epidemiological investigations of bathing water in the UK, Wyer *et al.* (1999) found a significant relationship between faecal indicator Streptococci concentration and the possibility of developing diseases such as gastroenteritis. These relationships, in addition to other factors such as a framework of the related health standards have been found to be important predictors of gastroenteritis. Nevertheless, the use of these

FIB to monitor recreational bathing waters without knowledge of the sources of pollution is questionable.

Researchers have often detected faecal pollution in water samples, but sediments may also act as a reservoir for faecal bacteria. Consequently, potential pathogens associated with bacterial indicators can be expected to be present in sediment as well as in water. Faecal material from land sources, including waste from wild and livestock animals enter water bodies and also the sediment. Several studies have stated that beach sediments may act as a reservoir for FIB and pathogens, and that they are liberated into the beach water by tides or runoff. Also, the survival or even the growth of these FIB and pathogens may be stimulated by nutrients in beach sediments. The occurrence of both FIB and pathogens in beach sediment provides possible further opportunities for negative human health effects to occur at non-point sources of pollution (Beversdorf *et al.*, 2007; Shah *et al.*, 2011). The level of this sediment pollution has been found to be directly related to the concentration of FIB in the water (Lee *et al.*, 2006b). On the other hand, sediment of the beaches which were studied in this project may act as a reservoir for FIB, and this was confirmed using fluorescence *in situ* hybridisation (FISH). FISH was used purely qualitatively in this study using sediment microcosms to determine the adhesion of *Bacteroides* cells onto particles of sediment. FISH is widely used in qualitative studies than quantitative approaches because the patchy distribution of bacteria (Rahalkar, 2007). However, further quantitative studies may have been possible following further optimization of FISH protocols and the use of

alternative fluorophores to distinguish between *Bacteroides* spp. and the total microbial population.

In all cases in this study, the numbers of traditional FIB and *Bacteroides* spp. were equivalent to each other in both water and sediment, suggesting that the use of *Bacteroides* spp. in a management scheme would be possible. In the current study, high positive correlations were observed between *E. coli* and *Bacteroides* spp. numbers isolated from water and sediment samples, respectively. In urban areas, a large impervious surface can lead to the accumulation of bacteria in sediments resulting from 'runoff' during rainfall. Moreover, prolonged persistence of FIB in sediments which can be subsequently re-suspended in water bodies has been reported (Boehm *et al.*, 2009). The bacterial counts in the water and sediment samples from each site in this study were fairly similar, which may be interpreted as follows. Firstly it could be a cause for concern, if the sediments are acting as a reservoir for bacteria. On the other hand, it may suggest that monitoring water is adequate if this reflects what is found in the sediment.

It is known that high levels of rainfall increase the FIB concentrations found in water (Ellis, 2004), and this was also found in this study. Overall, in the current study, higher levels of FIB were observed during or after rainfall occasions. Water samples should be collected during or 24 hours after of rainfall in order to identify faecal pollution source(s) associated with that event (EPA, 2011). Kay *et al.* (2012a) have stated that a high concentration of FIB in bathing water, particularly following severe rainfall events, may come from agricultural and animal farm lands near the coastal water and from urbanized areas, in the form of rainstorm water or sewer runoffs. Water

systems may be negatively affected in terms of bacterial numbers by such high flow events. It likely that significant variations are observed due to the nature of the catchments, composition of sewage effluents, timing and volume of sewage overflow spills, and sewage treatment efficacy (Stapleton *et al.*, 2009). Given this variety of sources, the need of environmental managers to be able to identify the origin of bacterial pollution is clear.

Additionally, *Bacteroides* spp. was used in this study as a faecal indicator of water and sediment pollution because:

i. it is an additional kind of FIB,
ii. it is found in abundance in the gut of warm-blooded animals, including human beings,
iii. it does not have a long survival rate outside the intestinal environment,
iv. it also can be used as a source of host-specific genetic or biochemical markers to track the faecal source(s) of pollution.

It also meets many of the criteria for selection of FIB to be used in the determination of bathing water quality by molecular methods, which Field (2002) has stated should:

i. be inexpensive, rapid, and reliable,
ii. have no need for culture-dependent isolates,
iii. not need a large amount of library data relating to reference strains,
iv. be flexible for easy field handling and storage, and
v. requires little specialized equipment.

Therefore, there is a clear scope for the use of *Bacteroides* spp. as a new reliable FIB that offers the additional benefits of source tracking.

6.2 Tracking the source(s) of faecal pollution

One of the basic objectives of this study was the application of MST to predict the source(s) of faecal pollution in bathing water. This project has explored these relationships and whether these bacteria are suitable for the MST analysis. It has found that MST methods based on *Bacteroides* spp. are promising tools for the identification of particular sources of faecal pollution.

To achieve an initial understanding about faecal pollution in water systems, FIB have been widely used, but the source(s) of this pollution problem may still not be known. MST methods can be used to provide essential information about possible source(s) of faecal pollution. MST is an approach aimed at identifying faecal source(s) impacting on water systems; it includes both library-independent and library-dependent methods (Field and Samadpour, 2007).

The quality of surface waters in any given catchment is influenced by several nonpoint sources that contribute to the bacterial load and the diversity of faecal indicators (Ahmed *et al.*, 2008a; 2009c). Molecular techniques are now widely used for the MST of pollution in the aquatic environment. Knowing the source of faecal pollution is important for the effective management of coastal bathing waters. The early detection of the source of faecal water pollution helps to increase the appropriate and effective remediation efforts, as well as to reduce cost and time (Bernhard and Field, 2000a; 2000b; Okabe *et al.*, 2007). PCR based on the host-specific *Bacteroides* 16S rRNA genetic marker method proved effective because it does not need a library database and can be performed rapidly. In this study it took only 6-8 hours from sampling to the obtaining the results.

The results of the current study demonstrated that faecal *Bacteroides* spp. have a host-specific distribution and so can be used to track the source of pollution. As this is one of the first studies to have used MST on bathing water in Devon, the method provided a valuable insight into a diverse range of potential inputs of bacterial pollution into bathing water, and established a solid basis for further studies that will support the targeting of host-specific sources. The general *Bacteroides* 16S rRNA genetic marker was detected in all water and sediment samples throughout this study. However, *Bacteroides* genetic marker PCR was able to differentiate between types of *Bacteroides* specific to different source animals, and as such could provide valuable information to water quality managers about possible action which could be taken (Boehm *et al.*, 2009). For example, human faecal pollution was confirmed in the sediment of the stream of South Sands beach using the human-specific *Bacteroides* 16S rRNA genetic marker. Mieszkin *et al.* (2009) and Layton *et al.* (2013) have previously stated that the human *Bacteroides* genetic marker perform well in to detecting faecal pollution from human waste.

In order to identify the source(s) of water pollution by faecal matter, it is necessary to use appropriate PCR primer sets. Whilst a variety of *Bacteroides* 16S rRNA host-specific genetic markers already exists for human, cow, horse and pig, no such highly sensitive primer exists for dog-specific *Bacteroides* spp. This may often be a cause for concern on bathing beaches which ban dogs. Because of a lack of studies about faecal pollution from dog faeces and a high number of dogs (9.5 million) in the UK, unique dog-specific *Bacteroides* 16S rRNA primer sets have been designed. Thus in

this study new dog-specific primer sets for tracking *Bacteroides* from dog faeces were developed. These new primer sets were developed based on PCR and qPCR assays that specifically targeted *Bacteroides* 16S rRNA sequences from dog to discriminate between dog and non-dog *Bacteroides* genetic markers. These were shown to be effective in identifying evidence of dog faeces on bathing beaches whilst none was found in the offshore control water sample. The new dog-specific *Bacteroides* PCR primer sets developed in this study are now available for public use, as dog-specific 16S rRNA *Bacteroides* genetic markers, and due to their high sensitivity and specificity can now be used effectively to track water pollution.

Furthermore, the advantage of applying host-specific indicator bacteria is that these can be studied by researchers to support the quality of other FIB, that have been identified as having possible uses in tracing the source(s) of water pollution (Scott *et al.*, 2002). MST might now best be defined as a semi-quantitative technique. It could enhance the general understanding of dominant faecal pollution sources when used in conjunction with other techniques. Moreover, Stapleton *et al.* (2009) stated that qPCR data relating to 16S rRNA *Bacteroides* genetic markers did not display a stable form of significant associations with FIB concentrations in different environmental samples. However, in this study a good correlation (r= 0.98) was found between *Bacteroides* 16S rRNA genetic marker concentrations and the concentration of culturable *Bacteroides* spp., which demonstrates the value of this approach for a variety of species, now including dog-specific *Bacteroides* genetic markers.

Using conventional PCR and qPCR, *Bacteroides* spp. was detected in water and sediment samples. *Bacteroides* DNA was detected for a longer period at low temperatures, suggesting a relationship between DNA degradation rate and environmental temperature. If *Bacteroides* 16S rRNA genetic markers are to become a standard indicator of faecal pollution and its sources, their survival and persistence should be studied in a variety of environmental conditions. Walters *et al.* (2009) have stated that pathogenic microbes behave differently in aquatic environments, thus leading to difficulty and confusion in health risk evaluations. MST cannot truly be considered quantitative until survival and persistence ratios of host specific genetic markers in the environment can be tested relative to the other host-specific genetic markers, faecal indicators and pathogenic microbes and also in response to waste treatment (Field and Samadpour, 2007).

Faecal indicators should exhibit survival and persistence profiles close to those of pathogenic microbes and be associated with the presence of pathogens. The use of *Bacteroides* species as faecal indicators requires the survival and persistence of their 16S rRNA genetic markers in environmental water and sediment to be known. Walters *et al.* (2009) have specified that host-specific *Bacteroides* 16S rRNA genetic markers are used as indicators of recent faecal pollution, but that their survival and persistence in environmental samples is variable. The survival and persistence profiles of *Bacteroides* genetic markers are comparable with persistence profiles for several faecal pathogens.

6.3 Implications for management of water pollution

A number of compulsory processes have been applied in developed countries to provide safe coastal bathing water, including guidelines, directives and legislation for acceptable microbiological quality in order to reduce cases of waterborne disease. The duties relating to the management of water pollution assume the acquisition of information that enables the identification, assessment and prioritising of potential water pollution problems. However, assigning priorities, such as according to the a risk to human health and/ or economic losses, is difficult (Larsen and Ipsen, 1997). Water quality management has focused on detecting and enumerating faecal water pollution based on FIB analysis results (Kinzelman and McPhail, 2012). Existing monitoring guidelines are based on epidemiological studies at sites affected by sewage pollution, which increase the risk and spread of infectious diseases between bathers (Shuval, 2003; Sinigalliano *et al.*, 2010).

European Union countries have a wide diversity of beaches and coastal bathing zones, and every year millions of tourists spend holidays at their local beaches. According to the European environment agency (EEA), in the UK in 2012, 93.6% from the total number (617) of coastal bathing beaches achieved the minimum quality standards established by the EU directives. 5.8 % were classified as poor bathing water quality and only 0.5 % failed or had insufficient sampling (EEA, 2013). In EU countries, water quality standards are often more severe than developing countries. Wide and strong monitoring programmes have applied on EU beaches for many years, and regular raw data has also been collected. Bertram and Rehdanz (2013) have stated that coastal ecosystems are considered an important site for human

‘entertainment’. An estimation by Pond (2005) observed that in the UK over 20 million people use the UK coastal water and beaches each year, in addition to inland waters and their surrounding areas.

The current EU legislation for bathing waters was approved in 2006, and will be completely in force by the end of 2014. It requires the creation of bathing water profiles, and the assessment of any potential impacts and threats to the water quality. These act as a basis of information for people and as a management tool for the relevant authorities. A monitoring schedule has to be established for every beach before the start of the bathing season. It sets fixed dates for the collection of water samples. Extra water samples may be taken, such as those collected to confirm water quality after rainfall or on the basis of notices from users (EEA, 2013).

Water quality may be a greater problem in developing countries because of the minimal monitoring, often poor sewage treatment, and limited tracking of water pollution. The improvement of water management systems requires world-wide intervention, not only in developed countries. However, differences are also found from country to country (Larsen and Ipsen, 1997). Good management to keep up water quality needs to be applied to water systems throughout the world. The important criteria include; safe, pathogen-free water systems, with consistent quality standards, as well as the identification of potential source(s) of pollution within watershed areas. Furthermore, environmental protection agencies (EPA) seek to make a complete plan for the improvement and conservation of all water resources as they supply standards for water quality. Classifications of water quality should also be established, as a useful link between water quality and

potential uses, as stated by environmental solutions Antigua limited (ESAL, 2008). This being said, in bathing waters, transmission of pathogens among users may occur directly, or contaminated bathing water and sediment may act as a vector of infection. The risk to human health arises from bathing water coming into contact with animal waste, including that from dogs. Notably, the regulations should place an obligation upon a dogs' owners to remove dog waste according to the report of World Health Organization (WHO, 2003).

More studies are needed in developing countries to determine the levels of faecal pollution and to discover the sources of this problem. In this regard, it is necessary to monitor the quality of water bodies and collate data from time to time to minimize such concerns. Mandatory water quality strategies should be established for all bathing waters around the world, especially water bodies located within watershed areas in developing countries. These guidelines are needed to maintain and, where necessary, to improve water quality so as to protect public health.

In relation to runoff from agricultural land into water systems, it is recommended that appropriate and effective best management is supported by periodical monitoring programmes (ESAL, 2008). This study provides information that could help the EA in the UK as well as in other EU countries to minimize the risk of water pollution such as:

i. The ease of recovery of FIB in water and sediment were fairly equivalent, which suggests that either water or sediment could be used to indicate faecal pollution in bathing beaches.

ii. The use of *Bacteroides* spp. could be utilized as faecal indicators for water pollution, because it has many useful characteristics as previously described. *Bacteroides* spp. can also be utilized to identify the source(s) of pollution and this assists the EPA in the prediction of potential risks that may result from the introduction of faecal waste into bathing waters from various sources.

iii. A high priority of environmental agencies is to distinguish and alleviate bacterial pollution in water systems. The source(s) of bacterial pollution can be tracked using host-specific genetic markers for FIB with conventional PCR and qPCR methods, as shown in this study. Environmental agencies should develop long-term molecular methods-based monitoring programmes for water quality to protect human health from the potential risks of water pollution (Sauer *et al.*, 2011).

6.4 Summary, conclusions and future work

The aim and objectives of this study have been achieved. Firstly, the quality of beach water on South Sands was monitored using the culture-dependent EU method and faecal pollution sourced using host-specific 16S rRNA *Bacteroides* genetic markers. Secondly, new dog-specific *Bacteroides* PCR and qPCR primer sets have been designed and successfully applied to water and sediment samples which were collected from beaches on Bigbury-on-Sea. Thirdly, the survival and persistence of *Bacteroides* and genetic markers have been investigated using small scale laboratory microcosms. However, several points still need further work to clarify the most appropriate method for accurately determining bathing water quality. MST methods

provide multiple opportunities to gather more in depth information about the exact nature of faecal pollution at a particular site, but a full understanding of their potential problems is in consequence far from complete. The continued development of techniques to discriminate between different sources of faecal pollution is therefore important for advancing the utility of the approach. Moreover, the effectiveness of sewage treatment in the reduction of bacterial diversity needs further research, so that management strategies based on the information in the MST database can lower faecal microorganism concentrations. In future applications, a suite of host-specific *Bacteroides* genetic markers could be design with a view to identify the source(s) of problem bacteria found on failing beaches. This study suggests various potential future projects to improve some aspects of the use of *Bacteroides* spp. and their markers as a tool in the MST method, such as:

i. *Bacteroides* spp. could be applied more widely as a faecal indicator for water pollution in both water and sediment. More host-specific *Bacteroides* primers for other sources such as sheep, duck, deer, cat etc. should be designed and applied to environmental water and sediment.

ii. *Bacteroides* spp. could also enable the determination of the source of faecal pollution, which may then facilitate work to remediate the problem of faecal pollution in a wide range of environments including drinking water and bathing beaches. In addition, this study recommends the involvement of *Bacteroides* spp. in future EU bathing water directives or other environmental legislation across the world as a faecal indicator for recent bacterial pollution.

iii. The effect of temperature and salinity on the survival and persistence of *Bacteroides* species and their genetic markers in environmental water has been studied here. However, further studies are needed in order to find out the influence of these factors and others, such as sunlight, lack of nutrients, ratio of oxygen, and pH, on survival of these bacteria in environmental waters and sediments, also compare nutrients to pathogens survival. A comparative microcosm study including *E. coli* (or Enterococci) could also be investigated.

iv. According to other studies (Tyagi *et al.*, 2006; Ahmed *et al.*, 2008b; 2009a), the correlation between the current FIB and the presence of pathogenic microbes such as enteric viruses and protozoa is not fully understood. Future work needs to determine this correlation between FIB and diverse pathogens which are found in environmental samples.

v. Another modern and accurate method which will be used in molecular source tracking to detect a whole diversity of bacterial water pollutions is pyrosequencing or next generation sequencing (NGS). Many current and potential applications use this technique. NGS could be used to investigate the community structure, temporal stability and major operational taxonomic units (OTUs) of bacterial diversity in bathing water and sediment samples (Navarro-Noya *et al.*, 2013; Zhu *et al.*, 2013). The original plan for this study included the use of this method to detect the whole community of microorganisms in the tested bathing water and beach sediment. However, budget and time constraints meant that this was not a viable approach.

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Appendix One
Publication, posters, accession numbers and additional Figures and Tables

Hydrology
Current Research

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http://dx.doi.org/10.4172/2157-7587.1000163

Research Article Open Access

Development and use of *Bacteroides* 16S rRNA Polymerase Chain Reaction Assay for Source Tracking Dog Faecal Pollution in Bathing Waters

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Abstract

Faecal pollution on bathing beaches poses a potential threat to human health and as a result may also negatively affect the local economy. In instances where the source of such pollution is not obvious, it may be necessary to track such sources using a host-specific genetic markers technique. *Bacteroides* species are potential indicators for source tracking of faecal pollution in bathing waters. This study designed specific primer sets to amplify sections of the 16S rRNA gene unique to *Bacteroides* from domestic dogs and used quantitative PCR (qPCR) to quantify such genetic markers in environmental samples. The sensitivity and specificity of the primer sets was determined; they were specific *in silico* against known dog *Bacteroides* sequences and *in vitro* against *Bacteroides* sequences originating from human and livestock faeces. Dog faecal *Bacteroides* contamination was then detected in sea water during the bathing season at a local beach where dogs are banned during the summer months, in spite of the fact that these waters had met EU directive standards based on the culture-based enumeration of faecal indicator bacteria. Quantitative PCR was used to determine the limit of detection (LOD) of the dog *Bacteroides* genetic markers in these water samples. The copy number of dog *Bacteroides* genetic markers in the water was low and the LOD of those markers was 4 copies per reaction. The use of these dog primers has the potential to supply important additional information when source tracking faecal pollution at bathing beaches and maintaining water quality.

Keywords: 16S rRNA marker; Dog-specific *Bacteroides* primer; Bathing water pollution

Introduction

Faecal indicator bacteria (FIB) such as Escherichia coli (E. coli) and Enterococci are currently used to determine faecal bathing water pollution; they are found in a variety of warm-blooded animals and are not unique to the intestinal flora of humans [1]. Determining the exact sources of faecal pollution is now of critical importance when attempting to comply with the EU bathing water directive 2006 [2]. Bacteria belonging to the genus *Bacteroides* are now used as additional source-tracking indicator bacteria, since they constitute a major part of the faecal bacterial population; as strict anaerobes they have little potential for growth in bathing waters and have a high degree of host specificity [3, 4]. Non-culture based, *Bacteroides*-based tracking methodologies are designed to target specific sequences within the *Bacteroides* 16S rRNA gene in order to differentiate human-derived contamination from that of other animals [5, 6]. The most commonly used tools for such studies are conventional PCR-based analysis [7] and quantitative PCR (qPCR) [8]. Coastal waters are frequently used for a variety of recreational and commercial activities. Faecal pollution may thus arise not only from human sources but also from farm livestock and other animals, which may contribute additional pathogens to bathing waters, including viruses and bacteria [9]. In urban areas there are many sources that may lead to the contamination of water supplies, such as urban runoff and negligent waste management, as well as discharge from domestic pets; these represent important potential sources of faecal pollution in aquatic systems [10-12]. In developed countries, the populations of domestic dogs (*canis lupus familiaris*) have grown significantly over the last two decades [13]. For example, according to a public survey the number of dogs in the UK is about 9.4 million [14]. Dog faeces that are not correctly disposed of can be washed directly by surface runoff into water systems. Moreover, dog faecal pollution poses a possible threat to public health because of the potential transmission to humans of zoonotic microbes [15-17]; such microbes can inhabit apparently healthy domestic dogs [12, 18, 19]. Dogs are now banned from various UK bathing beaches during the bathing season but there is currently no simple and/or inexpensive method for source-tracking faecal pollution from dogs on beaches and thus accurately assessing the actual effectiveness of such bans is difficult. Kildare et al. [4] have previously designed TaqMan® labelled assays to quantify dog-specific *Bacteroides*. In the current study, the authors report the development and use of specific and sensitive conventional PCR primer sets and qPCR assays based on SYBR® green fluorescent binding dye. Thus, this study aimed to design and test host-specific PCR primer sets to amplify a section of the 16S rRNA gene unique to *Bacteroides* spp. originating from dog faeces and to further determine the specificity and the sensitivity of these markers in bathing waters from two UK beaches with differing seasonal dog bans.

Materials and methods

Sampling and DNA extraction

Fifty eight faecal samples (10 dogs, 12 cows, eight horses, four pigs, eight sheep, four deer, two cats and six ducks) were collected from local sources in Devon, UK and four human faecal samples were obtained from adult volunteers. DNA was extracted from faecal samples (200 mg) using a QIA ampstool DNA mini kit (Qiagen, UK) according to

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AN EVALUATION OF BACTERIAL SOURCE TRACKING OF FAECAL BATHING WATER POLLUTION IN THE KINGSBRIDGE ESTUARY, UK

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1 INTRODUCTION

Vinten *et al.* [1] referred to certain areas of the UK such as South Wales, North Yorkshire and South West Scotland which have problems with bathing water compliance. Areas of intensive dairy farming with a high cattle concentration, cool, humid summers are prone to bathing water contamination [2]. Farm areas and cattle walkways are highly susceptible to direct runoff of microbial contaminated water to streams, and there is widespread direct use of streams to supply drinking water to stock in summer. Heavy rainfall can exacerbate these inputs from agricultural land and can also cause problems with sewage treatment systems, which if overloaded, may resort to the use of storm sewers overflows, discharging sewage and rainfall largely untreated[3]. Contamination of water systems can cause a higher risk to bathers, as well as economic losses as a result of closed beaches and shellfish harvesting areas[2, 4]. Faecal indicator bacteria (FIB) are commonly used to determine water contamination of public health significance [5]. The identification and enumeration of indicator bacteria has several advantages. However, these methods fail to detect the source of faecal contamination[4]. Detection of the source is a pre-requisite for the effective and efficient management of these aquatic environments. It also reduces the time and cost of implementing remedial measures[6]. The intestines of warm-blooded animals contain abundant indicator bacteria, and their presence in environmental waters indicates faecal contamination, including the potential presence of pathogenic micro-organisms[7]. Van Asperen *et al.* [8] identified the quality of water which is necessary to protect bathers from illness and the key feature is the concentration of faecal contamination in the water. The EU Bathing Water Directives 2006/7/EC are mainly focused on the protection of the health of bathers and those involved in coastal recreation[5, 9-11]. In the UK normally the bathing season starts from 15 May and ends on 30 September [12, 13]. The objectives of this study were to monitor FIB (Enterococci and *E. coli*) and *Bacteroides spp.* in and out of the bathing season at a site of known problems noting the trends and to evaluate *Bacteroides* PCR-based tracking to source human faecal contamination. Sediment was also monitored as a possible reservoir of water contamination.

Bacteroides spp. enrichment culture clone KH101 16S ribosomal RNA gene, partial sequence

GenBank: **JX431865.1**

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REFERENCE   1(bases 1 to 379)
  AUTHORS   Hussein,K.R., Nisr,R.B., Glegg,G. and Bradley,G.
TITLE       Polymerase Chain Reaction (PCR) for the detection of
            Bacteroides spp. specifically from dogs
  JOURNAL   Unpublished
REFERENCE   2(bases 1 to 379)
  AUTHORS   Hussein,K.R., Nisr,R.B., Glegg,G. and Bradley,G.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-MAY-2012) Biomedical and Biological
            Sciences,Plymouth University, Drake Circus, Plymouth,
            Devon PL4 8AA, United Kingdom
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61 attaggtagt aggcggggta acggcccacc tagccatcga tggatagggg ttctgagagg
121aaggtccccc acattggaac tgagacacgg tccaaactcc tacgggaggc agcagtgagg
181aatattggtc aatgggcgcg agcctgaacc agccaagtag cgtgaaggat gactgcccta
241tgggttgtaa acttcttttg tccgggaata aaaccgccta cgtgtaggcg cttgtatgta
301ccggtacgaa taagcatcgg ctaactccgt gccagcagcc gcggtaatac ggaggatgcg
361agcgttatcc ggatttatt
```

Bacteroides sp. enrichment culture clone **KH101** 16S ribosomal RNA gene, partial sequence
Sequence ID: gb|JX431865.1|Length: 379Number of Matches: 1 Related Information
Range 1: 1 to 379GenBankGraphics

Score	Expect	Identities	Gaps	Strand	Frame
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Features:

```
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      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Q61ATTAGGTAGTAGGCGGGGTAACGGCCCACCTAGCCATCGATGGATAGGGGTTCTGAGAGG 120
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S61ATTAGGTAGTAGGCGGGGTAACGGCCCACCTAGCCATCGATGGATAGGGGTTCTGAGAGG 120

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Bacteroides spp. enrichment culture clone KH102 16S ribosomal RNA gene, partial sequence

GenBank: **JX431866.1**

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ACCESSION JX431866
VERSION JX431866.1 GI:399139984
KEYWORDS ENV.
SOURCE *Bacteroides* sp. enrichment culture clone KH102
ORGANISM Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; *Bacteroides*; environmental samples.
REFERENCE 1(bases 1 to 572)
AUTHORS Hussein,K.R., Nisr,R.B., Glegg,G. and Bradley,G.
TITLE Polymerase Chain Reaction (PCR) for the detection of *Bacteroides* spp. specifically from dogs
JOURNAL Unpublished
REFERENCE 2(bases 1 to 572)
AUTHORS Hussein,K.R., Nisr,R.B., Glegg,G. and Bradley,G.
TITLE Direct Submission
JOURNAL Submitted (22-MAY-2012) Biomedical and Biological Sciences,Plymouth University, Drake Circus, Plymouth, Devon PL4 8AA,United Kingdom
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Bacteroides sp. enrichment culture clone **KH102** 16S ribosomal RNA gene, partial sequence
Sequence ID: gb|JX431866.1|Length: 572Number of Matches: 1
Related Information Range 1: 61 to 439GenBankGraphics

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Uncultured *Bacteroides* sp. clone KH103 16S ribosomal RNA gene, partial sequence

GenBank: **JX431867.1**

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DEFINITION  Uncultured Bacteroides sp. clone KH103 16S ribosomal RNA
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ACCESSION   JX431867
VERSION     JX431867.1  GI:399139985
KEYWORDS    ENV.
SOURCE      uncultured Bacteroides sp.
            Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales;
            Bacteroidaceae; Bacteroides; environmental samples.
REFERENCE   1(bases 1 to 639)
  AUTHORS   Hussein,K.R., Nisr,R.B., Glegg,G. and Bradley,G.
TITLE     Polymerase Chain Reaction (PCR) for the detection of
          Bacteroides spp. specifically from dogs
  JOURNAL   Unpublished
REFERENCE   2(bases 1 to 639)
  AUTHORS   Hussein,K.R., Nisr,R.B., Glegg,G. and Bradley,G.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-MAY-2012) Biomedical and Biological
            Sciences,Plymouth University, Drake Circus, Plymouth,
            Devon PL4 8AA,United Kingdom
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Uncultured *Bacteroides* sp. clone **KH103** 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|JX431867.1|Length: 639Number of Matches: 1Related Information

Range 1: 113 to 491GenBankGraphics

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Features:

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[A]:Query; [b]: subject

Data relevant for Chapters 4 and 5, designed of dog-specific *Bacteroides* PCR primer sets

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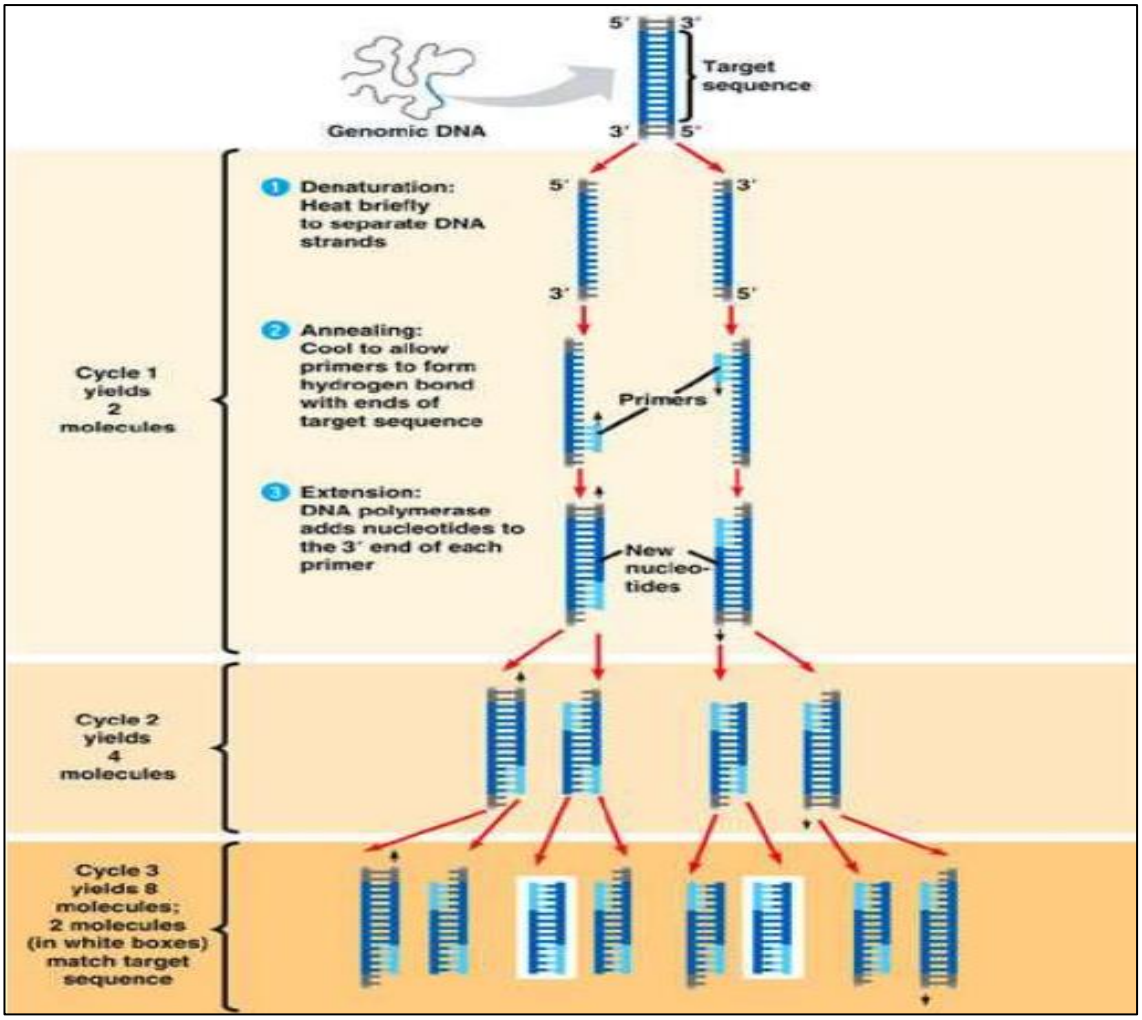

Figure 1 Schematic diagram illustrates PCR steps (Chapter 1), adapted from Domingo (2000).

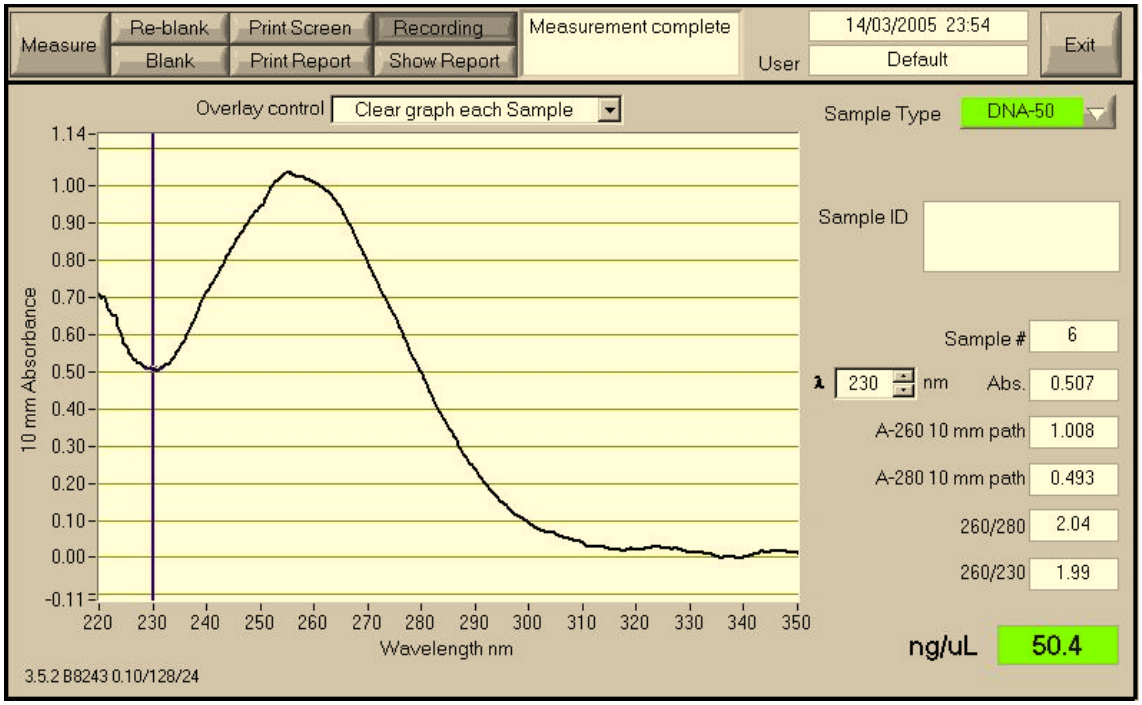

Figure 2 The concentration (ng μl^{-1}) and quality (A_{260}/ A_{280} and A_{260}/ A_{230}) of DNA product was measured by NanoDrop® UV spectrophotometer (Chapter 2).

The below Figures related to chapter 4 and 5

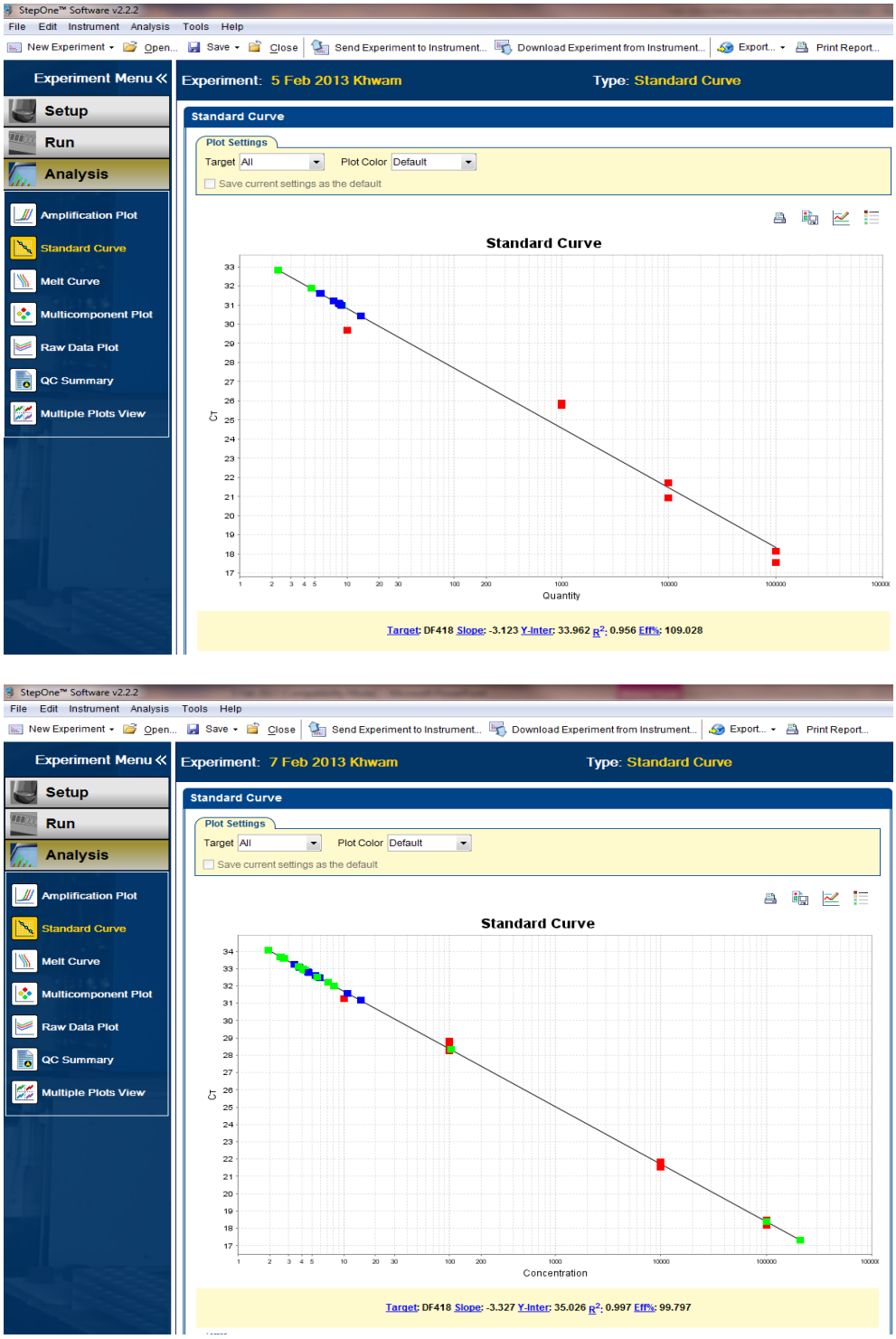

Figure 3 Example of standard curves obtained from qPCR runs

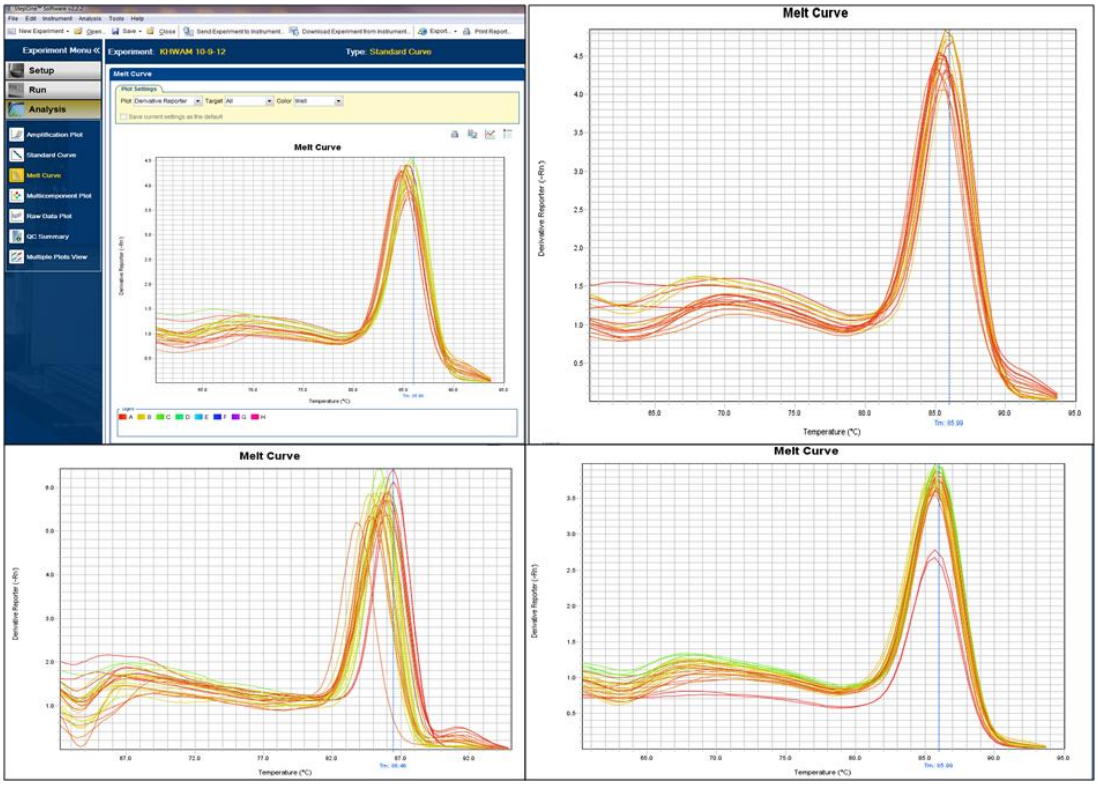

Figure 4 Example of melting curves obtained from qPCR runs.

Table 1 Limit of detection of *Bacteroides* genetic markers determined using qPCR by other authors (Chapter 4).

Author(s)	copy numbers per reaction
Stapleton *et al.* (2009)	Less than 20
Hospodsky *et al.* (2010)	5
Ahmed *et al.* (2010)	46
Labrenz *et al.* (2004)	20
Reischer *et al.* (2006)	6 to 20
Schwartz *et al.* (2003)	130
Okabe *et al.* (2007)	50 to 800
Savill *et al.* (2001)	1 to 60
Seurinck *et al.* (2005)	50 to 106
Yampara-Iquise *et al.* (2008)	9.3
Mieszkin *et al.* (2010)	1.6
Kildare *et al.* (2007)	2 to 3.7
Dick and Field (2004)	10
Nadkarni *et al.* (2002)	4.8 to 48
Sarma-Rupavtarm *et al.* (2004)	5
Ryu *et al.* (2012)	21
Sivaganesan *et al.* (2008)	100
Sauer *et al.* (2011)	15
Layton *et al.* (2013)	9.3 to 280
Mygind *et al.* (2002)	1
Lindecrona *et al.* (2002)	1
Schriewer *et al.* (2013)	57.5 to 381

Table 2 The decline rates CFU per day of *Bacteroides* spp. in the water and sediment microcosms (Chapter 5).

Day	River 10 °C		Sea 10 °C		River 17 °C		Sea 17 °C	
%	water	sediment	water	sediment	water	sediment	water	sediment
0	12108	8150	12010	7980	11880	8120	12040	7870
%	100	100	100	100	100	100	100	100
2	11490	8040	11720	7890	11696	8090	11860	7780
%	96.5	98	98	99	98	99.6	98.5	98.8
4	11190	7910	11370	7796	11375	7986	11566	7674
%	93	97	94.6	97.6	95.7	98	96	97.7
6	10820	7868	11030	7602	10422	7794	11024	7566
%	90.3	96.5	91.8	92.2	87.7	95.9	91.6	96
8	6658	7218	7026	7428	5134	7269	5066	7224
%	55.5	89.5	58.5	93	43.2	89.9	42	90.3
10	3879	5276	3822	4580	2882	4873	3027	3989
%	32.4	64.7	31.8	57.3	24.2	60	25.2	50.6
12	1980	2027	1312	1796	410	1510	198	1260
%	16.5	24.8	11.1	22.5	3.45	18.5	1.64	16
14	180	868	308	960	14	647	12	398
%	1.5	10.6	2.5	12	0.11	7.9	0.09	5
16	0	30	0	27	0	0	0	0
%	0	0.36	0	0.35	0	0	0	0
17	0	0	0	0	0	0	0	0
%	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0

Appendix Two
Molecular media and reagents

1 Luria-Bertani (LB) broth (1 l)

- NaCl 10 g
- Tryptone 10 g
- Yeast extracts 5 g

dH_2O was added to a final volume of one litre, pH adjusted to 7, then sterilised by autoclaving, ampicillin added to a final concentration 100 µg ml^{-1}

2 Luria Bertani agar (LB, 1 l)

- NaCl 10 g
- Tryptone 10 g
- Yeast extracts 5 g
- Agar 15 g

The above ingredients were dissolved in one litre of distilled water, pH adjusted to 7, autoclaved then chilled at room temperature to approximately 50 °C. Ampicillin (100 µg ml^{-1}), X-gal (80 µg ml^{-1}) and IPTG (0.5 mM) were added, and poured into the Petri dish to a depth of 10 mm of thickness, left to set, then stored at 4°C.

3 Lysis solution

- Tris/ Cl pH 8 50 mM
- Ethylenediaminetetraacetic acid (EDTA) 25 mM
- Sodium dodecyl sulfate (SDS) 3 %
- Polyvinylpyrrolidone (PVP) 1.2 %

4 Extraction solution

- Tris/ Cl, pH 8 10 mM
- EDTA 1 mM
- Na acetate 0.3 M
- PVP 1.2 % w/ v

5 X-gal solution

- 5-bromo-4-chloro-3-indolyl-β-D-galactoside 250 mg
- Indimethylformamide (DMF) 1 ml

X-gal was prepared as stock, sterilised by filtration and stored at 4 ºC.

6 IPTG solution

- Isopropyl-β-thiogalctopyranoside 0.5 M

The solution was sterilised by filtration and stored at 4 ºC.

7 Guanidine thiocyanate buffer (GITC)

- Guanidine Isothiocyanate 5 M
- EDTA (pH 8.0) 100 mM
- Sodium lauroyl sarcosinate 0.5 %

8 TE buffer

- Tris-Cl, pH 7.5 10 mM 10 ml
- EDTA 1mM 2 ml

9 AE buffer

- Sodium acetate (pH 5.2) 50 mM
- EDTA (pH 8) 10 mM

10 Louding buffer

- Glycerol 40 %
- Bromophenol Blue 0.25 %
- Xylene cyanol 0.25 %

11 TLE lysis buffer

- Tris/ Cl 10 mM
- EDTA 0.1 mM
  The pH was adjusted to pH 8

12 Tris-acetate-EDTA 10× (TAE) buffer

- Tris-base 0.4 M
- EDTA 0.5 M
- Acetic acid 1 M

The pH was adjusted to 8.5 and then diluted to make 1x TAE buffer.

13 Hybridization buffer (1 ml)

- NaCl 5 M 180 µl
- Tris/ HCL 1 M pH 8 20 µl
- Sodium Dodecyl Sulphate (SDS, 10 %) 1 µl
- ddH_2O 799 µl
- Formamide (varied 0 % or 35 %)

14 Washing buffer (50 ml)

- Tris buffer 1M pH 8.0 1 ml
- NaCl5 M 9 ml
- ddH_2O to 50 ml

15 Phosphate-buffered saline (PBS, 1 l)

- NaCl 8 g
- KCl 0.2 g
- Na_2HPO_4 1.44 g
- KH_2PO_4 0.24 g

These materials were dissolved in 800 ml of distilled water and adjusted the pH to 7.4, the volume completed up to one litter, then sterilized by autoclave.

16 Kovac's reagent

- Paradimethylamino-benzaldehyde 5 g
- Concentrated HCl 25 ml
- Amyl alcohol 75 ml

The reagent was stored in a screw-cap bottle at 4 ºC

Appendix Three
Ingredients and preparation of culture media

1 *Bacteroides* bile esculin agar/ 1 l

- Bile Salt Ox gall 20 g
- Pancreatic Digested Casein 15 g
- Peptic digests of soybean meal 5 g
- NaCl 5 g
- Esculin Hydrate 1 g
- Ferric ammonium citrate 0.5 g
- Hemin 0.0125 g
- Vitamin K solution (10 mM) 1 ml
- Bacteriological agar 15 g

Distilled water was added to a final volume of one litre, pH Adjusted to 7, sterilized by autoclaving for 15 minutes at 15 psi, and then added:

- Gentamicin 0.4 g 10 ml^{-1} dH_2O 2.5 ml
- Hemin 0.5 g 10ml^{-1} dH_2O 2.5 ml

2 *Bacteorides* phage recovery medium/ 1 l

- Peptone 10 g
- Tryptone 10 g
- Sodium chloride 5 g
- Yeast extract 2 g
- L-Cysteine monohydrate 0.5 g
- Magnesium sulfate heptahydrate 0.12 g
- Calcium chloride anhydrate 0.05 g

Distilled water 955 ml was added, pH adjusted to 7, sterilized by autoclaving for 15 minutes at 15 psi, then, to 95 ml broth aseptically added:

- Glucose 1M 1 ml
- Disodium Carbonate 1M 2.5 ml
- Hemin 1 % in 0.01 % NaOH 1 ml

3 Slanetz and Bartley medium/1 l

- Tryptose 20 g
- Yeast Extract 5 g
- Di-potassium hydrogen Phosphate 4 g
- Sodium Azide 0.4 g
- Glucose 2 g
- Tetrazolium Chloride 0.1 g
- Bacteriological agar 10 g

The ingredients were dissolved in one litre of distilled water by boiling, excessive heating avoided.

4 Membrane lauryl sulphate broth/ 1 l

- Peptone 39 g
- Yeast extract 6 g
- Lactose 30 g
- Phenol red 0.2 g
- Sodium lauryl sulphate 1 g

The pH was adjusted to 7.4, then distributed into final containers (100 ml), sterilised by autoclave at 121°C for 15 minutes at 15 psi.

5 Lauryl tryptose sulphate broth/ 1 l

- Tryptose 20 g
- Lactose 5 g

- Sodium chloride 5 g
- Dipotassium hydrogen phosphate 2.75 g
- Potassium dihydrogen phosphate 2.75 g
- Sodium lauryl sulphate 0.1 g

In one litre, dH_2O the ingredients were dissolved and distributed into small tubes with Durham tubes, sterilised by autoclave at 121°C for 15 minutes at 15 psi.

6 Brain heart infusion broth/ 1 l

- Beef heart infusion solids 17.5 g
- Proteose peptone 10 g
- Glucose 2 g
- Sodium chloride 5 g
- Di-sodium phosphate 2.5 g

The ingredients were dissolved in one litre dH_2O, sterilised by autoclave at 121°C for 15 minutes at 15 psi.

7 Rapid sodium chloride medium

- Brain heart infusion agar 52 g
- Dextrose 10 g
- Sodium chloride 60 g
- Bromocresol purple 20 g

In one litre volume, the above ingredients were dissolved by boiling and pH adjusted to 7. This medium was distributed (15 ml) into 100 ml screw-cap bottles, autoclaved at 121°C for 15 minutes at 15 psi, and allowed to cool in slant.

Appendix Four
Training courses, conferences and taught sessions attended

1 Courses and workshops

- English language summer school (intensive course), academic writing, 1st October 2009 to 30th March 2010. Plymouth University, UK.
- Postgraduate research skills and methods in biology (BIO 5124), October 2010 to January 2011. Plymouth University, UK.
- Laboratory based teaching methods and practices (ENV 5101), October 2010 to January 2011. Plymouth University, UK.
- General teaching associates course (GTAC), 27th January to 3rd March 2011. Plymouth University, UK.
- Geographic information system (GIS): Users workshop, Plymouth University, 8th December 2011.
- Real-time PCR training course, Dartington Hall Conference Centre, Totnes, Exeter University, UK. 23rd November 2011.
- European centre for environment and human health, 2nd south west microbiology group meeting (ECEHH), Truro, Cornwall, Plymouth University, UK. 24th January 2012.
- Getting the most from conference workshop, Plymouth University, UK. 30th March 2012.
- Training for better ergonomics, safety and pipetting results workshop, *Alfa* laboratories, Plymouth University, UK. 3rd May 2012.
- Molecular biology workshop, school of biomedical and biological sciences, Plymouth University, UK. 16th- 19th July 2012.
- Molecular ecology bioinformatics workshop for microbiologists part 1, Plymouth University, UK. 7th November 2012.

- Molecular ecology bioinformatics workshop for microbiologists part 2, Plymouth University, UK. 21st November 2012.
- Geographic information system (GIS): Users workshop, Plymouth University, 6th December 2012.
- Hands on genomics and biology techniques (PCR, qPCR bioinformatics data analysis), Plymouth University, UK. 26th -28th June 2013.
- Hands on proteomics workshop (protein structure, extraction and quantification, Western Blot, ELISA, 2D gel electrophoresis, mass spectrometry and data analysis), Plymouth University, UK. 1st - 3rd July 2013.
- Supporting English language classes (tutorial for academic writing), 1st September to 15th December 2013. Plymouth University, UK.

2 Taught sessions

- Inducted day for development of academic research. 27th April 2010.
- My research: Owning and using works. 6th May 2010.
- Preparing effective poster presentation. 10th May 2010.
- Information resources. 11th May 2010.
- Endnote session. 20th May 2010.
- International students' conference. 28th May 2010.
- Introduction of Endnote ×3. 9th June 2010.
- Preparing to transfer. 24th June 2010.
- Microsoft Excel 2007 introduction to essential features. 28th September 2010.
- SPSS session. 2nd November 2010.

- Introducing my Site. 4th November 2010
- Online collaboration services web conferencing. 11th November 2010.
- Microsoft PowerPoint creating presentation 2007. 15th November 2010.
- Introduction to R. 26th November 2010.
- Microsoft Word 2007 structuring your Thesis. 30th November 2010.
- Creating Graphic for Paint shop Pro Photo X2. 10th December 2010.
- SPSS session. 11th February 2011.
- Presentation to an audience part 1. 9th March 2011.
- LaTex introduction. 17th March 2011.
- Excel 2010 training course. 2nd November 2011.
- Conditional forming and chart Excel 2010. 29th February 2012.
- Research owning and using works. 17th January 2013.
- Preparing for your viva, 4th March 2014.

3 Conference registration

- The postgraduate society conference. Plymouth University, UK. 17th March 2011.
- International student conference, Plymouth University, UK. 27th May 2011.
- The postgraduate society conference. Plymouth University, UK. 29th June 2011.
- The postgraduate society annual conference. Plymouth University, UK. 26th June 2012.

- Ecotoxicology research and innovation centre, “Building international collaboration in environmental toxicology and chemistry”, 2nd annual meeting 2012, Plymouth University, UK. 13th July 2012.
- Marine institute annual research centre conference, Plymouth University, UK. 7th June 2012.
- Centre for research in translational biomedicine, (CRTB conference) annual research day. Plymouth University. UK, 5th April 2011.
- The postgraduate society annual conference, Plymouth University, UK. 26th June 2012.
- Riding the storm-storms surges and coastal ecosystems. Plymouth University, 30th September 2013.
- The postgraduate society conference, Plymouth University, UK. 21st November 2012.
- Annual marine conference, Plymouth University, UK. 4th June 2013.
- The postgraduate society annual conference, Plymouth University, UK. 27th November 2013.

4 Awards

- Travelling student grant ($ 500), American Society of Microbiology (ASM), San Francisco, USA. 16th- 19th June 2012.
- Travelling student grant (£ 315), Society of Applied Microbiology (SfAM), Edinburgh, UK. 2nd-5th July 2012.
- President’s fund grant (£ 676), Society of Applied Microbiology (SfAM), London to attend the V BioMicroWorld 2013 conference, Madrid, Spain.