Bacteriophages as surrogates of viral pathogens: A novel tool for the shellfisheries industry

Adewale Oluwasogo Olalemi

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School of Environment and Technology
The University of Brighton
United Kingdom

Abstract

Shellfish are filter-feeding aquatic animals that can bioaccumulate pathogens from contaminated water. Often, the sanitary quality of shellfish and their harvesting waters may meet national and international standards for faecal indicator bacteria (FIB) but still contain pathogenic enteric viruses at an infectious dose for humans, thereby posing a potential risk to the health of consumers. Currently, there are no standards or guidelines for acceptable levels of enteric viral pathogens in shellfish in Europe or elsewhere and the lack of affordable and reliable methods make this unlikely in the foreseeable future. This study focuses on the potential application of a novel low-cost surrogate approach to predicting and managing the risk of human viral disease among human consumers of shellfish. Specifically, the use of bacteriophages as indicators of human enteric viruses in shellfish and their harvesting waters have been investigated with the ultimate aim of offering an important new tool for public health protection.

The study investigated pathogen and phage ecology in shellfish from southern England, United Kingdom. Simple, low-cost culture-based methods and more advanced culture-independent genetic detection methods were used to monitor the sanitary quality of shellfish species (mussels and oysters) and their harvesting waters. Physicochemical properties of the shellfish-growing water as well as other hydrological and meteorological data were recorded and their influences on the behavioural dynamics of target indicators of faecal contamination were critically evaluated. Importantly, levels of FIB, proposed viral indicators (bacteriophages) and enteric viral pathogens were analysed in shellfish species and their harvesting waters. In addition, the rate of uptake, bioaccumulation and persistence of the faecal bacteria and phages in mussels and oysters tissue and intravalvular fluids were investigated under controlled conditions.

The results demonstrated elevated levels of FIB, phages and enteric viral pathogens in shellfish compared with their harvesting waters. Levels of FIB and phages showed a positive relationship with rainfall, river flow and turbidity, and an inverse relationship with temperature and salinity. The GB124 phages demonstrated positive correlations with total norovirus (0.761, $P < 0.05$) and adenovirus F and G (0.745, $P < 0.05$) while somatic coliphages correlated significantly with adenovirus F and G (0.703, $P < 0.01$). Levels of detected enteric viral pathogens and phage surrogates correlated significantly in shellfish and their harvesting waters. In a microcosm study, mussels and oysters bioaccumulated faecal bacteria and phages at varying rates. Mussels were demonstrated to bioaccumulate phages to a greater extent than the faecal bacteria, and in both shellfish species, faecal bacteria persisted for longer periods over 48 hours than the phages.

The findings of this study suggest that monitoring environmental parameters is a useful addition to surveillance plans for ensuring compliance of shellfish and their harvesting waters with public health protection regulations and as a component of predictive modelling in shellfish and water quality monitoring for human health protection. This study also highlights significant variation in the levels and the rate of accumulation and persistence with respect to both shellfish type and the indicators used to assess risk, and has demonstrated the effectiveness of bacteriophages as surrogates of enteric viral pathogens in shellfish and their harvesting waters. It is proposed that risk of human shellfish-related infections caused by enteric viruses can be reduced if analysis of the proposed phages is adopted as part of revised shellfish hygiene regulations in Europe and globally.

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Abbreviations

AF	Accumulation Factor
ANZECC	Australian and New Zealand Environment and Conservation Council
AQIS	Australian Quarantine and Inspection Service
ARMCANZ	Agriculture and Resources Management Council of Australia and New Zealand
ASP	Amnesic shellfish poisoning
ASQAAC	Australian Shellfish Quality Assurance Advisory Committee
ASQAP	Australian Shellfish Quality Assurance Program
AWQMS	Australian Water Quality Management Strategy
BPRMA	*Bacteroides* phage recovery medium agar
BPRMB	*Bacteroides* phage recovery medium broth
CDC	Centre for Disease Control
CEFAS	Centre for Environment Fisheries and Aquaculture Science
CFIA	Canadian Food Inspection Agency
CFU	Colony forming unit
CSO	Combined Sewer Overflow
CSSP	Canadian Shellfish Sanitation Program
CWA	Clean Water Act
DEFRA	Department for Environment, Food and Rural Affairs
DFO	Department of Fisheries and Oceans Canada
DNA	Deoxyribonucleic Acid
DSP	Diarrhoetic shellfish poisoning
EC	Environment Canada
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
ELISA	Enzyme-linked immunosorbent assay
EPHReG	Environment and Public Health Research Group
EQS	Environmental Quality Standards
EU	European Union
FSA	Food Standards Agency
FSANZ	Food Safety Australia New Zealand
GIT	Gastrointestinal tract
HAdV	Human Adenoviruses
HAV	Hepatitis A virus
HEV	Hepatitis E virus
HPA	Health Protection Agency
IAWPRC	International Association on Water Pollution Research and Control
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ISO	International Standards Organisation
ISSC	Interstate Shellfish Sanitation Conference
LV	Limit values
MLSA	Membrane lauryl sulphate agar
MMGB	Modified Mineral Glutamate Broth
MPN	Most probable number
MSA	Modified Scholtens' Agar
MSB	Modified Scholtens' Broth

MST	Microbial Source Tracking
MTB	Maximum Theoretical Bioaccumulation
ND	Non Detects
NMFS	National Marine Fisheries Service
NOAA	National Oceanic and Atmospheric Administration
NoV	Norovirus
NoV GI	Norovirus Genogroups I
NoV GII	Norovirus Genogroups II
NPDES	National Pollutant Discharge Elimination System
NSP	Neurotoxic shellfish poisoning
NSSP	National Shellfish Sanitation Program
NTU	Nephelometric Turbidity Unit
NWQMS	National Water Quality Management Strategy
PCR	Polymerase Chain Reaction
PFU	Plaque forming unit
PSP	Paralytic shellfish poisoning
QMRA	Quantitative Microbial Risk Assessment
qPCR	Real time - polymerase chain reaction
RASFF	Rapid Alert System for Food and Feed
RNA	Ribonucleic acid
RT-qPCR	Reverse-transcription quantitative polymerase chain reaction
SD	Standard Deviation
SE	Standard Error
SEPA	Scottish Environment Protection Agency
SPSS	Statistical Package for Social Sciences
TBX	Tryptone bile glucuronide agar
TYGA	Tryptone-yeast extract glucose agar
TYGB	Tryptone-yeast extract glucose broth
UK	United Kingdom
UNICEF	United Nations Children’s Fund
US EPA	United States Environmental Protection Agency
US FDA	United States Food and Drug Administration
US	United States
UV	Ultraviolet radiation
WHO	World Health Organisation

Dedication

Dedicated to God, my late mum Mrs Modupe Victoria Olalemi, my lovely wife Oluwakemi, my adorable son Samuel and my entire family.

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Author's Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not contain any material already submitted for a degree.

Signed…………………………………………

Dated………………………………………….

Chapter One: Introduction

Globally, numerous outbreaks of gastrointestinal disease including, many shown to be caused by viral pathogens, have been linked to the consumption of shellfish from coastal ecosystems (Lambertini *et al*., 2012). This is because shellfish have the ability to accumulate and retain microorganisms from their aquatic environment. A better understanding of waterborne pathogen transport, and consequently, improved risk assessment, is critical to the development of surveillance tools that will allow the shellfish industry to minimise health risk to consumers of seafood.

Studies have shown that faecal indicator organisms currently used for monitoring the sanitary quality of shellfish and their harvesting waters may be inadequate indicators of the presence of pathogenic enteric viruses. Shellfish may meet the *E. coli* standards for human consumption but still contain an infectious dose of human enteric viruses that cause gastroenteritis (Dore *et al*., 2000, 2003; Griffin *et al*., 2003). Bacteriophages (somatic coliphages, F-RNA coliphages and *Bacteroides fragilis* phages) have been proposed as indicators for these enteric viruses in shellfish and their harvesting waters because they appear to demonstrate a similar pattern of persistence in the environment (Burkhardt *et al.*, 1992). In addition, bacteriophages meet many of the requirements of an “ideal” surrogate because they demonstrate many characteristics that are similar to those of mammalian viral pathogens (i.e., their size, shape, morphology, surface chemistry, isoelectric points, and physiochemistry). Moreover, they are unlikely to replicate outside the host gut because of a lack of viable hosts and other limiting factors, pose little risk to the health of humans, plants, and animals, and are easier and less expensive to isolate and enumerate than enteric viruses (Tufenkji and Emelko, 2011).

1.1 Pollution of the aquatic environment

Water pollution can be described as the contamination of marine and freshwater bodies (such as lakes, rivers, oceans, aquifers and groundwater) with domestic sewage, agricultural pesticides and fertilizers, oils and oil dispersants, mercury and lead, solid wastes, industrial effluents, etc., and may occur when waste streams containing harmful compounds are not sufficiently treated prior to discharge into water bodies. The effect of pollution is generally damaging to the natural biological communities in the aquatic environment. From the earliest period of human history, humans have inhabited the margins of rivers, lakes and seas and, as a result of rapid urbanisation, the quality of surface waters in highly industrialised nations has become compromised. The exploitation of the seas for food, transportation and recreation also contribute to concentration of waste in the aquatic environment.

Sources of surface water pollution may be grouped into two categories based on their origin, namely point sources and non-point sources. Point source water pollution refers to contaminants that are discharged directly into a body of water from a single, identifiable source, such as effluent discharges from wastewater treatment plants, factories and industrial plants, latrines, septic tanks, barnyards or livestock confinement, construction sites, etc. Non-point source pollution refers to diffuse contamination that does not originate from a single discrete source. It is usually the cumulative effect of small amounts of contaminants gathered from a large area that reduces water quality. Agricultural waste runoff into rivers or streams both on the surface and through the soil, is an example of diffuse pollution.

1.2 Faecal pollution

Many aquatic environments are prone to pollution from the indiscriminate discharge of municipal wastewater to surface and coastal waters. Often, these wastewaters contain faecal matter of either human or non-human origin and are consequently a source of waterborne pathogens. Waterborne pathogens from faecal pollution continue to be a major cause of infectious disease in many parts of the world (WHO, 2010). Low-, middle- and a few high-income countries are still facing the problem of outbreaks of infectious diseases resulting from faecal contamination of source water for drinking, recreation and the rearing of shellfish. The incidence of these infections are higher in low- and middle-income countries than they are in high-income countries, this is because of inadequate water, sanitation and hygiene (WHO, 2014).

Identifying the source of faecal contamination in aquatic environments is a key component of effective pollution control and health protection strategies. The field of microbial source tracking (MST) has developed rapidly over the past twenty years and is based on the assumption that, using appropriate methods and faecal source identifiers, the source of faecal pollution can be detected (US EPA, 2005).

1.3 Excreta-related pathogens

Pathogens are microorganisms (bacteria, viruses, protozoa and helminths) that have the ability to cause infection and potentially disease. Many pathogens of human health significance enter water bodies as a component of human or non-human faeces and one gram of human faeces may contain 10 million viruses, 1 million bacteria, 1,000 parasite cysts and 100 parasite eggs (UNICEF, 2008).

The use of faecal indicator bacteria (intestinal enterococci, total coliforms, faecal coliforms and *E. coli*) as indicators of faecal pollution in water quality measurement is widely accepted. Sidhu *et al*. (2012) conducted an investigation into the presence of human enteric bacteria and viruses from urban stormwater runoff and found levels of faecal indicator bacteria exceeding the guidelines for managing risks in recreational waters. Gonzalez *et al*. (2012) developed and applied an empirical predictive modelling tool for estimating real-time faecal indicator bacteria in coastal and estuarine waters since the quality of these waters has a direct impact on human health when used for recreational activities or aquaculture. Wilkes *et al*. (2013) assessed the occurrence of bacteria, viruses, parasites, the concentration of faecal indicator organisms and quantitative risk of *E. coli* 0157:H7 infection in humans using over 3500 water samples collected over a seven year period from four sites along an intermittent stream running through a small livestock pasture system. The authors found the densities of total coliform, faecal coliforms and *E. coli* to reduce significantly downstream in the restricted pasture system with seasonal and flow conditions contributing to greater densities of indicator bacteria.

Enteric pathogenic viruses infect the intestinal tract of humans through ingestion of food and water contaminated with viruses of faecal origin. They are excreted in enormous quantities in the faeces of infected persons (Fong and Lipp, 2005). Viral contamination of surface waters may derive from untreated or partially-treated wastewaters discharged into the water body, or from surface run-off following open defecation by an infected person. The most commonly reported human pathogenic viruses that may be waterborne include poliovirus, hepatitis A and E viruses, coxsackie A and B viruses, echovirus, astrovirus, sapovirus, enterovirus, adenovirus, norovirus, and rotavirus (Pond, 2005).

Parasitic protozoa that may be transmitted through the faecal-oral route, such as *Cryptosporidium parvum, Giardia lamblia, Toxoplasma gondii, Entamoeba histolytica, Acanthamoeba* spp.*, Cyclospora cayetanensis, Microsporidia, Isospora, Blastocystis hominis, Sarcocystis* spp.*, Naegleria spp. and Balantidium coli,* have been implicated in waterborne infections worldwide. In a review of worldwide disease outbreaks caused by parasitic protozoa between 2004 and 2010, Baldursson and Karanis (2011) suggested that 60% of human protozoan diseases may be caused by *Cryptosporidium parvum*, 35% by *Giardia lamblia* while other protozoa were among the remaining 5%.

Helminths are parasitic worms that may cause a wide range of infectious diseases in humans. The eggs are excreted by the infected host individual and transmitted to others through the faecal-oral route or through contact with contaminated soil or recreational waters. Infectious helminths of public health significance include *Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus*, *Ancylostoma duodenale*, *Schistosoma* spp., *Diphyllobotrium latum*, *Hymenolopsis nana*, *Enterobius vermicularis*, *Fasciolopsis buski*, *Taenia* spp., *Fasiola* spp., *Wuchereria bancrofti* etc. Inadequate wastewater disposal, inadequate sanitation facilities, households living in close proximity to sanitation facilities, discharge of untreated effluents from wastewater treatment plants, etc., are major factors contributing to helminthic infections in humans (Ziegelbauer *et al*., 2012).

1.4 The public health impacts of water pollution

Waterborne infectious diseases are transmitted primarily through contamination of water sources with the excreta of humans and other animals that are either active cases or carriers of disease. Carriers do not show any signs of disease, although they have disease-causing agents in their body that can be transferred to others, whereas active cases are those

individuals who are displaying visible signs of disease. Use of contaminated water for drinking or cooking, or contact with contaminated water during washing or bathing, may result in infection. The dose or amount ingested that is necessary to induce illness depends on the type of pathogen. Exposure to a single pathogenic organism does not always result in infection and disease. The minimum infectious dose also varies with the age, health, nutritional and immunological status of the exposed individual. Infants and young children, people who are debilitated, people who are living in unsanitary conditions, people who are sick and the elderly are at greatest risk of waterborne diseases (WHO, 2000).

1.5 The aetiology of waterborne diseases

Waterborne diseases are caused by pathogenic organisms that are most commonly transmitted via contaminated water environments (Martia *et al*., 2013). Infection commonly results from contact with, or consumption of, contaminated water during bathing, washing, drinking, preparation and consumption of food. The various forms of waterborne diarrhoeal disease account for an estimated 3.6% of the total global burden of disease, and cause about 842,000 human deaths annually. The World Health Organization estimates that most of this burden is attributable to unsafe water supply, sanitation and hygiene. Microorganisms causing waterborne diseases prominently include bacteria, viruses, protozoa and helminths (Table 1.1) and most of these pathogenic microorganisms are thought to contaminate water and shellfish through municipal discharge of wastewater into coastal systems (WHO, 2014).

Table 1.1: Infectious microbial waterborne diseases (Nwachcuku and Gerba, 2004; Nwachcuku *et al.,* 2005; Dziuban *et al*., 2006; Petrini, 2006)

Microorganisms	Disease
Bacteria	
Clostridium botulinum	Botulism
Campylobacter jejuni	Campylobacteriosis
Vibrio cholerae	Cholera
Escherichia coli	*E. coli* infection
Mycobacterium marinum	*M. marinum* infection
Shigella dysenteriae	Dysentery
Legionella pneumophila	Legionellosis
Leptospira	Leptospirosis
Some bacterial species	Otitis externa (swimmer's ear)
Salmonella	Salmonellosis
Salmonella typhi	Typhoid fever
V. vulnificus	Vibrio illness
V. alginolyticus	
V. parahaemolyticus	
Viruses	
Coronavirus	SARS (Severe Acute Respiratory Syndrome)
Hepatitis A virus (HAV)	Hepatitis A
Poliovirus	Poliomyelitis (Polio)
Polyomavirus	Polyomavirus infection
JC virus, BK virus	
Norovirus	Stomach flu
Adenovirus	Gastroenteritis
Protozoa	
Entamoeba histolytica	Amoebiasis
Cryptosporidium parvum	Cryptosporidiosis
Cyclospora cayetanensis	Cyclosporiasis
Giardia lamblia	Giardiasis
Microsporidia	Microsporidiosis
Helminthes	
Schistosoma spp.	Schistosomiasis
Dracunculus medinensis	Dracunculiasis
Taenia spp.	Taeniasis
Fasciolopsis buski	Fasciolopsiasis
Hymenolepis nana	Hymenolepiasis
Echinococcus granulosus	Echinococcosis
Multiceps	Coenurosis
Ascaris lumbricoides	Ascariasis
Enterobius vermicularis	Enterobiasis

1.6 Microbial indicators of faecal pollution

1.6.1 Total coliforms – This is a group of aerobic and facultative anaerobic, Gram-negative, non-sporulating, rod-shaped bacteria that ferment lactose to produce acid and gas within 48 hours at 35°C. The total coliform group includes bacteria of faecal and non-faecal origin. Bacteria of faecal origin are found in the faeces of humans and other warm-blooded animals, while those of non-faecal origin are found in soil or on plants, e.g., species of *Citrobacter. Enterobacter, Klebsiella* and *Aeromonas.*

1.6.2 Faecal coliforms – This is a group of facultative anaerobic, rod-shaped, Gram-negative, non-sporulating bacteria that ferment lactose to produce acid and gas within 24 hours at 44.5^{o}C. Faecal coliform bacteria are also referred to as thermotolerant coliform as a result of their ability to survive relatively high ambient temperature. They are found in the faeces of humans and other warm-blooded animals. These organisms enter rivers through direct discharge from mammals and birds; from agricultural and storm runoff containing mammalian and bird wastes; and from sewage discharge. Even though most faecal coliform bacteria are not pathogenic, they may co-present with pathogenic organisms of faecal origin; therefore, their presence suggests the potential presence of disease-causing organisms. The shellfish water quality standard (2006/113/EC) for faecal coliform in the European Union (EU) is less or equal to 100 colony forming unit (CFU) per 100 ml of water.

1.6.3 *Escherichia coli* (*E. coli*) – Is a Gram-negative, facultative anaerobic, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. It is a type of faecal coliform bacterium that is normally specific to faeces. In many parts of the world, *E. coli* has been used to indicate faecal pollution of the environment for over a

century, since the origin of the bacterium is the faeces of warm-blooded animals, its presence is interpreted to suggest the potential presence of human enteric pathogens (Ashbolt *et al*., 2001). Many organisations involved with legislation on water quality standards such as Commission of the European Union (CEU), Environment Canada (EC), United States Environmental Protection Agency (USEPA) etc., uses *E. coli* measurements to determine whether fresh water is considered safe for recreational use. Disease-causing bacteria, viruses and protozoans may be present in water that has elevated levels of *E. coli*. The concentration of *E. coli* in streams can vary greatly and rain events usually increase the levels of bacteria in the water (Nnane *et al*., 2011). *E. coli* is normally measured as CFU per 100 ml. The US EPA recreational water quality guideline for *E. coli* is 394 CFU per 100 ml (USEPA, 2012), whereas in the EU, the water quality guideline (2006/113/EC) for *E. coli* is less than or equal to 100 CFU per 100 ml in bathing or recreational waters as well as shellfish growing and harvesting waters and less than 300 CFU per 100 ml in shellfish flesh and intravalvular fluid (CEU, 2006).

1.6.4 Intestinal enterococci - This is a group of Gram-positive, facultatively anaerobic bacteria of the family *Streptococcaceae*, formerly classified in the genus *Streptococcus*. They are a subset of faecal streptococci and grow at pH 9.6, between 10°C to 45°C, under aerobic conditions. *Enterococcus faecalis* and *Enterococcus faecium* are normal inhabitants of the human intestinal tract. Enterococci demonstrate the ability to survive in saline (6.5% sodium chloride) and fresh water environment making them useful indicators of health risk in waters used for recreational purposes or growing shellfish (Ashbolt *et al*., 2001; Wyer *et al*., 2012).

1.7 Microbial contamination of shellfish

'Shellfish' is a term commonly used for exoskeleton-bearing aquatic invertebrates harvested from marine and freshwater environments (Festing and Tyas, 1999). They are sedentary filter feeders that pump large quantities of water through their bodies. This process can concentrate microbial pathogens within their tissues, causing little or no harm to the animal, but posing substantial risks to human consumers (NOAA, 1998). Shellfish are classified into two groups: molluscs and crustaceans. The molluscs may possess a pair of shells (bivalves), a single shell (univalves) or no shell (cuttlefish and octopuses). Bivalves are filter-feeding aquatic molluscs that have two-part shells, which are symmetrical along a hinge line. The class has about 30,000 species, including scallops (*Argopecten* spp., *Chlamys* spp., *Patinopecten* spp., *Pectinopectin* spp.), clams (*Mercenaria mercenaria*), oysters (*Ostrea* spp., *Crassostrea* spp.), carpet shell (*Venerupis* spp., *Ruditapes* spp., *Venus* spp.,), cockle (*Katelysia* spp., *Anadara* spp., *Glycymeris* spp.), razor shell (*Ensis arcuatus*), tellin (*Tellina radiata*) and mussels (*Mytilus* spp.). Examples of univalves include: limpet (*Patella vulgata*), cowrie (*Cypraea* spp.), tower shell (*Turritella communis*), tusk shell (*Antalis longitarsus*), whelk (*Busycon* spp.), winkle (*Nucella lapillus, Littorina littorea*), etc. The crustaceans have external skeletons and examples include: barnacles (*Balanus glandula*), lobster (*Homarus americanus*), crayfish (*Pacifastacus leniusculus*, *Austropotamobius pallipes, Cambarus* spp.), crabs (*Callinectes sapidus, Stenorhynchus seticrnis*), prawn (*Fenneropenaeus indicus, Penaeus* spp.), shrimp (*Metapenaeus dobsoni, Palaemon serratus, Crangon crangon*).

Shellfish are a valuable human food resource in many parts of the world (such as the United States, United Kingdom, China, Canada, France, Australia, etc.). Commercial

shellfish industries grow and harvest these sea animals from aquaculture or as wild stock from marine, estuarine or fresh waters (NMFS, 1997).

1.7.1 Oysters - The major types of oysters of high commercial value are *Ostrea edulis* (Figure 1.1), Pacific oysters (*Crassostrea gigas*) (Figure 1.2), Eastern oysters (*Crassostrea virginica*), and Olympia oysters (*Ostrea lurida*). Of these, the Pacific oysters are the most widespread species in the world (Chew, 1990), probably as a result of their ability to adapt to various environments.

Figure 1.1: *Ostrea edulis*

Figure 1.2: Pacific oysters (*Crassostrea gigas*)

1.7.2 Clams - Most clams are oval-shaped with two symmetrical shells. They are bivalve molluscs consisting of several broad groups. The common clams harvested commercially are: surf clams (*Spisula solidissima*), quahog clams (*Mercenaria mercenaria* and *Arctica islandica*), softshell clams (*Mya arenaria*), manila clams (*Ruditapes philippinarum*),

geoduck clams (*Panopea abrupta*, *P. generosa*) and razor clams (*Siliqua patula*) (Meschke and Boyle, 2007).

1.7.3 Mussels - These are bivalve molluscs that secrete a byssal thread for attachment to substrates in their harvesting waters. The commercially important species of mussels (family - *Mytillidae*) is the blue mussel (*Mytilus edulis*) (Figure 1.3) (Meschke and Boyle, 2007).

Figure 1.3: Mussels (*Mytilus edulis*) (Source: Seafood.fabriko.co.uk)

1.7.4 Scallops - These have a fan-shaped shell with fluted edges and a central adductor muscle which is responsible for their swimming ability. The commercially important species is the New England sea scallop (*Placepten magellinacus*) (Meschke and Boyle, 2007).

The microbial quality of shellfish and their harvesting waters is normally measured using indicator organisms rather than specific pathogens (Table 1.2). Routine monitoring for pathogens that cause disease outbreaks may be costly so, the use of microbial indicators is considered an important component of actions to prevent human disease (Payment and Locas, 2011).

Table 1.2: Terms used to describe microbial indicator, index organism and faecal indicator, to measure microbial contamination in the environment (Ashbolt *et al*., 2001; Sinclair *et al*., 2012)

Term	**Definition**
Process indicator	Used to demonstrate the efficacy of a process or if the process has been compromised
Index organism	A group/or species indicative of pathogen presence. e.g. *E. coli* as an index for *Salmonella*
Model organism	A group/or species indicative of pathogen behaviour e.g. F-RNA coliphages as models of human enteric viruses
Faecal indicator	An organism that indicate the presence of faecal contamination e.g. *E. coli*
Surrogate organism	An organism or substance used to study the fate of a pathogen in a specific environment

1.8 Shellfish-borne human diseases

Human infectious diseases, most especially gastroenteritis, have long been associated with the consumption of shellfish (Mead *et al*., 1999), which is mainly because shellfish are eaten raw or partially cooked (Furuta *et al*., 2003). Pathogenic microorganisms associated with shellfish-borne diseases may occur in the shellfish- harvesting waters as a result of faecal pollution of the harvesting waters (Meschke and Boyle, 2007) and Table 1.3 shows some of the more common shellfish-borne diseases.

Vibrio spp. are often found in the water column attached to phytoplankton, sediments and a few shellfish species and of all the species, *V. cholerae,* which can cause cholera, has been reported to be implicated in most *Vibrio*-related infections (Potasman *et al*., 2002). Other species include *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. fluvialis*, *V. furnissii*, *V. damsela*, *V. hollisae,* etc. Similarly, *Aeromonas* spp. are opportunistic pathogens and have been associated with outbreaks of shellfish-related illness (Merino *et al*., 1995). Those species implicated include *A. hydrophila* and *A. caviae.*

Faecal pollution of the aquatic environment from human or non-human sources has been identified as a major source of pathogenic microorganisms associated with shellfish-borne diseases. Viral illnesses are the most commonly encountered shellfish-related illness (Koopmans and Duizer, 2004). Enteric viruses, such as norovirus and hepatitis A virus, have been observed to pose the greatest risk to public health and they are the most common type of viruses associated with human gastroenteritis from shellfish (Koopmans *et al*., 2002). Other types of viruses associated with illness resulting from consumption of shellfish include enteroviruses, adenoviruses, rotaviruses and astroviruses.

Table 1.3 Common shellfish-borne diseases (Lipp and Rose, 1997)

Shellfish-borne diseases caused by		**Illnesses**
Bacteria	*Vibrio cholerae*	Cholera
	Escherichia coli	Diarrhoea
	Salmonella typhi	Typhoid fever
	Campylobacter spp.	Campylobacteriosis
	Bacillus cereus	Bacterial toxin infection
	Staphylococcus aureus	Bacterial toxin infection
	Aeromonas spp.	Gastroenteritis
	Plesiomonas spp.	Gastroenteritis
	Shigella spp.	Dysentery
	Salmonella	Salmonellosis
	Vibrio spp.	Vibrio illness
	V. vulnificus,	
	V. alginolyticus,	
	V. parahaemolyticus	
	Yersinia enterolitica	Diarrhoea, vomiting, fever
	Listeria spp.	Spontaneous abortion, stillbirth, septicaemia, meningitis
Viruses	Norovirus	Gastroenteritis
	Hepatitis A virus	Infectious hepatitis
	Enteroviruses:	Paralysis, diarrhoea, myocarditis, fever, rash, nephritis, pericarditis, respiratory illness, etc.
	Poliovirus	
	Coxsackievirus	
	Echovirus	
	Adenovirus	Gastroenteritis
	Rotavirus	Gastroenteritis
	Astrovirus	Gastroenteritis
Protozoa	*Cryptosporidium parvum*	Cryptosporidiosis
	Giardia lamblia	Giardiasis
Toxins		
Tetramine	Red whelk	Paralytic shellfish poisoning (PSP)
Saxitoxin	Marine dinoflagellates	PSP
Neurotoxin	Dinoflagellates:	PSP
	Alexandrim catenella	
	A. tamarensis	
Okadiac acid	Dinoflagellates:	Diarrhoetic shellfish poisoning (DSP)
	Dinophysis fortii	
	D. acuminata	
Neurotoxin	Dinoflagellates:	
	Gymnodinium breve	Neurotoxic shellfish poisoning (NSP)
Histamine	Diatoms:	
	Nitzschia pungens	Amnesic shellfish poisoning (ASP)

Enteric bacteria of the family *Enterobacteriaceae* have also been isolated from shellfish grown in waters contaminated with faeces, either from human or non-human origin. These enteric bacteria include *Salmonella* spp., *Shigella* spp., *Escherichia coli* and *Yersinia enterocolitica* (Farmer, 2003). *E. coli* has been used as an indicator organism for monitoring faecal pollution in many environmental regulatory organisations and public health laboratories that carry out water quality analysis. Enteric protozoan parasites have also been isolated from oysters and mussels in different studies and *Cryptosporidium* spp. and *Giardia* spp. are known to cause infections that are the most common forms of gastroenteric parasitosis (Fayer *et al*., 1998; Graczyk *et al*., 1999).

The consumption of shellfish has also been linked to forms of human toxicosis, such as red whelk poisoning and paralytic shellfish poisoning (Tian *et al*., 2014). Red whelk poisoning occurs when the salivary glands of red whelk (*Neptunea antiqua*), containing a toxin known as tetramine, are consumed. Similarly, paralytic shellfish poisoning is caused by toxins produced by marine dinoflagellates (*Gonyaulax*), a form of plankton on which molluscs feed. This toxin is known as saxitoxin (Suikkanena *et al*., 2013).

Outbreaks of shellfish-associated infections have been reported on all continents except Africa. This may be as a result of the prevailing warm climate in the region, relatively low levels of shellfish consumption or because outbreaks are under-reported. Figure 1.4 shows the percentage of disease associated with shellfish reported over three decades in various parts of the world. Most reports involved oysters (Figure 1.5), clams, mussels, and other types of shellfish (Potasman *et al*., 2002).

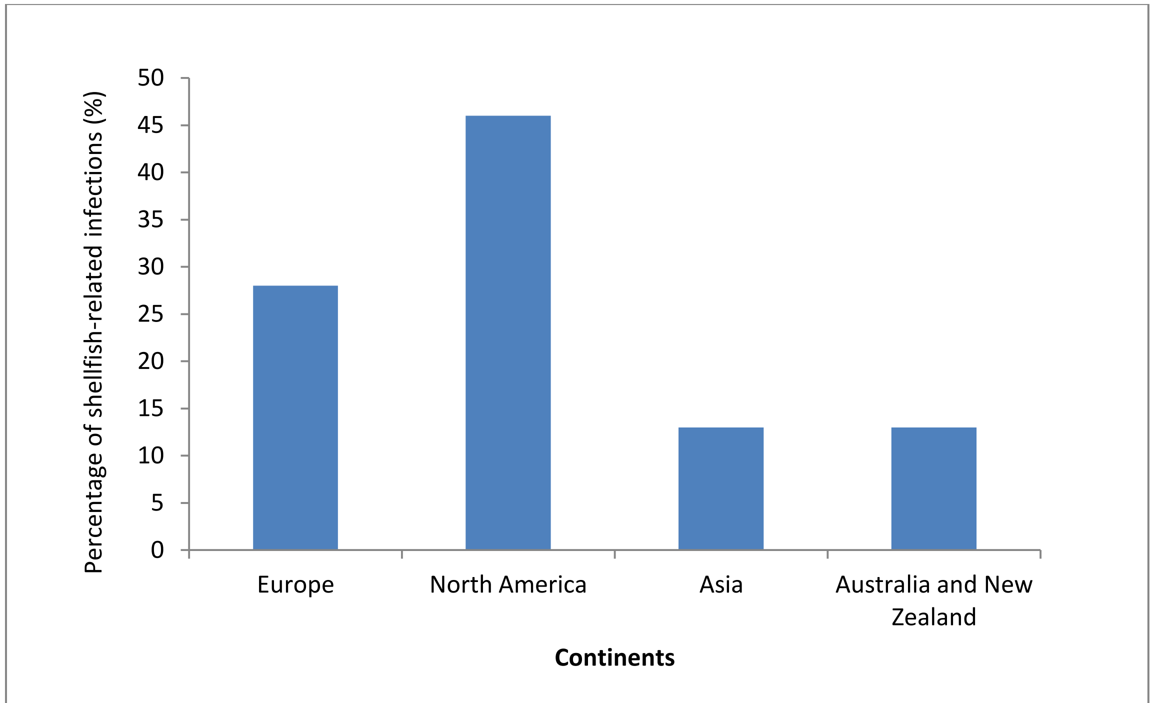


Figure 1.4: Reports (n = 35) indicating infections associated with shellfish in some parts of the world (1969-2000) (Potasman *et al*., 2002)

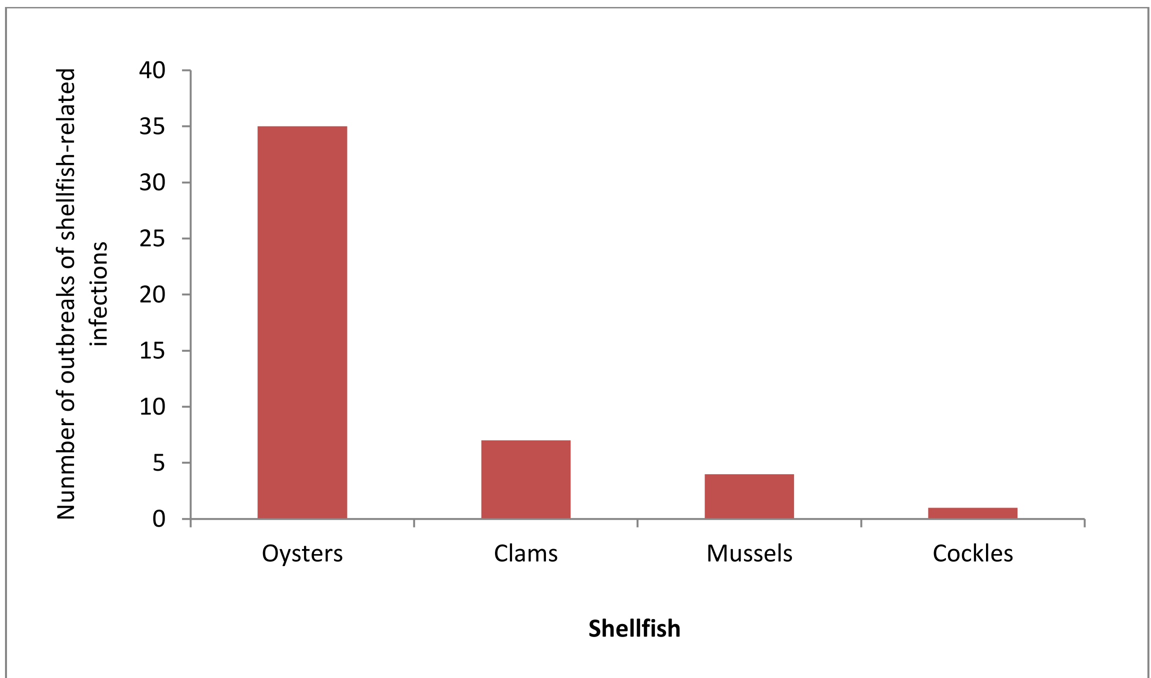


Figure 1.5: Some reports (n = 35) of shellfish-related infections (1969-2000) (Potasman *et al*., 2002)

1.9 Legislation on water quality, shellfish-harvesting waters and hygiene

Globally, many organisations are involved in monitoring and setting standards for water and shellfish quality (Table 1.4). Legislation on environmental water quality standards is made by these competent bodies in order to protect human health through microbiological monitoring of surface waters, recreational waters, bathing waters, shellfish waters, etc. In this section, legislation on water and shellfish quality in the European Union (EU), United States (US), Canada and Australia is examined.

Table 1.4: List of organisations involved in setting standards for water and shellfish quality

Organisation / Programmes	Abbreviation	Region
European Union European Commission Food Standards Agency Centre for Environment Fisheries and Aquaculture Science Health Protection Agency Scottish Environment Protection Agency	EU EC FSA CEFAS HPA SEPA	Europe
United States Environmental Protection Agency United States Food and Drug Administration	US EPA US FDA	United States
Environment Canada Canadian Shellfish Sanitation Program	EC CSSP	Canada
Australian Water Quality Management Strategy	AWQMS	Australia
World Health Organisation United Nations Children's Fund	WHO UNICEF	Global

1.9.1 European Union (EU) legislation on water and shellfish quality

In the EU, water and shellfish microbial quality are assessed against standards that are considered indicative of levels of faecal pollution, within the EU Bathing Water Directive, EU Shellfish Water Directive and EU Food Hygiene Regulations (CEU, 2003; 2006). The EU legislation ensures that bathing waters and shellfish waters meet the set standards. Similarly, shellfish sold for consumption must comply with the legislation and shellfish growing and harvesting waters are also classified according to legislation that sets out limits of certain bacteria (*E. coli* and faecal coliforms) in samples of shellfish flesh.

1.9.1.1 The EU Shellfish Water Directive. The Shellfish Waters Directive 79/923/EC (CEU, 1979) was first adopted in 1979 and transcribed into UK legislation in 1997 under the Surface Waters Shellfish Classification Regulations. The Directive outlines the requirements for the quality of waters in which shellfish are grown and harvested. The ultimate aim of this Directive is to protect shellfish populations from contamination resulting from discharges of public or private sewage, run off from agricultural lands or industries into their harvesting waters. The Shellfish Water Directive 2006/113/EC concerns the quality of shellfish waters, i.e., coastal and brackish waters that needs protection or improvement so as to support shellfish life and growth, thereby contributing to assuring high quality shellfish for direct human consumption (CEU, 2006). Figure 1.6 shows areas that are classified for shellfish production in United Kingdom.

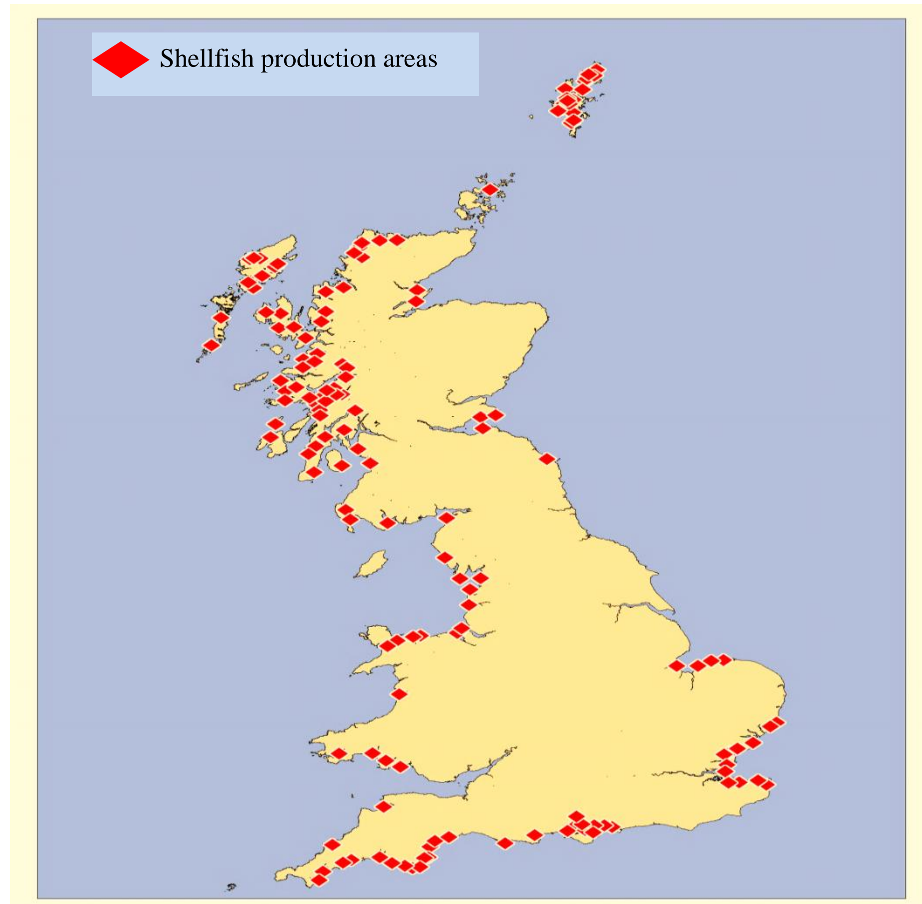


Figure 1.6: United Kingdom classified shellfish production areas
(Source: CEFAS, 2013)

1.9.1.2 The EU Shellfish Hygiene Directive. The EU Shellfish Hygiene Directive, under regulation EC (No) 854/2004, was established to protect human health, especially those who consume shellfish (CEU, 2004). The legislation ensures that shellfish sold for consumption meet the food safety health and hygiene (i.e., *E. coli*) standard and is also used for classifying shellfish growing or harvesting waters as having a Class A, Class B, Class C or prohibited status (Table 1.5), based on the number of faecal indicator bacteria (faecal bacteria, *E. coli*, enterococci) in the shellfish flesh and waters (Table 1.6).

Table 1.5: The EU classification categories of shellfish waters and microbiological criteria (Source: Regulation (EC) No. 854/2004)

Classification category	Microbiological criteria	Recommendation
Class A	Shellfish must contain ≤ 230 *E. coli* /100 g of flesh and intravalvular fluid	It can be consumed without treatment
Class B	Shellfish must contain ≤ 4600 *E. coli* /100 g of flesh and intravalvular fluid in 90% of samples	It must be depurated or relayed in clean water (Class A) for 2 months or treated by an approved process e.g. use of heat or Ultraviolet (UV) radiation
Class C	Shellfish must contain ≤ 46000 *E. coli* /100 g of flesh and intravalvular fluid	It must be depurated or relayed in clean water (Class A) for longer periods or treated by an approved process e.g. use of heat or Ultraviolet (UV) radiation
Prohibited		Shellfish must not be harvested for consumption in this area

Table 1.6: The EU quality of shellfish waters (Adapted from EC Directive 2006/113/EC)

Parameter	Guide	Mandatory standard	Minimum sampling and measuring frequency
pH		7 – 9	Quarterly
Temperature	Discharge must not cause the temperature of shellfish waters to increase by more than 2°C	≤ 40%	Quarterly
Salinity	12 to 38%	Discharge must not increase the salinity by more than 10%	Monthly
Dissolved oxygen (Saturation %)	≥ 80%	≥ 70%	Monthly
Faecal coliforms per 100 ml	≤ 300 in the shellfish flesh and intravalvular fluid		Quarterly

1.9.2 United States (US) legislation on water and shellfish quality

1.9.2.1 The Clean Water Act (CWA). In 1948, the Federal Water Pollution Control Act was enacted in the United States (US) to maintain water quality. This Act was amended in 1972 and became the 'Clean Water Act – 33 United States Code §1251 et seq. (1972)'. The objective of the CWA is to restore and maintain the chemical, physical and biological integrity of waters in the US. It establishes the basic structure for regulating discharges of pollutants into surface waters, thereby maintaining water quality standards that are highly comparable with standards in EU, Canada, Australia, etc.

The US Environmental Protection Agency has implemented pollution control programmes by setting wastewater standards for industries and water quality standards for contaminants in surface waters under the CWA. Municipal or industrial wastewaters cannot be discharged into navigable waters unless a National Pollutant Discharge Elimination System (NPDES) permit is obtained (US EPA, 2013).

1.9.2.2 US Shellfish waters. The National Shellfish Sanitation Program (NSSP) is the federal/state cooperative programme recognised by the United States Food and Drug Administration (FDA) and the Interstate Shellfish Sanitation Conference (ISSC) for the sanitary control of shellfish produced and sold for human consumption (US FDA, 2013). The purpose of the NSSP is to promote and improve the sanitation of shellfish by setting bacteriological standards for shellfish, shellfish waters and the classification of their harvesting waters into different categories, as provided by the Ordinance. The regulation emphasises that shellfish waters are 'not subject to contamination from human or non-human faecal matter at levels that, in the judgement of the Authority, presents an actual or potential public health hazard; and not contaminated with pathogenic organisms, poisonous

or deleterious substances; biotoxins; or that it's bacterial concentration exceeds bacteriological standards'.

Similarly, the Ordinance also states that shellfish- harvesting waters 'are subjected to sanitary survey that shall be correctly classified based on twelve (12) year sanitary survey, and its most recent triennial or annual re-evaluation will be expressed as only one of the following: approved; conditionally approved; restricted; conditionally restricted; or prohibited' (Figure 1.7). The bacteriological standard for shellfish- harvesting waters to be classified as 'approved' is 14 CFU per 100 ml of water samples collected regularly and analysed using membrane filtration or most probable number (MPN) method (NSSP, 2011).

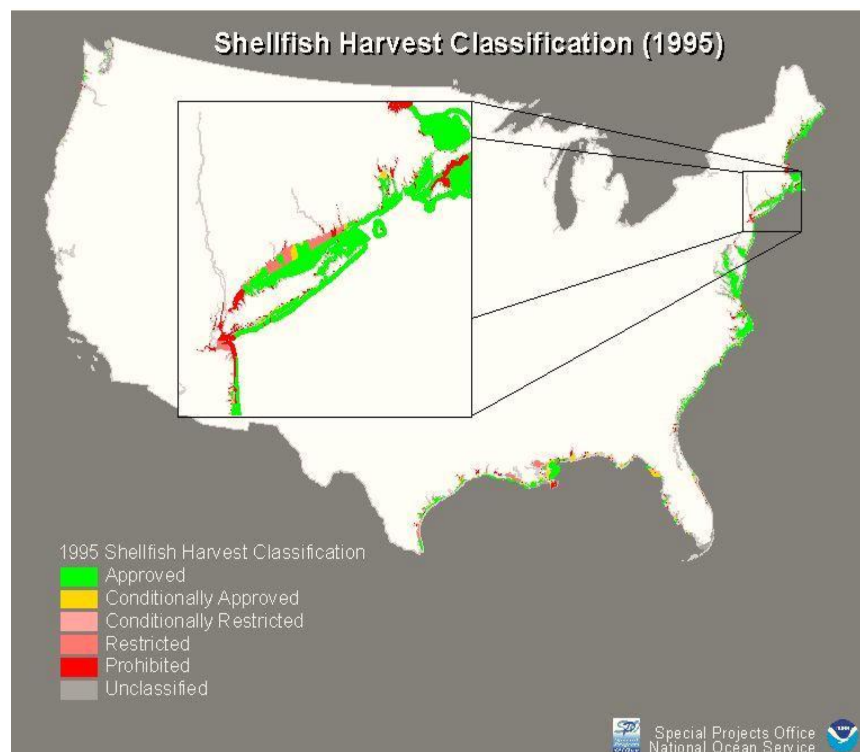


Figure 1.7: United States classified shellfish production areas
(Source: NOAA Coastal Geospatial Data – Background, 1995)

1.9.3 Legislation on water and shellfish quality in Canada

1.9.3.1 The Federal Water Policy. The Federal Government of Canada monitors scientific research and provides leadership on the development of guidelines for water quality, although provinces and territories in Canada are responsible for managing water quality by testing and monitoring shellfish waters at regular intervals. This is stated under the Constitution Act (1867), which states that the provinces are 'owners' of water resources and that they have wide responsibilities for their management. In 1970, the Federal Government established the 'Canada Water Act' (Environment Canada, 2013a) which is water legislation administered by Environment Canada that contains provisions for formal consultation and agreements with the provinces, especially with regard to water-related activities.

1.9.3.2 The Canadian Shellfish Sanitation Program (CSSP) is Canada's Federal food safety programme established to provide assurance that shellfish are safe for human consumption by focusing on water sanitation, biotoxins control, shellfish harvesting and processing. The programme is jointly administered by three federal organisations: The Canadian Food Inspection Agency (CFIA), the Department of Fisheries and Oceans Canada (DFO) and Environment Canada (EC). The legal authority for CSSP is provided by the Fisheries Act, Management of Contaminated Fisheries Regulations, Fish Inspection Act and Fish Inspection Regulations. These Acts and Regulations enable CFIA, EC and DFO to carry out their roles and responsibilities. Canadian Food Inspection Agency controls the handling, storage, transportation, labelling, marketing, import and export of shellfish; manage the marine biotoxins control programme and liaise with foreign governments. Environment Canada monitors environmental conditions that affect the sanitation of shellfish- harvesting waters upon which classification of the waters are based.

The bacteriological standard for an approved classification is 230 *E. coli* per 100 g of raw molluscan shellfish (Table 1.7). The Department of Fisheries and Oceans controls relaying, depuration and harvesting of shellfish from classified harvesting waters (Figure 1.8) (Environment Canada, 2013b).

Table 1.7: Classification categories of shellfish waters in Canada (Source: CSSP, 2012)

Classification category	Microbiological criteria	Recommendation
Approved	Water must contain ≤ 14 MPN faecal coliform/100 ml. Shellfish must contain ≤ 230 MPN faecal coliform/100 g of flesh and intravalvular fluid	Shellfish from this site can be consumed without treatment
Conditionally approved	Initially meets all the standards of an approved classification and later predisposed to intermittent pollution	Shellfish must not be harvested from this area until criteria for an approved classification are met.
Restricted	Water exceeds 14 MPN faecal coliform/100 ml.	Decontamination plan accepted by shellfish control authority such as depuration, natural relaying, container relaying or canning must be carried out.
Conditionally restricted	Initially meets the minimum criteria for restricted classification for a predictable period before intermittent pollution	Harvesting of shellfish is permitted when it meets all the requirements of a restricted classification otherwise closed
Prohibited	Highly contaminated with faecal material, pathogenic microorganisms, etc.	Shellfish must not be harvested from this area

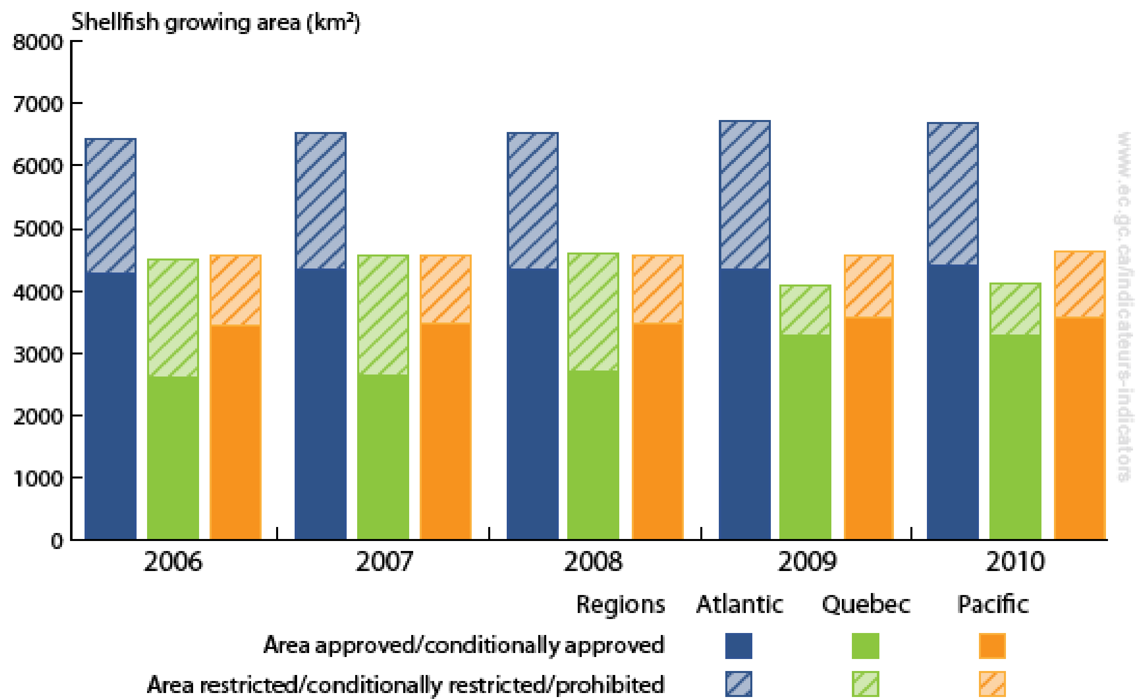


Figure 1.8: Classified shellfish-harvesting waters in Canada
(Source: Environment Canada – Shellfish growing area quality indicator, 2010)

1.9.4 Legislation on water and shellfish quality in Australia

In 1992, the Australian and New Zealand Environment and Conservation Council (ANZECC) released a document outlining water quality guidelines for fresh and marine waters as part of the National Water Quality Management Strategy (NWQMS), jointly developed by the Agriculture and Resources Management Council of Australia and New Zealand (ARMCANZ) and ANZECC. The document outlines the management framework recommended for applying water quality guidelines to natural and semi-natural marine and fresh water resources in Australia and New Zealand; and provides a summary of the water quality guidelines proposed to protect and manage the environmental values supported by the water resources (NWQMS, 2000).

The NWQMS aimed to achieve sustainable use of Australia's and New Zealand's water resources by protecting and enhancing their quality while maintaining economic and social development. The overall objective of the NWQMS is to provide an authoritative guide for setting water quality objectives required to sustain current or likely future environmental values for natural and semi-natural water resources in Australia and New Zealand (NWQMS, 2000).

1.9.4.1 The Australian Shellfish Quality Assurance Program (ASQAP) provides procedures and guidelines for the control of shellfish-harvesting waters, harvesting, processing and distribution of shellfish in Australia. The program was based on the United States National Shellfish Sanitation Program and is overseen by the Australian Shellfish Quality Assurance Advisory Committee (ASQAAC), which is made up of representatives from Food Safety Australia New Zealand (FSANZ) and Australian Quarantine and Inspection Service (AQIS). The federal, state and local government agencies are involved in the management of shellfish resources and sanitation controls of shellfish-harvesting waters in Australia and New Zealand (ASQAP, 2009).

The objective of the ASQAP is to protect the health of shellfish consumers through the administration and application of procedures that assess the risk of shellfish contamination by pathogenic bacteria and viruses, biotoxins and chemicals derived from the growing water; control the harvesting of shellfish with regards to the assessed risks and protect shellfish from contamination after harvesting. Shellfish-harvesting waters are classified as approved, restricted and prohibited for waters with no significant public health risk, moderately polluted and heavily polluted respectively (ASQAP, 2009).

In the EU, US, Canada and Australia, the microbiological criteria used to set standards for legislation on water and shellfish quality are based on the use of standard methods (membrane filtration and most probable number) to identify faecal indicator bacteria (such as faecal coliform, *E. coli*, intestinal enterococci). However, it has been observed that these organisms do not adequately predict the presence of human enteric viral pathogens in water and shellfish (Dore *et al*., 2003). Many studies have proposed direct detection methods, such as molecular techniques to identify enteric viral nucleic acids in water and shellfish, but these methods are often expensive for routine monitoring of shellfish quality and their harvesting waters. Currently, there is limited information on less expensive but effective methods to identify these viral pathogens in environmental and food samples.

Better tools to monitor shellfish and their overlying water are urgently needed to protect human health. The research described in this thesis therefore, for the first time, critically evaluates the novel application of relatively low-cost bacteriophage-based techniques to predict levels of enteric viral pathogens (validated by molecular techniques) in shellfish. The research is intended, to demonstrate whether these phage-based methods could be adopted by policy-makers as new microbiological criteria to identify and predict enteric viral pathogens in shellfish and their harvesting waters.

1.10 Aim of the study

To develop an improved understanding of the behavioural dynamics of enteric viral pathogens, bacteriophages and faecal indicator organisms in shellfish, in order to provide new management tools for the shellfisheries industry.

1.11 Objectives of the study

- To test the potential usefulness of low-cost phage-based approaches and culture-independent molecular-based approaches to monitor shellfish safety (Chapter 3).

- To assess the role of physico-chemical characteristics (temperature, dissolved oxygen, turbidity, conductivity, redox potential, pH, salinity) in the survival of enteric viruses, bacteriophages and faecal indicator organisms in shellfish (Chapter 4).

- To determine whether, and if so in which part of the organism enteric viruses, bacteriophages and faecal indicator bacteria are concentrated in commonly-consumed shellfish species after uptake and bioaccumulation. To this effect, for the first time, it will be determined in which part of oysters and to what levels phages infecting human specific *Bacteroides fragilis* - GB124 are concentrated following bioaccumulation (Chapter 3 and 5).

- To test the rate of uptake, retention and persistence of bacteriophages and faecal indicator organisms by a range of commonly-consumed shellfish species (Chapter 5).

- To determine whether the use of bacteriophages as pollution markers is adequate to predict enteric viral pathogen concentration of shellfish and their harvesting waters for the improved protection of the health of consumers of shellfish (Chapter 6).

Chapter Two: Literature Review

2.1 Enteric viruses of human health significance

Viruses are obligate parasites that require a living host to support their growth and replication. Their hosts may either be prokaryotic or eukaryotic. Enteric viruses are found in the gastrointestinal tract (GIT) of warm-blooded animals, including humans (human enteric viruses). The group of enteric viruses that causes gastroenteritis in humans infect and replicate in the epithelial cells of the gastrointestinal tract and are excreted in large numbers in stools of infected individuals (Melnick and Gerba, 1980), while those that cause inflammation of the liver (hepatitis) are referred to as hepatitis virus (Grabow *et al*., 1999a).

Viral gastroenteritis, also known as 'stomach flu' (DiCaprio *et al*., 2013) is the inflammation of the stomach, small and large intestine. The symptoms are watery diarrhoea, vomiting, nausea, abdominal cramps and pain, occasional muscle aches or headache and sometimes low-grade fever (CDC, 2011). Viral hepatitis is the inflammation of the liver, i.e., formation and accumulation of damaged liver cells in the liver tissue. Enteric viral hepatitis is usually acute (i.e., lasting less than six months) rather than chronic, with symptoms such as tiredness, malaise, slight fever, nausea, pains below the right ribs, aching muscles and joints, headache, etc., at the early stages, and yellowing of the eyes, dark urine, light-coloured stools at the jaundiced (diseased) stage. In a mini-review, cited by Bosch (2010) highlighted the mortality rate from hepatitis A in high-income countries as 0.6%.

Enteric viruses implicated in gastroenteritis include: *Reovirus, Rotavirus, Adenovirus, Norovirus, Astrovirus, Enterovirus, Coronavirus, Torovirus*, and *Picobirnavirus*; while

those that cause hepatitis are classified as: Hepatitis A virus and Hepatitis E virus (Masclaux *et al*., 2013; Polo *et al*., 2014). The nucleic acids in enteric viruses may either be ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) and are either single- or double-stranded. Usually these viruses are without membranes, which enhances their survival by making them resistant to stressful or unfavourable environmental conditions in the GIT and during their transmission through the faecal-oral route. In addition, studies have shown that enteric viruses persist longer than bacteria in environmental samples, probably as a result of their simple structure and lack of membrane (Fong and Lipp, 2005). Table 2.1 highlight some outbreaks of viral gastroenteritis associated with water and shellfish consumption.

Table 2.1: Recent outbreaks of viral gastroenteritis associated with water and shellfish

Year	Virus	Cases	Vehicle	Location	Reference
2007	Norovirus	33	Food	Sweden	Nordgren *et al*., 2010
2007	Rotavirus			Australia	Donato *et al*., 2012
2008	Norovirus		Groundwater	Korea	Koh *et al*., 2011
2008	Norovirus, Sapovirus	38	Carpet shell	Japan	Iizuka *et al*., 2010
2010	Norovirus		Oysters	Denmark, Sweden, Norway, UK, France	Westrell *et al*., 2010
2010	Norovirus	30-300	Oysters	UK	Baker *et al*., 2011
2010	Astrovirus	7		Hungary	Pankovics *et al*., 2011
2011	Sapovirus	9	Food	Puerto Rico	Hassan-Rios *et al*., 2013
2012	Rotavirus	3600	Water	Central Greece	Mellou *et al*., 2014
2012	Norovirus	11,150	Strawberries	Germany	Bernard *et al*., 2014

The transmission of human enteric viruses via the faecal-oral route may occur through various environmental pathways. Common routes of transmission are through contact with surface waters such as coastal, estuaries, rivers, lakes and groundwater prone to faecal contamination from indiscriminate discharge of wastewater, solid wastes or land runoff. Outbreaks of infection occurs when contaminated waters are used for irrigation purposes in agriculture, as sources of drinking water supply, for recreational activities (swimming, bathing, surfing, canoeing, etc.) and shellfish harvesting (Lipp and Rose, 1997; Jiang *et al*., 2001; Sinclair *et al*., 2012). Of all these vehicles of transmission, contaminated drinking water and shellfish have been reported to be the main vehicle of outbreaks of infection caused by enteric viruses. When assessing the risks associated with the transmission of infectious viruses and identifying the source of faecal contamination in waters, enteric viruses have shown great potential as indicators of faecal contamination in water quality measurements (Fong and Lipp, 2005). Table 2.2 highlight some report for enteric virus alerts associated with shellfish in Europe between 2008 and 2014.

Table 2.2: Report for enteric virus alerts associated with shellfish in Europe (Rapid Alert System for Food and Feed (RASFF), Annual Reports, 2008; 2009; 2010; 2011; 2012; 2013; 2014)

Year	Month	Virus	Shellfish	Notifying Country	Product Origin	Persons affected	Distribution
2008	January	Norovirus GI	Oysters	France	Spain	Outbreak	
	January	Norovirus	Oysters	Netherlands	France	Outbreak	
	April	Norovirus	Oysters	Norway	UK	6	
	September	Hepatitis A	Tellina clams	Spain	Peru	5	
2009	March	Norovirus	Oysters	Norway	Sweden	19	
2010	February	Norovirus	Oysters	Norway	France	37	Belgium, Hong Kong, Netherlands, Norway, Singapore, Switzerland, United Arab Emirates, Thailand
	February	Norovirus	Oysters	Ireland	Ireland	4	UK
	February	Norovirus	Oysters	Ireland	Ireland	Large outbreak	Ireland and UK
	March	Norovirus	Oysters	Denmark	France	23	Belgium, Italy, Denmark, Luxembourg
	March	Norovirus	Oysters	Denmark	France and Ireland	2	Belgium, Denmark, Germany, Italy Hong Kong, Netherlands, Russia Sweden
	June	Norovirus	Scallops	France	Chile	4	France and Italy
2011	January	Norovirus	Oysters	Denmark	France	11	Denmark
	March	Norovirus	Mussels	France	Netherlands, Ireland, UK	16	France, Denmark, Switzerland
	March	Noro.GI & GII	Oysters	Norway	Netherlands	16	Belgium, Germany, Norway
2012	February	Noro.GI & GII	Oysters	Norway	Ireland, via France	18	Austria, Denmark, Germany, Russia Italy, Hong Kong, Switzerland
	February	Noro.GI & GII	Oysters	Denmark	Ireland, via France	20	Belgium, Denmark, France, Polynesia, Germany, Hong Kong, Netherlands, Italy, Russia, Sweden
	February	Norovirus	Oysters	Denmark	Ireland, via Netherlands	4	Austria, Belgium, Denmark, Norway, Germany
	December	Norovirus	Oysters	Netherlands	"	59	Denmark
	December	Noro.GI & GII	Oysters	Denmark	France	15	Belgium, Hong Kong, Italy, Norway, Spain, Thailand

Year	Month	Virus	Shellfish	Notifying Country	Product Origin	Persons affected	Distribution
2013	January	Norovirus	Clams	France	Portugal, via Spain	5	France, Spain
	January	Norovirus	Oysters	France	Spain	8	France
	January	Norovirus	Oysters	Denmark	France	9	Belgium, Czech Republic, Denmark, Germany, Italy, Netherlands, Russia Sweden
	January	Norovirus	Oysters	Italy	France	3	Italy
	February	Norovirus	Oysters	Denmark	France	9	Denmark
	February	Norovirus	Oysters	Netherlands	France	7	France and Netherlands
	February	Noro.GI & GII	Oysters	Denmark	France	5	France
	March	Noro.GI & GII	Oysters	Denmark	Netherlands	10	Denmark and Netherlands
	March	Noro.GI & GII	Oysters	Norway	Spain, via Netherlands	37	Austria, Belgium, Denmark, Germany Norway
	April	Norovirus	Clams	Spain	Portugal	12	Spain
	April	Hepatitis A	Mussels	Italy	Slovenia		Italy
	May	Hepatitis A	Oysters	Italy	France, Netherlands	1	Italy
	July	Noro.GI	Oysters	Italy	France	9	Italy
2014	January	Norovirus	Oysters	France	Spain	3	France
	February	Noro.GI & GII	Oysters	Denmark	Netherlands	9	Denmark
	March	Norovirus	Oysters	Denmark	France	9	Denmark
	March	Norovirus	Oysters	Denmark	France	13	
	April	Norovirus	Oysters	Sweden	France, via Netherlands	8	Sweden
	July	Norovirus	Mussels	Spain	Spain		Italy, Romania and Spain
	September	Noro. GI & GII	Oysters	Denmark	UK	7	Denmark
	November	Noro. GII	Oysters	Norway	Ireland, via France		Norway
	December	Noro. GI & GII	Oysters	Norway	Ireland	10	Germany and Norway

Persons affected – reported at the time of the original notification

2.1.1 Adenovirus

Adenoviruses are medium-sized (90-100 nm), non-enveloped, double-stranded DNA-containing viruses belonging to the family *Adenoviridae*. Their nucleocapsid has an icosahedral shape (Figure 2.1). Of all the viruses implicated to date in water-borne diseases, adenoviruses are the only DNA viruses. The human adenoviruses (HAdV) have been classified into 57 serotypes in seven species (A-G), causing a wide range of illnesses (Jones *et al*., 2007). However, the species and serotypes associated with gastroenteritis are HAdV-F types 40, 41 and HAdV-G type 52. Adenoviruses are highly infectious and as low as one to ten viral particles are sufficient to induce infection with an incubation period of between 4 and 24 days. The mode of transmission of adenovirus is the faecal-oral route (Pond, 2005) and they may persist in the environment for a relatively long period following excretion in human faeces. This may be due to their structural properties, which enhance their resistance to different physical and chemical treatments, such as chloroform and ether. They have also been shown to be up to 60 times more resistant to ultraviolet irradiation than RNA viruses, probably as a result of their high molecular weight as well as the ability of their double-stranded DNA to serve as a template for repair by host enzymes (Fong and Lipp, 2005).

The detection of adenovirus in environmental samples has been described by many authors. Puig *et al*. (1994) described a nested polymerase chain reaction (PCR) amplification method used to detect adenovirus in nine river water and sixteen sewage samples. Pina *et al*. (1998) detected adenovirus in urban and slaughterhouse-wastewater, river water, seawater and shellfish samples using a DNA amplification technique by PCR. Calgua *et al*. (2011) developed an immunofluorescence assay for the detection and quantitation of infectious human adenoviruses and compared the assay with other

quantitative techniques such as plaque assays, tissue culture infectious dose-50 and quantitative PCR (qPCR) and found the immunofluorescence assay to have a higher sensitivity for the detection of infectious viral particles than other cell culture techniques evaluated.

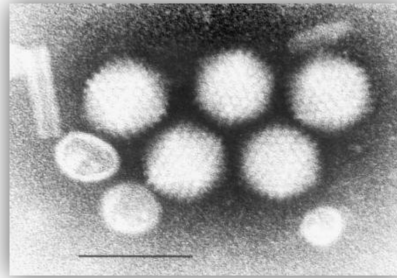


Figure 2.1: Electron micrograph of human adenovirus (Source: Ackermann, 2006)

2.1.2 Norovirus

Norovirus belongs to the family *Caliciviridae*. They are small (33 nm), non-enveloped, single-stranded RNA-containing viruses (Carstens, 2011). Their capsid is spherical and the genus contains various strains of a particular species, formally known as Norwalk virus (Figure 2.2). Noroviruses are the most common cause of viral gastroenteritis in humans, especially those associated with contact with food or water contaminated with faeces (Goodgame, 2006). Noroviruses can also be transmitted by contact with an infected person or contaminated surfaces (Said *et al*., 2008). The viruses are highly infectious with as few as five to twenty viral particles capable of causing an infection. Their incubation period is usually less than 24 hours and clinical symptoms include nausea, vomiting, and watery diarrhoea (Morillo and Timenetsky, 2011).

Noroviruses are genetically classified into five genogroups (GI-V); genogroups I, II and IV infect humans while genogroups III and V infect other, non-human, animals. However, most noroviruses associated with human gastroenteritis belong to genogroups I and II

(Vinjé *et al*., 2000). The detection of norovirus has been described by many authors. Jothikumar *et al*. (2005) used TaqMan-based one-step reverse transcription PCR to detect noroviruses in naturally contaminated shellfish samples. Le Guyader *et al*. (2009) detected and quantified noroviruses in shellfish by comparing a modified one-step reverse transcription PCR with previously established method in which Nuclisens magnetic silica-based guanidine was used for viral nucleic acid extraction. Similarly, Bosch *et al*. (2011) detected noroviruses in shellfish, soft fruits and water using reverse transcription PCR.

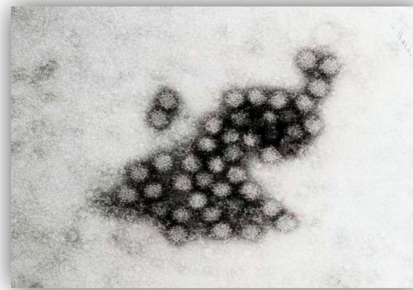


Figure 2.2: Electron micrograph of norovirus (Source: Schramlova *et al*., 2010)

2.1.3 Hepatitis virus

Hepatitis is a human disease in which the liver becomes inflamed. It is most commonly caused by hepatitis viruses though autoimmune disease and various toxic substances, including alcohol, may also be the cause (Gao and Bataller, 2011; Liberal *et al*., 2013). One of the causative viral agents of hepatitis is Hepatitis A virus (HAV). HAV is a small (40-60 nm), positive-sense, non-enveloped, single stranded RNA virus (Figure 2.3) that belongs to the *Picornaviridae* family (Minor, 1991). The mode of transmission of the virus is the faecal-oral route, especially via contaminated food and drinking water and its incubation period is between 15 and 45 days (Connor, 2005). Clinical symptoms are non-specific and include malaise, lassitude, myalgia and fever, which could lead to jaundice within two to seven days when not treated. Hepatitis E virus (HEV) has also been

implicated in waterborne disease outbreaks in tropical countries with problems associated with safe water supplies. HEV has similar clinical features to HAV but with a longer incubation period (15 to 60 days) (Hunter, 1997; Hughes *et al*., 2010). The detection of HAV and HEV is based on demonstration of specific immunoglobulin M and G (IgM and IgG) antibodies in clinical samples by cellular culture or enzyme-linked immunosorbent assay (ELISA). Molecular techniques using reverse transcription polymerase chain reaction have been described as a suitable method of detection after extraction and purification of viral RNA from food and environmental samples (Sanchez *et al*., 2007; Chigor and Okoh, 2012).

The occurrence of HAV in the environment is associated with human faecal contamination, since the virus is restricted to the human host and can only replicate within this host. Outbreaks of infection associated with drinking water, recreational water and raw or undercooked shellfish harvested from polluted waters have been described (Pinto *et al*., 2009; Sinclair *et al*., 2009; ECDC, 2013).

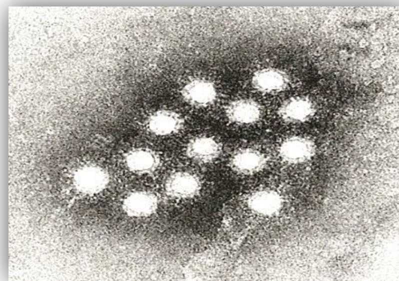


Figure 2.3: Electron micrograph of hepatitis A virions (Source: CDC, 2009)

2.2 Bacteriophages (phages)

The genome of phages is surrounded by a protein coat (capsid) that is made up of morphological subunits called capsomeres. The capsomeres consist of a number of protein subunits or molecules called protomers. Additional structures such as tails and spikes are present in some phages (Figure 2.4) (Grabow, 2001).

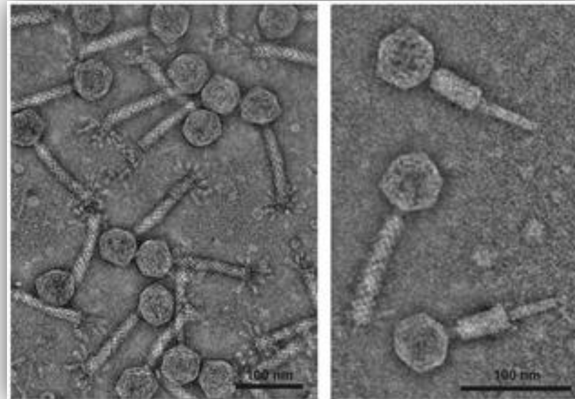


Figure 2.4: Transmission electron micrographs of phage PV94
(Source: Pryshliak *et al.*, 2014)

Phages can only multiply in metabolising host bacteria using the ribosomes, protein-synthesising factors, amino acids, and energy-generating systems of the host cell (Goyal *et al.*, 1987). Some phages have 30 to100 genes and are less dependent on the cellular functions of the host bacterium, whereas others have fewer than 10 genes and are dependent on the host's cellular functions (Freifelder, 1987). Host specificity is a common phenomenon observed in phages. It is a phenomenon in which a particular phage can only attach and infect a certain species of bacterium. Host specificity is always determined by the receptor sites on the surface of the bacteria and only certain phages will recognise these receptor sites and attach to them (Goyal *et al.*, 1987). Phage receptor sites are located on various parts of the bacterial host, particularly their cell wall. Somatic phages have the ability to recognise, attach and infect these host bacteria, as well as to attach to the host

including dead bacteria. Somatic phages include members of the families *Myoviridae, Siphoviridae, Podoviridae* and *Microviridae*. Male-specific phages can only recognise and attach to receptor sites located on the sex (fertility) fimbriae of host bacteria. These fimbriae are only produced by bacteria in the logarithmic growth phase under optimal growth conditions. Male-specific phages include members of the families *Inoviridae* and *Leviviridae* (Grabow, 2001). The replication of both somatic and male-specific phages in the gastrointestinal tract of humans and other warm-blooded animals is due to the optimal condition found in these gastrointestinal tracts, usually recording temperatures of not less than 30°C. Similarly, most human enteric viruses are released into the environment via the gastrointestinal tract, thus, suggesting a possible relationship between the presence of these phages and certain human viruses in terms of origin and release into the environment.

Phages are divided into two groups based on their mode of replication. These are: lytic (virulent) and lysogenic (temperate) phages. The lytic phages replicate after infecting the host cell and new viruses are released in large numbers by lysis (rupture) of the host cell within a few minutes of infection. These phages produce clear plaques on a lawn of susceptible host bacteria (Davies *et al.*, 1990). On the other hand, the lysogenic phages do not cause immediate lysis of host cells. When the nucleic acids of the phage are integrated into that of the host cell, they remain there for some time until they become induced to produce plasmids (episomes). Then, replication starts followed by cell lysis (Brock and Madigan, 1991). During lysogenic infection, normal harmless bacteria may become pathogenic because some lysogenic phages may convey new properties to the host bacteria. For example, Muniesa and Jofre (1998; 2000) examined the Shiga toxin-converting phages involved in the pathogenicity of *E. coli* O157:H7 and found that the

number of phages infectious for *E. coli* O157:H7 and carrying the Shiga toxin gene was in the range of one to ten per millimetre (approximately 1% of all phages infecting *E. coli* O157:H7) of sewage from two different origins. Phages may be grouped based on their host range, morphology, nucleic acid, strategies of infection, morphogenesis, phylogeny, serology, sensitivity to physical and chemical agents and dependence on the properties of the hosts and the environment (Abeles *et al*., 1984). Goyal *et al*. (1987) classified phages into three groups according to the location of receptor sites on the host bacterium:

i. Appendage phages – attach to host bacterium appendages, such as pili and flagella.
ii. Capsule phages – attach to the outer layer of the host bacterium, such as the polysaccharide capsule.
iii. Somatic phages – attach to the host bacterium cell wall.

Most of these phages, as listed in Table 2.3, have been detected in a variety of aquatic environments (Tartera and Jofre, 1987; Havelaar *et al*., 1990; Grabow *et al*., 1993, 1998).

Phages have been used as indicators of faecal pollution in water quality assessments for over three decades. Typical examples include somatic coliphages, male-specific F-RNA coliphages and phages infecting *Bacteroides fragilis.* These groups of bacteriophages are part of the numerous tools suggested for potential use in microbial source tracking (MST) i.e., distinguishing sources of faecal pollution within a particular body of water (Malakoff, 2002; Jofre *et al*., 2011; 2014). Also included in these MST groups are bacteriophages infecting certain *Enterococcus* strains (Yasmin *et al.*, 2010; Purnell *et al.*, 2011; Santiago-Rodriguez *et al.*, 2010; 2013).

Table 2.3: Important phages in water quality assessment classified according to their morphology and nucleic acid content (Maniloff and Ackermann, 1998; Grabow, 2001).

Family	Morphology	Nucleic acid	Member
Myoviridae	cubic capsid (icosahedral or elongated), long contractile tail	linear dsDNA	coliphage T4, P1, Mu
Siphoviridae	cubic icosahedral capsid long non-contractile tail	linear dsDNA	coliphage T5, 1
Podoviridae	cubic icosahedral capsid short non-contractile tail	linear dsDNA	coliphage T7, enterobacter phage P22
Microviridae	cubic icosahedral capsid	circular ssDNA	coliphage £X174
Inoviridae	filamentous or rod-shaped	circular ssDNA	coliphage f1, fd and M13
Levividae	cubic icosahedral capsid	linear ssRNA	enterobacterio-phages MS2, Qß
Cystoviridae	enveloped icosahedral capsid	segmented dsRNA	
Lipothrixviridae	enveloped rod-shaped capsid	linear dsDNA	
Corticoviridae	lipid-containing icosahedral capsid	circular dsDNA	
Tectiviridae	lipid-containing double icosahedral capsid	linear dsDNA	
Plasmaviridae	enveloped pleomorphic capsid	circular dsDNA	
Rudiviridae	non-enveloped rod-shaped capsid	linear dsDNA	
Fuselloviridae	non-enveloped lemon-shaped capsid	circular dsDNA	

DNA–Deoxyribonucleic acid; RNA–Ribonucleic acid; ds–double strand; ss–single strand.

2.2.1 Somatic coliphages

These are lytic phages that are members of the families *Myoviridae, Siphoviridae, Podoviridae* and *Microviridae*. They infect the bacterial host by attaching to receptor sites on the cell wall of the host, replicate in large numbers and then lyse the host cell. Their hosts include *Escherichia coli* and closely-related members of the bacterial family Enterobacteriacea (Hayes, 1968). Studies have shown that somatic coliphages in wastewater and raw water sources generally outnumber F-RNA coliphages as well as cytopathogenic human viruses (Grabow *et al.*, 1993). Somatic coliphages are thus

potentially useful indicators of the presence of enteric viruses in aquatic environments (Grabow *et al*., 1993). They are also detectable by relatively simple, inexpensive and rapid plaque assays (Grabow *et al*., 1998; Anon., 2012).

2.2.2 Male-specific F-RNA coliphages

F-RNA coliphages are single-stranded RNA phages that adsorb specifically to fertility (F) fimbriae (sex-pili), coded by the F-plasmid of *E. coli* K-12 (Havelaar and Pot-Hogeboom, 1988), which has been successfully transferred to *Salmonella typhimurium* as well as *Shigella* and *Proteus* species making recipient cells susceptible to male-specific coliphages. However, since F-encoded pili are synthesised at temperatures above 30°C, F-RNA coliphages are not likely to replicate in environments other than that of the gastro-intestinal tract of warm-blooded animals (Birge, 1981).

The physical structure, composition, and morphology of F-RNA coliphages, as well as their inability to replicate in water environments, closely resemble the characteristics of many human enteric viruses. These features therefore qualify them as potential surrogates for human enteric viruses contaminating environmental samples (IAWPRC, 1991; Havelaar *et al.,* 1993). The detection of F-RNA coliphages is relatively difficult compared with that of somatic coliphages since the F-fimbriae with receptor sites for the phage are produced only by the host bacteria during the exponential phase of growth. Therefore, the preparation of host culture for plaque assays must be timed carefully to ensure that the host cell concentration is within the exponential growth phase (Grabow *et al*., 1998).

2.2.3 Phages infecting *Bacteroides fragilis*

Bacteroides fragilis is an obligate anaerobic, Gram-negative, non-sporulating bacterium found in the gastrointestinal tract of humans. The genus *Bacteroides* is found in large quantities (more than 10^9-10^{10}/g) in human faeces (Salyers, 1984) and is rapidly inactivated by environmental oxygen levels. However, phages infecting *Bacteroides fragilis* are resistant to unfavourable conditions (Jofre *et al*., 1995) and are also useful for water quality monitoring, especially in differentiating faecal sources from human origin to those of non-human origin.

Jofre *et al.* (2014) critically reviewed the use of bacteriophages infecting *Bacteroides* as a marker for microbial source tracking and noted that phages infecting *Bacteroides fragilis* HSP40 (Tartera and Jofre, 1987; Gantzer *et al*., 1998b), GA17 (Payan, 2006, Jebri and Yahya, 2013) and GB124 (Ebdon *et al*., 2007; 2012) were detected in 100% of standard secondary effluents from wastewater treatment plants. Plaque assays for detecting *Bacteroides fragilis* phages are more complex than those for somatic and F-RNA coliphages. Their growth media must be supplemented with antibiotics and plates have to be incubated under strictly anaerobic conditions (Anon., 2001a).

2.3 The choice of host bacterial strain for phage enumeration

Phages are highly specific to the type of bacterium they attack and a wide variety of host bacterial strains have been used for the enumeration of phages (Grabow, 2001). Several strains of *E. coli* had been used for detecting somatic coliphages as a result of their exposed receptor sites, which makes them highly susceptible to phages (Havelaar and Pot-Hogeboom, 1983). These include: wild-type *E. coli, E. coli* B, *E. coli* C (WG4) and *E. coli*

K-12. Of all these laboratory host strains of *E. coli,* the most commonly-used strain for detecting somatic coliphages in water environments is *E. coli* WG5 (Anon., 2000a).

E. coli K-12 produces fimbriae as it carries a fertility (F) plasmid in its genes and the receptor sites for male-specific coliphages are located on the fertility fimbriae of the *E. coli* strain (Primrose *et al*., 1982). This strain was originally used for the detection of male-specific F-RNA coliphages, until the F plasmid was transferred to a strain of *Salmonella typhimurium* through genetic engineering (Havelaar *et al*., 1984). The genes coding for pathogenicity in *S. typhimurium* were also deleted for health and safety reasons as the organism became a host strain used for routine analysis in most laboratories. Presently, the strain of *S. typhimurium* WG49 is now widely used for detecting male-specific F-RNA coliphages (Anon., 2001b).

Bacteroides fragilis strain RYC2056 has been used as a host for detecting phages of both human and non-human origin in water quality assessments (Puig *et al*., 1999). Also, phages that infect certain strains such as *Bacteroides fragilis* HSP40, were detected in the faeces of human but not in those of other warm-blooded animals (Tartera and Jofre, 1987; Grabow *et al*., 1993). Similarly, Ebdon *et al*. (2007; 2012) isolated human-specific phages of a *Bacteroides fragilis* host (GB124) for the identification of faecal pollution from human sources in aquatic environments and suggested that these phages are useful for distinguishing between faecal pollution of human and non-human origin. Other potential human-specific host strains, isolated by Jofre *et al.* (2014), include *B. tethaioataomicron* GA17 (Payan *et al*., 2005; Blanch *et al*., 2006; Jebri and Yahya, 2013), *B. fragilis* HB13 (Payan *et al*., 2005) and *Bacteroides* sp. ARABA 84 (Wicki *et al*., 2011).

2.4 Phages in their natural environment

Phage ecology refers to the occurrence and interaction of bacteriophages (viruses that infect bacteria) and their natural environment (Clokie *et al*., 2011). A large number of these phages occur in both human and non-human animal wastes as well as in water environments, such as fresh water and marine water environments (Bergh *et al*., 1989). Phages commonly isolated from aquatic environments belong to the groups somatic coliphage, F-RNA coliphage and phages infecting *Bacteroides fragilis*.

The survival, replication and behaviour of phages in water environment are affected by many varying factors. These include: the presence and densities of host bacterial strains; the presence of organic matter, which influences the metabolic activity of the host bacterium; organic compounds such as humic and fulvic acids, which influence the attachment of phages to their host bacterium as well as to sediments and other solids; the concentration and type of ions, e.g., cations such as calcium and magnesium ions promote phage adsorption to host bacteria; ultraviolet and visible light; temperature; and pH level (Havelaar and Pot-Hogeboom, 1983; Sobsey and Hickey, 1985; Goyal *et al*., 1987).

Studies have demonstrated high counts of somatic and F-RNA coliphages from lake and river waters exposed to human and non-human faeces (Araujo *et al*., 1997; Grabow *et al*., 1998). Similarly, Tartera *et al*. (1989) isolated high counts of phages infecting *Bacteroides fragilis* from wastewater polluted river. Phages infecting *Klebsiella pneumoniae* and *Salmonella* spp. have also been recovered from fresh water environments (Goyal *et al*., 1987).

Seawater contains high concentrations of salt (sodium chloride), providing an ionic environment among other factors, such as anaerobic conditions, temperature and pressure, which influence the survival of phages in marine environments. Somatic and F-RNA coliphages, including phages of *Bacteroides fragilis* have been recovered from sea water (Tartera and Jofre, 1987; IAWPRC, 1991).

Wastewaters are a mixture of water and wastes from domestic, industrial or agricultural activities and often contain solids, chemicals, organic matter, microorganisms, etc. High counts of somatic coliphages, F-RNA coliphages and phages of *Bacteroides fragilis* have been observed in wastewaters (Tartera *et al*., 1989; IAWPRC, 1991).

Shellfish are of great economic value in the United Kingdom, France, United States, Canada, Australia and many other parts of the world. These sea animals (oysters, mussels, clams, scallops, cockles, etc.) feed by filtering large volumes of water and consequently bioaccumulate microorganisms together with food particles causing little or no harm to the animal, but posing substantial risks to human consumers (NOAA, 1998). Enteric viruses, bacteria and phages have been isolated from contaminated shellfish samples (Rippey, 1994; Fong and Lipp, 2005; Bosch, 2010). Depuration, a process used for purifying contaminated shellfish has shown that enteric viruses and phages persist longer than bacteria in the shellfish and often exhibit similar patterns of behaviour (Havelaar *et al*., 1993; Payment and Locas, 2011). However, these phages have been proposed as tools for assessing the virological safety of shellfish intended for human consumption (Grabow *et al*., 1999b).

2.5 Phages as surrogates of enteric viral pathogens

Bacteriophages have been used as surrogates of viral contamination in surface waters because they have many characteristics that are similar to those of mammalian viral pathogens and they pose little risk to human health (Tufenkji and Emelko, 2011). Ballester *et al*. (2005) evaluated a two-step enrichment procedure to detect coliphages and an integrated cell culture-nested polymerase chain reaction to detect human astrovirus, enteroviruses, rotavirus and adenoviruses in water samples and found significant correlation between the coliphages and the human enteric viruses. Ogorzaly *et al*. (2009) also demonstrated a positive correlation between bacteriophages (F-specific RNA) and enteric virus (human adenovirus) in river water. Phages may therefore be regarded as conservative indicators of the presence of enteric viruses.

2.6 Methods of detecting phages in the environment

The internationally accepted method to date for the enumeration of phages in environmental and food samples, such as water, sewage and shellfish samples, is by direct quantitative plaque assays with double-agar layers in petri dishes of 90 mm diameter with 1 ml of sample tested (Anon., 2000a; 2001a; 2001b). The upper agar layer is a semi-solid agar containing nutrients specific for the phage to be quantified, mixed with 1 ml of test sample and a culture of a host bacterium specific for the phage, while the lower agar layer is a solidified agar containing the same nutrients as the semi-solid agar. To enhance the visibility of plaques and to prevent interference with smooth growth of host bacterium by other bacterial contaminants, antibiotics such as nalidixic acid and kanamycin monosulphate may be added to the agar media. Other reagents, such as calcium glucose solution, calcium chloride, sodium carbonate, 35% hydrochloric acid and haemin solutions

(iron source) are added to the basal agar medium depending on the type of phage investigated (Anon., 2000a; 2001a; 2001b).

2.6.1 Molecular detection methods: In recent years, advanced culture-independent genetic detection methods have also been developed for the detection and enumeration of phages and viruses in different samples. These molecular techniques normally involve the use of real- time polymerase chain reaction (qPCR) and can also be used for quantitative detection of enteric viruses, such as norovirus and hepatitis A virus in shellfish (CEFAS, 2012). In general, methods for the recovery of phages should be rapid, inexpensive and, simple, with high efficiency, maximum yield and be suitable for a wide range of environmental samples (Grabow, 2001; Ebdon *et al*., 2012). Molecular detection methods are rapid but costly compared with culture-based phage detection and they do not indicate the infectivity of phages.

2.6.2 Adsorption-elution: In this technique, the sample to be assayed for phages is filtered through a filter medium (47 mm diameter, 0.45 µm pore size) to which the phages adsorb. This adsorption is possible because filter media are negatively charged, whereas the phages have electrostatic charges that become positive at an acidic pH level of 3.5 (Grabow, 2004). Following the filtration process, the filter is then rinsed with an eluent, usually a buffer solution, at pH 9.5. This enhances the release of phages adsorbed to the filters, which may then be detected using a conventional method, such as a quantitative plaque assay (Anon., 2012). A wide range of filter media have been used successfully, namely:

i. Electronegative adsorbent filters: Such as cellulose nitrate and fibre-glass acrylic resin membrane filters with 0.45 µm pore sizes.

ii. Glass powder: An electronegative filter that uses a buffer with a high pH level (11.5) for phage elution from the glass powder. This high pH level is a limitation as most phages are often inactivated at extreme pH levels (Seeley and Primrose, 1982).

iii. Electropositive membrane filters: These filters have been reported to have higher yield and recovery rates than negatively charged filters. Examples include: Plus 50-S, Plus 60-S, CUNO Zeta, etc. (Grabow *et al*., 1993).

iv. Charge modified glass powder: Treating glass powder with polyethylenimine changes its surface charge from negative to positive (Gajardo *et al*., 1991). The modified glass powder has been demonstrated to have a relatively good recovery rate but shortcomings have been observed with adsorption-elution technique (Grabow, 2001), as a result of potential interference by dissolved and colloidal substances in water competing with viruses for adsorption on filters.

v. Glass wool: Columns of glass wool are suitable for routine recovery of viruses from large volumes of water using beef extract-glycine buffer at pH 9.5 (Vilaginès *et al*., 1997).

2.6.3 Ultrafiltration: Particles of diameter greater than 0.02 nm cannot pass through membrane filters, which are usually made of polysulphonate material in the ultrafiltration process. Phages and viruses have diameters greater than the pore sizes of the filters. Thus, they are retained on the filters. In the ultrafiltration setup, the water to be filtered is kept in constant motion using a recirculating pump and a stirring apparatus to prevent clogging of the filters (Jansons and Buccens, 1986). The volume of water sample is restricted as the filters have been observed to clog rapidly. Ultrafiltration is relatively expensive but results in high efficiency of recovery of phages and viruses from water samples. However, its efficiency may be impaired if the water is highly turbid because viruses adsorb to particulate matter. This may be prevented by pre-treating the membranes with beef extract,

which has the ability to block potential adsorption sites for viruses (Divizia *et al*., 1989, Brenner, 2013).

2.6.4 Flocculation: Flocculation involves the use of flocculants, such as aluminium hydroxide (Al $(OH)_3$), for the recovery of viruses from water samples. Viral surfaces are negatively-charged while aluminium hydroxide surfaces are positively-charged, causing an electrostatic interaction to occur that leads to the formation of flocs. Viruses are recovered by centrifugation or filtration following vigorous shaking to disintegrate the flocs (Anon., 2012).

2.6.5 Hydro-extraction: In hydro-extraction, the water sample is placed in a cellulose dialysis bag and exposed to hygroscopic polyethylene glycol, which extracts the water and micro-solutes through the semi-permeable membrane. During this process, viruses and macro-solutes are trapped in the membrane (Anon., 2012).

2.6.6 Secondary concentration methods: These procedures aim to reduce further the volume of concentrate generated initially from a viral or phage recovery method such as by using smaller filters to reduce the volume of adsorption-elution liquid from 100 ml to 10 ml (Anon., 2012). Similarly, concentrates generated from other methods, such as hydro-extraction, flocculation and ultrafiltration can also be reduced using secondary concentration procedures.

2.6.7 Direct plaque assays on large sample volumes: This method involves mixing about 100 ml of water sample with concentrated agar medium in a molten state, adding the bacterial host-culture and pouring the mixture into 140 mm diameter petri dishes. The

plates are allowed to set before incubating (aerobically or anaerobically) at an appropriate temperature in an inverted position. Plaques are enumerated and recorded, usually after 24 hours. This method was later modified into the double-agar layer plaque assay by pouring the mixture into equal volumes on top of a bottom agar layer in ten large Petri dishes (Grabow *et al*., 1998; Hayward, 1999).

2.6.8 Qualitative presence-absence enrichment tests: In qualitative presence-absence tests, nutrients and bacterial host culture are added to the volume of water sample to be tested for phages. The mixture is incubated appropriately depending on the type of phage being investigated. Following incubation, replicated phages are detected easily using simple methods. Although the number of phages present in the water sample cannot be quantified, this method yields relatively good qualitative results and has been widely used by researchers and water standard regulation agencies (Grabow *et al*., 1993; WHO, 1997; Hayward, 1999).

2.7 Influence of environmental factors on microbial behaviour and survival

Environmental factors, such as biotic and abiotic factors, can affect the behaviour and survival of an organism in the aquatic environment (Table 2.4). The physical and chemical composition of the surrounding liquid, air or solid necessarily influences the survival of organisms (Sinclair *et al*., 2012). In this section, some factors that influence the survival and behavioural dynamics of faecal indicator bacteria, enteric viruses and bacteriophages are critically evaluated.

Table 2.4: Influence of environmental factors on microbial behaviour and survival

Parameter	Effect	Reference
pH	Optimum pH is between 6 and 7. Acidic and alkaline pH levels reduces microbial count	Solic and Krstulovic, 1992 Fong and Lipp, 2005 Sinclair *et al*., 2012
Temperature	Negative effect on microbial growth when temp. is above or below optimum. Temp. requirement for mesophilic microbes (20-45°C), thermophilic microbes (>45°C), psychrophilic microbes (<20°C).	Melnick and Gerba, 1980 Jiang *et al*., 2001 Fong and Lipp, 2005
Salinity	High salinity reduces microbial count, except halophilic microbes.	Solic and Krstulovic, 1992 Gantzer *et al*., 1998a
Ultraviolet (UV) radiation	Exposure to UV radiation inactivates microorganisms	Gerba *et al*., 2002 Diston *et al*., 2012
Nutrients	Dissolved organic matter increases microbial growth	Melnick and Gerba, 1980
Microbial antagonism	Microorganisms may be inactivated by substances produced by other organisms	Wang *et al*., 2012
Adsorption to solids and sediments	Solids and sediments to which microbes attach in water systems may offer protection against ultraviolet radiations or other inactivating factors	Gantzer *et al*., 1998a Brookes *et al*., 2004
Dilution and osmotic shock	Microorganisms in wastewater may experience osmotic shock upon sudden exposure to seawater, causing their cytoplasmic membrane to rupture	Grabow *et al*., 1999a
Bioaccumulation	Microbial counts have been shown to be far greater in shellfish than in overlying waters	Observed in this study. Trajano Gomes Da Silva, 2013 Grodzki *et al*., 2014
Biofilms	Formation of biofilms by microorganisms aid their survival and resistance to predation and external attacks	Whitehead and Verran, 2009
Turbidity	Turbid waters shield microbes from effects of ultraviolet radiations	Sinclair *et al*., 2012

2.7.1 pH level: Solic and Krstulovic (1992) observed that the optimum pH level for the survival of faecal coliform bacteria is between pH 6 and 7 with a rapid decline in their counts both above and below these pH values. Most organisms exhibit the greatest stability at near-neutral pH (Sinclair *et al*., 2012). However, enteric viruses can survive over a wide pH range, usually between pH 3 and 10, for extended periods at low temperatures (Kocwa-Haluch, 2001; Fong and Lipp, 2005). Moreover, bacteriophages have limited chances of survival at acidic pH levels close to the isoelectric point of the viral particle. Langlet *et al*. (2007) observed a significant aggregation process in the reduction of plaque-forming unit counts of MS2 phage when the pH level was reduced from 3.9 (isoelectric point) to 2.5.

2.7.2 Temperature: In aquatic environments, a temperature above 37°C is not favourable to the survival of mesophilic bacteria (optimum growth at moderate temperatures between 20 to 45°C) as it denatures their proteins, except spore-producing bacteria or thermotolerant bacteria that thrive at relatively high temperatures between 45 and 122°C, e.g., *Bacillus stearothermophilus* and psychrophilic microorganisms that grow best at temperatures below 20°C, e.g., *Listeria* sp. (Melnick and Gerba, 1980). Viral capsid and nucleic acids are also damaged by temperatures above 45°C, thus, preventing the adsorption of the virus to its host and enzymes required for replication may also be inactivated (Bitton, 1980). Burkhardt *et al*. (2000) investigated the inactivation of faecal indicator microorganisms such as *E. coli*, *Clostridium perfringens* and male-specific bacteriophages in estuarine waters. The authors examined the effects of a range of physicochemical factors on the survival of the selected indicator microorganisms and observed that temperature/sunlight exhibited the most significant effect on the decay rates of the faecal indicator microorganisms. Male-specific bacteriophage, *Clostridium perfringens* and faecal coliforms had 83, 84 and 99% density reductions, respectively,

upon exposure to high temperature and/or sunlight. Similarly, Seo *et al*. (2012) examined the effect of temperature on the inactivation kinetics of murine norovirus and coliphage MS2. The authors observed that both murine norovirus and coliphage MS2 were rapidly inactivated at temperatures above 60°C

Faecal indicator bacteria are inactivated at relatively high temperatures but persist in the environment at temperatures below 37°C, whereas, enteric viruses and bacteriophages have been reported to survive and still retain their infectivity for up to four or five months in seawater, freshwater and wastewater at 20 to 30°C (Jiang *et al*., 2001). Viruses however generally persist longer than bacteria in the environment, although they might not replicate since they are obligate parasites and require a host (Fong and Lipp, 2005).

2.7.3 Salinity: Salinity refers to the amount of dissolved salt in a body of water and is expressed in parts per thousand (ppt) or grams per litre (g/l). The aquatic environment is categorised as seawater or marine, estuarine or brackish water and fresh water based on the degree of salinity. The major chemical ions contributing to salinity include: chloride, sodium, sulphate, magnesium, calcium, potassium, bicarbonate, bromide, borate, strontium and fluoride (Anderson, 2008). Generally, most microorganisms persist longer in fresh water than marine water environments except halophilic organisms that live in highly saline environments. However, increasing salinity is detrimental to faecal indicator bacteria (Solic and Krstulovic, 1992; Gantzer *et al*., 1998a). Darakas *et al*. (2008) investigated the effect of wastewater dilution in seawater on the concentration of faecal indicator bacteria in sewage and compared their decay rate to those in the effluent from a secondary biological treatment. The authors observed that the dilution of wastewater in seawater reduces the counts of faecal indicator bacteria in the wastewater at a faster rate than that of

the secondary biological treatment. Similarly, Jabari *et al*. (2015) investigated the effect of salinity on the bacterial diversity shift of anaerobic batch cultures treating abattoir wastewater. The authors observed that bacterial diversity varies depending on the culture conditions, and they reported that an increase in salt concentration from zero to 20 and 40 g/l at thermophilic condition caused a significant reduction in bacterial diversity. The authors suggested that the observed reduction in the bacterial community diversity may be due to a pressure generated by elevated salinity, thus eliminating salt-sensitive bacterial species.

2.7.4 Ultraviolet radiation / sunlight: Ultraviolet (UV) solar radiation has wavelengths (200-400 nm) and vibrates at very high frequency. Wavelengths are grouped as UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (200-280 nm). They have been shown to inactivate most microorganisms found in the aquatic environment but the surface of a water body receives more and direct UV light compared with the deeper zones. This explains why microorganisms at the bottom of a water column or those attached to sediments are less liable to inactivation than those at the surface (Gantzer *et al*., 1998a).

The concentration of faecal indicator bacteria generally reduces when exposed to sunlight and has been used in engineered systems to measure the UV light dose for water disinfection (USEPA, 2003). Studies have also shown that enteric viruses and bacteriophages inactivated in sunlight are ten times higher than those inactivated in the dark (Sinton *et al*., 2002). Diston *et al*. (2012) examined the effect of UV-C radiation (254 nm) on candidate microbial source tracking phages infecting a human-specific strain of *Bacteroides fragilis* (GB124) and found that phages infecting GB124 were inactivated by the levels of UV-C radiation routinely delivered during tertiary wastewater treatment

processes. Viruses are less susceptible to the effect of UV radiation than many other pathogens and indicator bacteria as a result of their low molecular weight (Fujioka and Yoneyama, 2002). However, those with double-stranded DNA or RNA are particularly stable since their undamaged nucleic acid may serve as a template for repair by host enzyme (Gerba *et al*., 2002).

2.7.5 Nutrients: Organic matter dissolved in aquatic environments is a source of nutrient to the microbial community in that environment. In addition to this, it may also offer protection to these organisms during disinfection interventions by buffering the effect of the chemical processes (Carpenter *et al*., 1999). The pollution of a water environment by indiscriminate discharge of wastewater may increase the population of faecal indicator bacteria in that environment. This may also trigger the replication of viruses, as their host bacteria become abundant. Antagonistic substances may also be neutralised by the organic matter (Melnick and Gerba, 1980).

2.7.6 Microbial antagonism and predation: Enteric viruses and some other pathogens may be inactivated by compounds, such as polysaccharides produced by certain marine organisms (Wang *et al*., 2012). Predation on microorganisms by higher organisms is also a common feature of the aquatic environment (Melnick and Gerba, 1980; Grabow *et al*., 1999b).

2.7.7 Adsorption to solids and sediments: Solids and sediments offer protection to viruses in natural water environments, thus enhancing their persistence in the environment. Viruses that adsorb to sediments are more resistant to inactivation by ultraviolet radiations, enzymes or other degrading factors (Gantzer *et al*., 1998a). Counts of viruses have also

been shown to be far greater in marine sediments than in overlying waters, irrespective of the season (Grabow *et al*., 1999b). Pathogenic microorganisms that adsorb to suspended solids and sediments in water environments have been suggested as warranting particular consideration during water quality assessment for public health risks, since they are likely to be resuspended or redistributed in the water column by natural or anthropogenic disturbance (Brookes *et al*., 2004).

2.7.8 Dilution and osmotic shock: Dilution of wastewater normally occurs as soon as it is discharged into the aquatic environment and the extent of dilution is obviously dependent on the volume of the wastewater and that of the receiving water. Microorganisms in wastewater may experience osmotic shock upon sudden exposure to seawater, causing their cytoplasmic membrane to rupture. Bacteria are the group of microorganisms mostly affected by osmotic shock and viruses are less affected since they do not possess a cytoplasmic membrane (Grabow *et al*., 1999a).

2.7.9 Bioaccumulation in shellfish: Microorganisms survive longer in the gastrointestinal tract and tissues of sea animals such as molluscs (shellfish) than in seawater environments (Grabow *et al*., 1999b). Shellfish are filter-feeding aquatic animals that can bioaccumulate pathogens from contaminated water, thereby posing a potential risk of infection to human consumers. Nappier *et al*. (2009) examined how co-localisation of *Crassostrea virginica* and *C. ariakensis* (oysters) in tanks of seawater differ in bioaccumulation, retention and depuration of microbial indicators and enteropathogens such as norovirus and hepatitis A virus. The authors observed that *C. ariakensis* was more likely to harbour microbial indicators and enteropathogens compared to *C. virginica* and suggested that *C. ariakensis*

may present a major public health threat to human consumers especially when the oysters are consumed raw from contaminated sites.

The biological property of the shellfish tissues influences the pattern of accumulation of different pathogens observed in different shellfish species (Polo *et al*., 2014). Several studies have shown that bioaccumulation of pathogens in shellfish (either experimentally under controlled conditions, or naturally in their harvesting waters) is dependent on a number of factors; including physiology of the shellfish and/or pathogen, the rate of metabolism in the shellfish, the duration of exposure of the shellfish to the pathogen or its source, the exposure dose, along with other factors such as salinity and temperature that can also influence the filtration rate in the shellfish (Graczyk *et al*., 2006; Nappier *et al*., 2008, 2009). Grodzki *et al*. (2014) examined the bioaccumulation efficiency, tissue distribution, and environmental occurrence of hepatitis E virus in shellfish. The authors compared the bioaccumulation efficiencies of oyster, flat oysters, mussels and clams at different periods during the year, and they observed that most of the viruses were concentrated in the digestive tissues of the four shellfish species, and mussels and clams were observed to be more sensitive to sporadic contamination events as demonstrated by their rapid bioaccumulation of the virus.

There appears to be a differential selection process involved in bioaccumulation, based on the size and shape of particles, which is influenced by the uptake efficiency of the shellfish species as well as the ability of the labial palp and gills to reject or filter certain particles (Espinosa *et al*., 2008; Willis *et al*., 2014). Roslev *et al*. (2009) investigated the uptake, accumulation, and persistence of human associated *Enterococcus* in mussels. The rationale for the study was that microorganisms and molecular markers for microbial source tracking

in coastal waters are often present in low quantities with significant variation with time. The authors observed that the molecular markers were often not detectable in seawater but were detectable in mussels within four to six hours of faecal contamination and were bioaccumulated to levels up to 300 times greater than in the seawater. The authors suggested that mussels should be considered as additional targets in microbial source tracking studies in coastal waters.

2.7.10 Biofilms: The formation of biofilms by microorganisms is common in the aquatic environment and it plays a major role in the survival and resistance of the organisms against predation and external attacks. A biofilm is a microbial community composed of cells attached to a substrate or an interface or to each other by extracellular polymeric substances such as slime (Whitehead and Verran, 2009). Matz *et al.* (2008) observed evidence of chemically-mediated resistance against protozoan predators as a common feature among biofilm populations in a diverse set of marine bacteria.

2.7.11 Turbidity: The volume of suspended organic or inorganic particles in the water environment determines its level of turbidity. Studies have shown that microorganisms are inactivated when exposed to solar ultraviolet radiation in water systems (Kokjohn *et al*., 1994). However, in highly turbid waters these organisms are shielded from the effects of ultraviolet radiations (Sinclair *et al*., 2012).

Chapter Three: Materials and Methods

3.1 The fieldwork study area

The principal field study site was situated at Piddinghoe, on the estuary of the River Ouse, which is the second largest river in the county of East Sussex in southern England, draining 396 km^2 to its tidal limit. The river is one of the four rivers that cut through the South Downs and flows directly into the English Channel. The river catchment contains tributaries that flow through urban areas, such as Haywards Heath, Lindfield, Uckfield, Lewes and Newhaven (Figure 3.1). However, the catchment is predominantly rural and is located in a region that encompasses a diverse range of contaminant sources. The water from the river is used as a source of raw drinking water (abstracted by Southeast Water at Barcombe Mills); for discharge of treated wastewater (e.g., by Southern Water at Scaynes Hill); and for urban

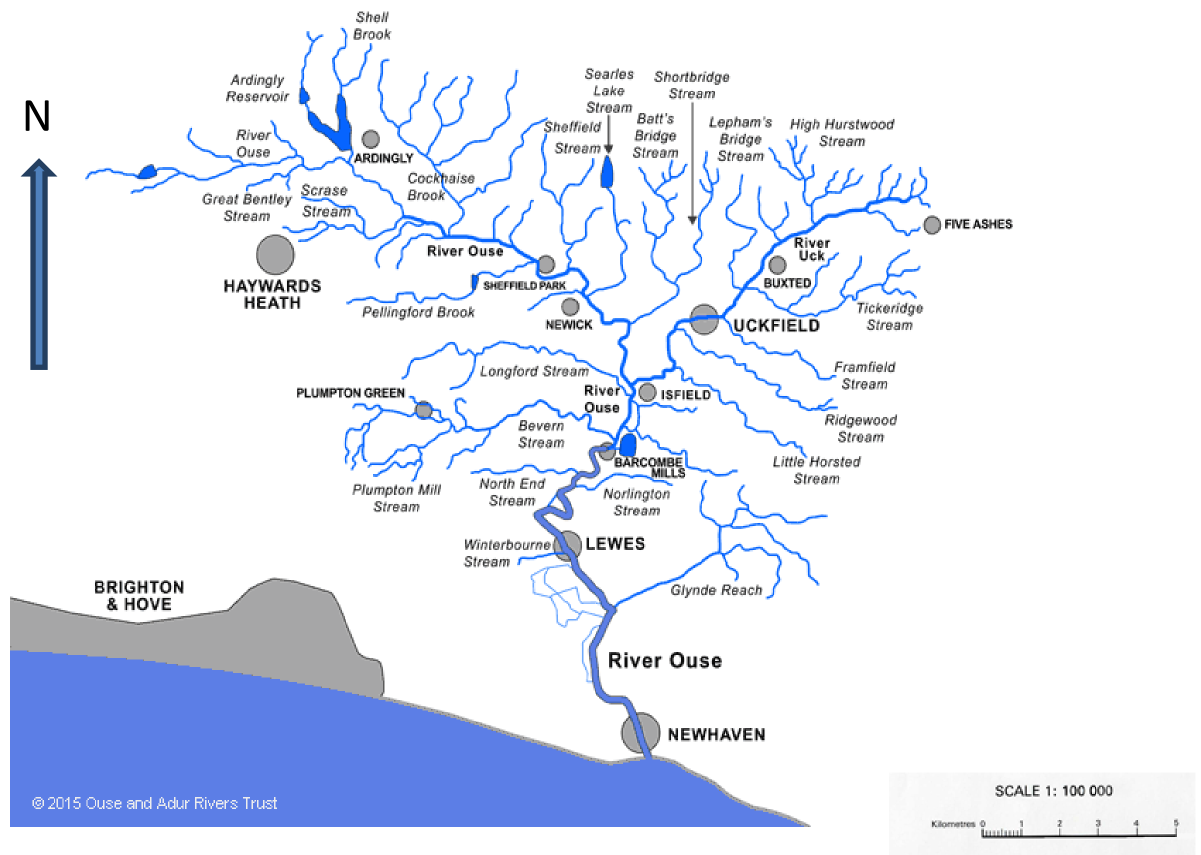


Figure 3.1: The River Ouse catchment
(Adapted from Sussex Ouse Map – Ouse and Adur Rivers Trust)

and rural drainage. In terms of land use, agriculture accounts for approximately 65% of the land area, with 56% of this being used for livestock grazing. Poultry and sheep constitute by far the greatest numbers of farm animals, accounting for 89% and 9.7% of total farm animals, respectively (Environment Agency, 2006; Ebdon *et al*., 2007; Nnane *et al*., 2011).

3.2 Collection of shellfish samples on River Ouse

The Piddinghoe site (Figure 3.2) is a non-commercial site and was selected because of its rich mussel (*Mytilus edulis*) bed and its proximity to the laboratory facilities used for this research.

Figure 3.2: Close-up of the mussel bed at Piddinghoe during low tide

On each sampling occasion (every two weeks over a period of twenty-four months) shellfish and overlying water samples were collected from Piddinghoe at low tide and tested for microbial and physicochemical parameters in order to provide a robust database that was suitable for predictive modelling purposes. Hydrological information such as rainfall, river flow and meteorological data, was also obtained during this period. Additional shellfish samples (Pacific oysters and mussels) were obtained by hand-piking or raking from sites in southwest England (courtesy of the Centre for Environment, Fisheries

and Aquaculture – CEFAS, as part of method validation and exchange of samples within the RiskManche project) and were analysed, as above.

3.2.1 Shellfish samples

Live shellfish samples – mussels (*Mytilus edulis*) that were about six to ten centimetres in length; Pacific and native oysters (*Crassostrea gigas* and *Ostrea edulis*) that were about ten to fifteen centimetres in length (Figure 3.3) were collected in accordance with EU Regulation 854/2004 (CEFAS, 2009). These samples were collected by either hand-picking/raking (Figure 3.4) or dredging. The sample sizes collected, following the recommendations of CEFAS, were 18-35 for mussels and 12-18 for oysters, to avoid inherent animal to animal variation in microbial concentration.

3.2.2 Overlying water samples

River water samples (Figure 3.5) were collected in pre-sterilised one litre polyethylene bottles in accordance with standard protocol (Anon., 2012). Shellfish and overlying water samples were transported in a cool box to the Environment and Public Health Research Group (EPHReG) laboratory at the University of Brighton for immediate analysis, normally within one hour.

Figure 3.3: Pacific oysters from southwest England

Figure 3.4: Collection of shellfish samples

Figure 3.5: Collection of overlying water

3.3 Microbial analysis of samples

Microbial analyses were carried out using simple-culture-based methods for the isolation and identification of *E. coli*, intestinal enterococci, faecal coliforms, phages of *Bacteroides fragilis*, F-RNA coliphages, somatic coliphages; and molecular detection approaches (quantitative polymerase chain reaction (qPCR) and reverse-transcription quantitative polymerase chain reaction (RT-qPCR)) for the detection and identification of human enteric viruses – hepatitis A virus, norovirus, and adenovirus in the various shellfish species and their harvesting waters.

3.4 Culture-based methods

3.4.1. Bacteriological analysis of water samples by membrane filtration

3.4.1.1 *E. coli*

Membrane lauryl sulphate agar (MLSA), tryptone bile glucuronide (TBX) agar, membrane faecal coliform '*m*-FC' agar and membrane *Enterococcus* '*m-Enterococcus*' agar (all from Difco, BDMS, UK) were prepared according to the manufacturer's instructions.

The membrane filtration process was set up, connected to a vacuum (Fisherbrand®) and sterile filter units were rinsed with ¼ strength Ringer's solution. A sterile membrane

cellulose nitrate filter, of pore size 0.45 µm (Fisher, UK), was placed aseptically within the filter unit. Approximately 20 ml of Ringer's solution was then poured into the cup and 100 ml of water sample (or dilution thereof) were added and filtered through the membrane. The membrane cellulose nitrate filters was then removed and placed onto freshly prepared selective media. MLSA plates were incubated at 37 (±0.5) ^{o}C for 18-24 hours (presumptive test) and colonies with yellowish coloration were subcultured onto TBX agar and incubated at 44 (±0.5) ^{o}C for 24 hours for confirmation of the number of *E. coli* colony-forming units (cfu) present in 100 ml of the water sample. Confirmed positive colonies of *E. coli* exhibit dark or light blue or blue-green colouration on this agar.

Calculation of positive *E. coli* per 100 ml: $\frac{100}{A} \times B = C$

$$C \times D = E$$

A = Volume of water sample tested

B = Average cfu count

C = Total cfu count per 100 ml of water sample tested

D = Number of positive colonies / number of colonies tested

E = Total positive cfu count per 100 ml

3.4.1.2 Faecal coliforms

Inoculated *m*-FC agar plates were incubated at 44 (±0.5) ^{o}C for 24 hours. Presumptive positive colonies of faecal coliforms exhibit purple coloration on this agar and were reported as colony-forming units per 100 ml of water sample.

3.4.1.3 Intestinal enterococci

Inoculated *m-Enterococcus* agar plates were incubated at 37 (±0.5) ^{o}C for 48 hours. Presumptive positive colonies of enterococci exhibit purple, maroon colouration on this agar and were reported as colony-forming units per 100 ml of water sample.

3.5 Bacteriological analysis of shellfish samples by most probable number (MPN)

Media preparation: Double- and single-strength Modified Mineral Glutamate Broth (MMGB), tryptone bile glucuronide (TBX) agar, membrane faecal coliform '*m*-FC' agar, membrane *Enterococcus* '*m-Enterococcus*' agar (all from Difco, BDMS, UK) were prepared according to the manufacturer's instructions.

Fifteen sterile test tubes were placed in a test tube rack (arranged for three dilutions and five replicates for each dilution). Ten ml of double-strength MMGB were then pipetted into each of the five test tubes in the first row for the first dilution. Ten ml of single-strength MMGB were pipetted into each of the remaining ten test tubes in the second and third rows for the second and third dilutions respectively. All equipment and media were prepared under aseptic conditions.

3.5.1 Sample preparation and homogenisation

Shellfish samples to be analysed (10-15 mussels or 8-10 oysters) were cleaned with running cold water and placed in a stainless steel shucking tray. Using a shucking knife and a chainmail-shucking glove for protection, the shellfish samples were separated from their shells and the whole flesh and intravalvular fluid were weighed using an electronic balance (Salter-AND FX-300). Diluent (0.1% peptone) was added at a ratio of 1:2 (sample:diluent) and poured into a sterile polythene stomacher bag for homogenisation. Homogenisation was carried out in a Seward stomacher® (400 Lab System) for four

minutes. The homogenised shellfish samples were poured into 250 ml Schott bottles and placed on an electronic flask shaker (Stuart Scientific Ltd) for 15 minutes at 600 shakes per minute.

Using a pipette, 30 ml of the homogenate were added to 70 ml of 0.1% peptone to make a 'master mix'. A dilution of 10^{-2} was made by adding 1 ml from the master mix to 9 ml of 0.1% peptone. Ten millilitres (10 ml) of master mix (equivalent to 1 g of tissue per tube and 10^{0} dilution) were inoculated into the five test tubes containing 10 ml of double-strength MMGB. 1 ml of master mix (equivalent to 0.1 g of tissue per tube and 10^{-1} dilution) was inoculated into five test tubes containing 10 ml of single-strength MMGB and 1 ml of 10^{-2} dilution of the master mix (equivalent to 0.01 g of tissue per tube) was inoculated into the remaining five test tubes containing 10 ml of single-strength MMGB. Inoculated tubes were incubated at 37 (±0.5) ^{o}C for 24 hours.

3.5.2 Confirmation of *E. coli*

The test tubes were examined for acid production after incubation, which is demonstrated by a yellow colouration throughout the medium. Presence of *E. coli* was confirmed by subculturing from tubes that showed production of acid onto freshly prepared (TBX) agar plates by streaking to obtain single colonies after incubation at 44 (±0.5) ^{o}C for 24 hours. Positive colonies of *E. coli* exhibit dark or light blue or blue-green colouration on this agar and were counted as most probable number (MPN) per 100 g of shellfish sample.

3.5.3 Confirmation of faecal coliforms

The presence of faecal coliforms in shellfish flesh and intravalvular fluid was confirmed by subculturing from tubes that showed production of acid unto freshly prepared *m*-FC agar plates by streaking to obtain single colonies after incubation at 44 (±0.5) ^{o}C for 24 hours.

Positive colonies of faecal coliforms exhibit purple colouration on this agar and were reported as MPN per 100 g of shellfish sample.

3.5.4 Confirmation of intestinal enterococci

Intestinal enterococci were confirmed by subculturing from tubes that showed production of acid onto freshly prepared *m-Enterococcus* agar by streaking to obtain single colonies after incubation at 37 (±0.5) ^{o}C for 24 hours. Positive colonies of intestinal enterococci exhibit maroon or red colouration on this agar and were reported as MPN per 100 g of shellfish sample.

Calculation: The number of positive plates for each dilution was observed and recorded. This gives a three figure tube combination from which the most probable number of *E. coli*, faecal coliforms and intestinal enterococci were determined using CEFAS protocol (2014) (Table 3.1).

In summary, the detection of faecal indicator bacteria in overlying waters and shellfish by membrane filtration and most probable number methods is shown as a flow chart in Figure 3.6 and references to these methods are shown in Table 3.2.

Table 3.1: ***E. coli*, faecal coliforms and intestinal enterococci most probable number: Multiple tube method using 5 × 1g, 5 × 0.1g, 5 × 0.01g** (Source: CEFAS protocol, 2014)**.**

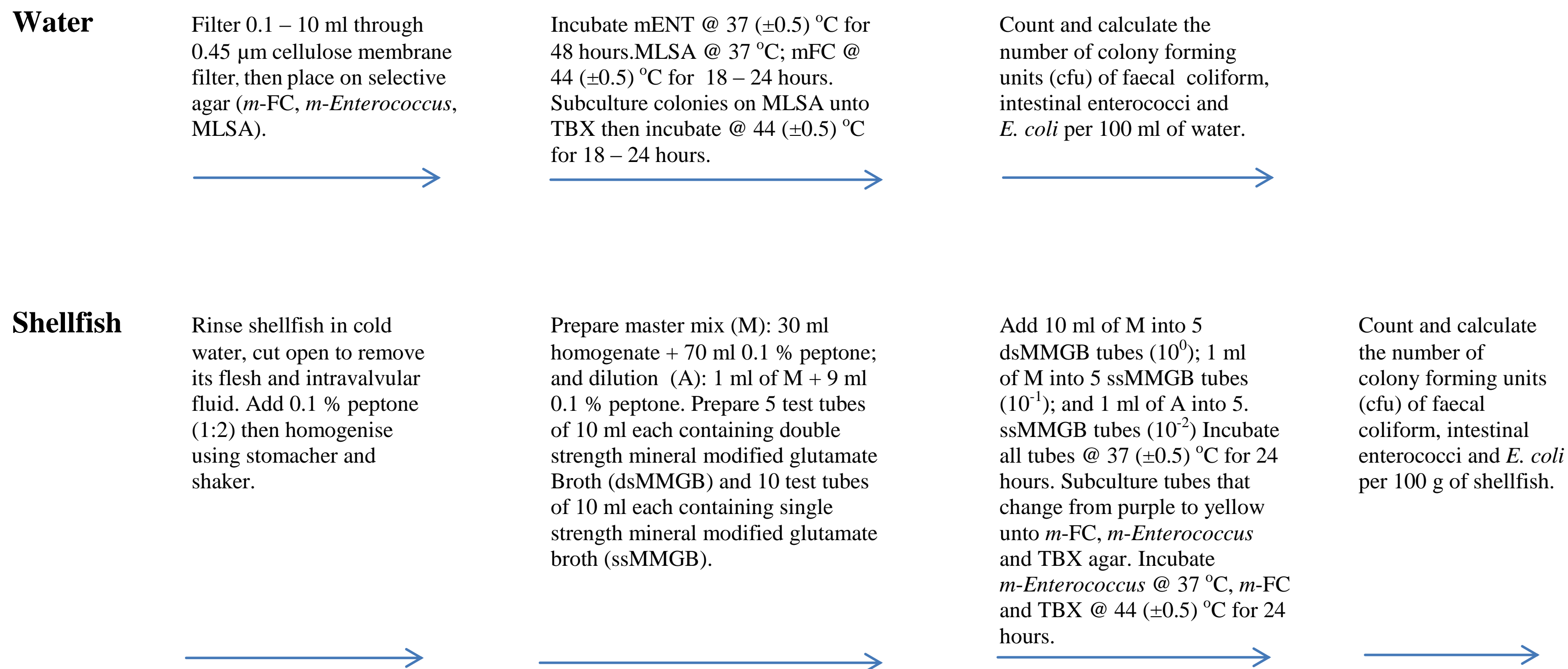
1g	0.1g	0.01g	MPN/100g	Category
0	0	0	<18	1
0	1	0	18	1
1	0	0	20	1
1	0	1	40	2
1	1	0	40	1
1	2	0	61	2
2	0	0	45	1
2	0	1	68	2
2	1	0	68	1
2	1	1	92	2
2	2	0	93	1
3	0	0	78	1
3	0	1	110	1
3	1	0	110	1
3	1	1	140	2
3	2	0	140	1
3	2	1	170	2
3	3	0	170	2
4	0	0	130	1
4	0	1	170	1
4	1	0	170	1
4	1	1	210	1
4	2	0	220	1
5	0	0	230	1
4	2	1	260	2
4	3	0	270	1
4	4	0	340	2
5	0	1	310	1
5	1	0	330	1
5	1	1	460	1
5	1	2	630	2
5	2	0	490	1
5	2	1	700	1
5	2	2	940	1
5	3	0	790	1
5	3	1	1100	1
5	3	2	1400	1
5	3	3	1700	2
5	4	0	1300	1
5	4	1	1700	1
5	4	2	2200	1
5	4	3	2800	1
5	4	4	3500	2
5	5	0	2400	1
5	5	1	3500	1
5	5	2	5400	1
5	5	3	9200	1
5	5	4	16000	1
5	5	5	>18000	1

Key: Category – Most probable number tube combination probability category 1 or 2 (Source: CEFAS protocol, 2014)

Table 3.2: Summary of faecal indicator bacteria used as indicators of faecal contamination of water and shellfish in this study (including methods of analysis and references).

Faecal indicator bacteria	Samples	Method	References
E. coli	Overlying river water	Membrane filtration	Anon., 2000b
	Shellfish (mussels, native and pacific oysters)	Most Probable Number	CEFAS protocol, 2014
Intestinal enterococci	Overlying river water	Membrane filtration	Anon., 2000c
	Shellfish (mussels, native and pacific oysters)	Most Probable Number	Developed in EPHReG (following CEFAS protocol, 2014)
Faecal coliforms	Overlying river water	Membrane filtration	Anon., 2000b
	Shellfish (mussels, native and pacific oysters)	Most Probable Number	Developed in EPHReG (following CEFAS protocol, 2014)

Figure 3.6: Flow chart for detection of faecal indicator bacteria by membrane filtration and most probable number methods
(Anon., 2000b; 2000c and CEFAS protocol, 2014)



Key: MLSA - Membrane lauryl sulphate agar, TBX - Tryptone bile glucuronide agar, *m*-FC - Membrane faecal coliform agar, *m*-ENT – Membrane *Enterococcus* agar, dsMMGB – Double-Strength Mineral Modified Glutamate Broth, ssMMGB – Single-Strength Mineral Modified Glutamate Broth

3.6 Phage assays for overlying waters and shellfish

3.6.1 Preparation of samples

Water samples to be analysed for the various phage groups were first filtered through a 0.22 μm membrane cellulose nitrate filter (Millipore) into 10 ml plastic vials (Sterilin). This was undertaken to remove bacterial contamination before phage assay (Ebdon *et al*., 2007).

Shellfish samples were prepared as for faecal indicator bacteria (see section 3.5.1) but the digestive glands were separated from the whole flesh and intravalvular fluid of the shellfish and were weighed on an electronic balance (Salter-AND FX-300). In this study, the digestive glands were used for phage analysis following preliminary analysis, which showed that glands had about 60% concentration of phages greater than those in shellfish flesh and intravalvular fluid. This is in agreement with previously published studies on bacteriophages in shellfish (Jofre, 1992; Lowther *et al*., 2012; Trajano Gomes Da Silva, 2013). The glands were chopped finely to expose the content and phages were eluted using 0.25 M glycine buffer at pH 9.5 at a ratio of 1:5. The mixture was then poured into a centrifuge tube and placed on an electronic flask shaker (Stuart Scientific Ltd) for 20 minutes at 600 shakes per minute. Thereafter, the mixture was transferred into a centrifuge (Heraeus megafuge 16R) at 2000 $\times$ g for 15 minutes at 4°C. The supernatant was filtered through a 0.22 μm membrane cellulose nitrate filter into plastic vials. Glycine buffer was used, based on the studies of Muniain-Mujika *et al*. (2000) who evaluated various methods for bacteriophage analysis and human viruses in shellfish and concluded that glycine buffer seems to be the most efficient eluent for the recovery of phages.

3.6.2 Enumeration of F-RNA coliphages

The host strain used in this study was *Salmonella typhimurium* WG49 (Havelaar *et al*., 1993). Media preparation: Tryptone-yeast extract glucose broth (TYGB), tryptone-yeast extract glucose agar (TYGA) and semi-solid tryptone-yeast extract glucose agar (ssTYGA) were prepared in accordance with ISO 10705-1 (Anon., 2001b).

One vial of the host strain working culture was removed from the freezer (-70 ±10°C) and allowed to thaw at room temperature. 50 ml of TYGB were then added to a sterile Schott bottle and 2.0 ml placed into a cuvette (Fisher Scientific, UK) and used to adjust the reading of the spectrophotometer (Hach Lange DR 3900) to zero (0) at 600 nm wavelength. 0.5 ml of the working culture was then inoculated into the 50 ml of pre-warmed TYGB, then incubated at 37 (±0.5) ^{o}C while shaking (110 rpm) in a water bath (Grant OLS 200). Optical density readings were taken every 30 minutes until a density of 0.33 was attained; this had previously been found to be the turbidity corresponding to a cell density of approximately 10^{8} colony-forming units per millilitre (cfu/ml) (Anon., 2001b).

Fifty ml ssTYGA was melted in the microwave (Cuisina) and allowed to a cool temperature between 45 and 50°C in the water bath (Fisherbrand®). 0.5 ml of calcium glucose was then added to make a complete medium. 2.5 ml aliquots of the complete medium were distributed into plastic culture tubes (Fisher Thermo Scientific, UK) placed in the water bath (Fisherbrand®). 1 ml of the prepared sample to be assayed was pre-warmed at room temperature and was added to each of the tubes and 1 ml of the cultured host strain was also added to each of the tubes. This was mixed thoroughly using a vortex mixer (Fisherbrand®) for 2 to 3 seconds and thereafter poured aseptically onto TYGA plates. The mixture was swirled gently for even distribution and then allowed to solidify on

a horizontal cool surface. All assays were carried out in duplicate and inoculated plates were incubated in an inverted position at 37 (±0.5) ^{o}C for 24 hours.

Following incubation, the visible plaques observed (Figure 3.7) were enumerated and the results expressed as plaque-forming units per millilitre (pfu/ml) of water sample or plaque-forming units per gram (pfu/g) of shellfish sample.

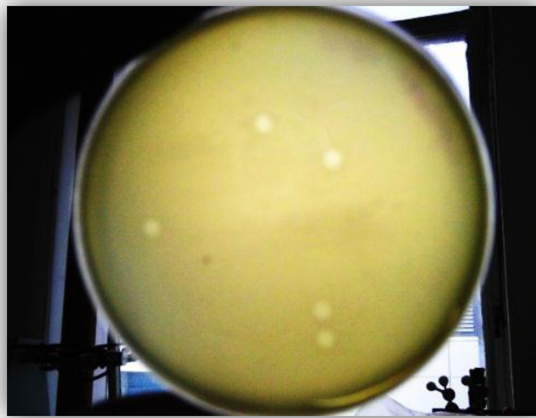


Figure 3.7: Visible plaques of F-RNA coliphages on TYGA plate

3.6.3 Enumeration of somatic coliphages

The host strain used in this study was *Escherichia coli* WG5.

Media preparation: Modified Scholtens' Broth (MSB), Modified Scholtens' Agar (MSA) and semi-solid Modified Scholtens' Agar (ssMSA) were prepared in accordance with ISO 10705-2 (Anon., 2000a).

One vial of the host strain working culture was removed from the freezer (-70 ±10°C) and allowed to thaw at room temperature. 50 ml of MSB were added to a sterile Schott bottle and about 3 ml were transferred to a cuvette (Fisher Scientific, UK) and used to adjust the reading of the spectrophotometer (Hach Lange DR 3900) to zero (0) at a wavelength of

600 m. 0.5 ml of the working culture was inoculated into the 50 ml MSB previously pre-warmed to room temperature, then incubated at 37 (±0.5) ^{o}C while orbitally shaking in a water bath (Grant OLS 200). Optical density readings were taken every 30 minutes until an optical density of 0.33 was attained; which corresponded to a cell density of approximately 10^{8} colony forming unit per millilitre (cfu/ml) (Anon., 2000a).

Fifty ml of ssMSA were melted in a microwave (Cuisina) and allowed to cool to between 45 and 50°C in the water bath (Fisherbrand®). 0.3 ml of calcium chloride was added to make a complete medium. A 2.5 ml aliquot of the complete medium was distributed into plastic culture tubes (Fisher Thermo Scientific, UK) and placed in the water bath (Fisherbrand®) for no longer than two hours. 1 ml of the prepared sample to be assayed was pre-warmed to room temperature and was added to each of the tubes and 1ml of the cultured host strain was also added to each of the tubes. This was mixed thoroughly on a vortex (Fisherbrand®) for two to three seconds and thereafter poured aseptically onto MSA plates. The mixture was swirled gently for even distribution and allowed to solidify on a horizontal cool surface. All assays were carried out in duplicate and inoculated plates were incubated in an inverted position at 37 (±0.5) ^{o}C for 24 hours.

The numbers of visible plaques observed (Figure 3.8) were counted and the results were expressed as plaque forming units per millilitre (pfu/ml) of water sample or plaque forming units per gram (pfu/g) of shellfish samples.

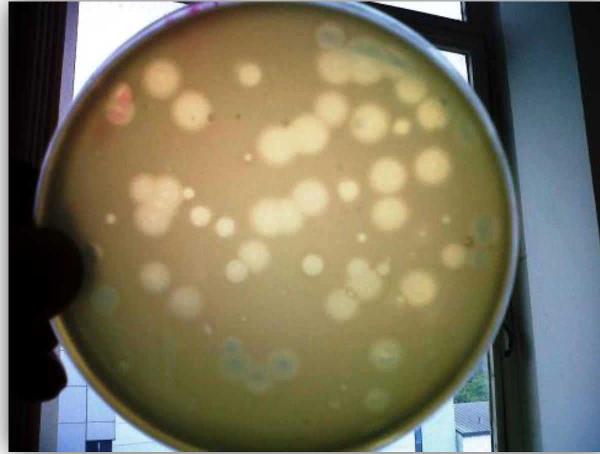


Figure 3.8: Visible plaques of somatic coliphages on MSA plate

3.6.4 Enumeration of bacteriophages infecting a human-specific strain of *Bacteroides fragilis*

In this study, phages that lysed a previously isolated (Payan *et al.*, 2005; Ebdon *et al.*, 2007; 2012) human-specific bacterial host strain (*Bacteroides fragilis* GB124) were also detected and enumerated in shellfish and their overlying waters. Phages that infect this bacterial host strain have been demonstrated to be human specific, i.e., restricted to the human gastrointestinal tract (Payan *et al.*, 2005; Ebdon *et al.*, 2007; 2012; Ogilvie *et al.*, 2012; McMinn *et al.*, 2014).

Media preparation: *Bacteroides* phage recovery medium broth (BPRMB), *Bacteroides* phage recovery medium agar (BPRMA) and semi-solid *Bacteroides* phage recovery medium agar (ssBPRMA) were prepared in accordance with ISO 10705-4 (Anon., 2001a).

One vial of the host strain working culture was removed from the freezer (-70 ±10°C) and allowed to thaw at room temperature. Additives to make a complete broth were added to 50 ml BPRMB and 10 ml of the mix were placed into five screw-capped glass culture

tubes (Pyrex®) each. 1 ml of the host strain was added to one of the five tubes, topped up with more BPRMB until filled to the brim, to avoid aerobic condition, then incubated at 37 (±0.5) ^{o}C for 24 hours. 1 ml of the overnight grown host was added to three other screw-capped glass culture tubes (Pyrex®) in the same process to enhance anaerobic condition while the fifth screw capped glass culture tube (Pyrex®) was left without adding the inoculum and used to adjust the reading of the spectrophotometer (Hach Lange DR 3900) to zero (0) at a wavelength of 620 nm. Inoculated screw-capped glass culture tubes (Pyrex®) were incubated at 37 (±0.5) ^{o}C and optical density readings were taken every 30 minutes until an optical density of 0.33 was attained; this corresponded to a cell density of approximately 10^8 colony forming unit per millilitre (cfu/ml) (Anon., 2000a).

Fifty ml of ssBPRMA were melted in a microwave (Cuisina) and allowed to cool to a temperature between 45 and 50°C in a water bath (Fisherbrand®). Additives (haemin, sodium carbonate, hydrochloric acid, kanamycin monophosphate and nalidixic acid) were added to make a complete medium and a 2.5 ml aliquot of the complete medium was distributed into plastic culture tubes (Thermo Fisher Scientific®) and placed in the water bath (Fisherbrand®). 1 ml of the prepared sample to be assayed was pre-warmed to room temperature and added to each of the tubes. 1 ml of the cultured host strain was also added to each of the tubes. This was mixed thoroughly using a vortex mixer (Fisherbrand®) for two to three seconds and thereafter poured aseptically onto BPRMA. The mixture was swirled gently for even distribution and then allowed to solidify on a horizontal cool surface. All assays were carried out in duplicate and the inoculated plates were placed in an anaerobic jar (Oxoid) containing an anaerobic pack (AnaeroGen™) placed in an inverted position, and the jar was incubated at 37 (±0.5) ^{o}C for 24 hours.

The visible plaques observed (Figure 3.9) were enumerated and the results expressed as plaque-forming units per millilitre (pfu/ml) of water sample or plaque-forming units per gram (pfu/g) of shellfish sample.

Calculation of number of plaques forming units per millilitre (pfu/ml) or gram (pfu/g):

$$X = \frac{N}{(n1\ V1\ F1)+(n2\ V2\ F2)} \quad OR \quad X = \frac{N}{nVF}$$

X = Actual number of plaques forming units per millilitre (pfu/ml) or gram (pfu/g)

N = Total number of visible plaques counted on plates

n_1, n_2 = Number of replicates

V_1, V_2 = Volume of sample tested

F_1, F_2 = Dilution factor

Note: The dilution factor for water when 1 ml is added to 9 ml distilled water is 10^{-1}, i.e., 1/10; whereas 1 g of digestive gland in 5 ml glycine buffer (1:5) is 1/6 for shellfish.

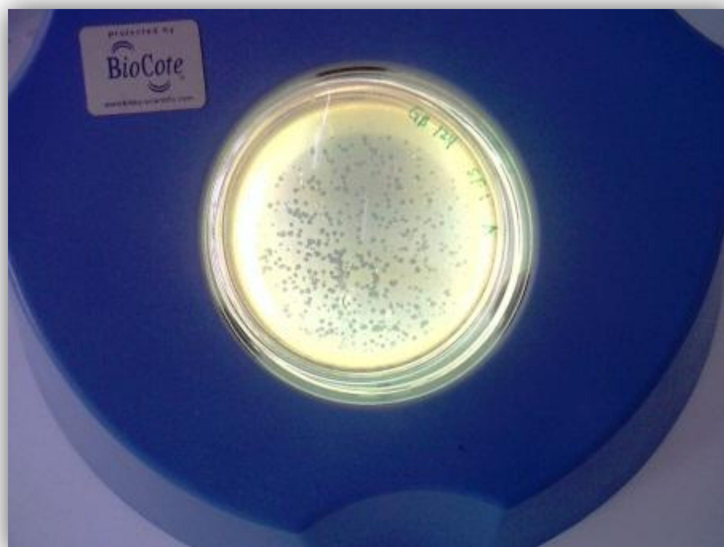


Figure 3.9: Visible plaques of bacteriophages infecting human specific *Bacteroides fragilis* (GB124) on BPRMA plates

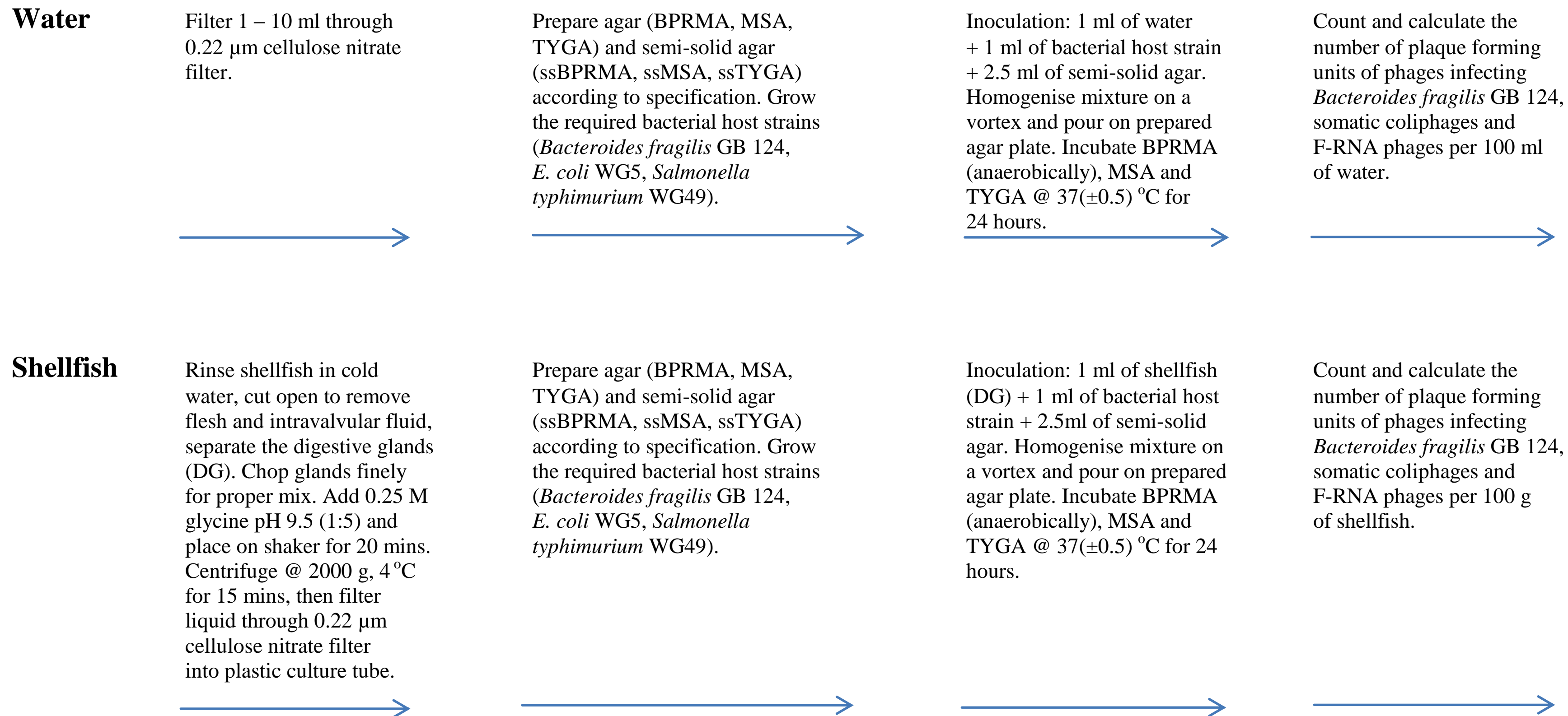
In summary, the detection of phage–based indicators in overlying waters and shellfish by the double-agar layer method is shown as a flow chart in Figure 3.10 and references to the method are given in Table 3.3.

Table 3.3: Summary of bacteriophages used as surrogates of viral pathogens in monitoring water and shellfish sanitary quality, methods of analysis and references.

Bacteriophage	Host bacteria	Samples	Method	References
Somatic coliphages	*E. coli* WG5	Overlying river water and shellfish (mussels, native and pacific oysters)	Double-agar layer	Anon., 2000a
F-RNA coliphages	*Salmonella enterica typhimurium* WG49	Overlying river water and shellfish (mussels, native and pacific oysters)	Double-agar layer	Anon., 2001b
Human-specific *Bacteroides fragilis* bacteriophages	*Bacteroides fragilis* GB124	Overlying river water and shellfish (mussels, native and pacific oysters)	Double-agar layer	Anon., 2001a

Figure 3.10: Flow chart for detection of phage-based indicator by double-agar layer method

(Anon., 2000a; 2001a; 2001b)



Key: TYGA - Tryptone-yeast extract glucose agar, ssTYGA - Semi-solid tryptone-yeast extract glucose agar, MSA - Modified Scholtens' Agar, ssMSA - Semi-solid Modified Scholtens' Agar, BPRMA - Bacteroides phage recovery medium agar, ssBPRMA - semi-solid Bacteroides phage recovery medium agar, DG - Digestive glands

3.7 Molecular detection and quantitation of enteric viruses in overlying waters and shellfish using real-time polymerase chain reaction (qPCR)

3.7.1 Preparation of water samples

The basic steps for virological analysis of water include; sample concentration, nucleic acid extraction and molecular detection (Harwood *et al*., 2013).

3.7.1.1 Concentration of viruses in water samples

Adenovirus, norovirus and hepatitis A virus were concentrated from 300 ml overlying river water samples filtered through 0.45 µm membrane cellulose nitrate filters. Magnesium chloride (5 M $MgCl_2$) was prepared by dissolving 475 g of its crystals in 1000 ml of distilled water, then filter-sterilizing the solution through a 0.22 µm membrane cellulose nitrate filters. Prepared $MgCl_2$ was added to the water samples in a filter cup before the filtration process to increase viral recovery by facilitating and enhancing virus attachment to the filters (Mendez *et al*., 2004). In detail, 6 ml of 5 M $MgCl_2$ was added aseptically to 300 ml of water sample to make a final concentration of 0.1 M $MgCl_2$ before filtration. The filters were stored at -80°C prior to nucleic acid extraction.

3.7.1.2 Nucleic acid extraction from concentrated samples

The frozen filters were equilibrated at room temperature and the surface was scraped using a fresh laboratory razor blade (Fisher Scientific) into a 1.5 ml microcentrifuge tube. Viral nucleic acids were extracted using the QIAamp MinElute Virus Spin Kit (Qiagen GmbH) according to the following procedure. 25 µl QIAGEN protease resuspension buffer was placed in a 1.5 ml microcentrifuge tube. 0.9% sodium chloride solution was added to bring the volume of protease and sample up to a total of 225 µl. 200 µl buffer AL containing 6.2 µl carrier RNA in buffer AVE were added to the tube and pulse-vortexed for 15 seconds

for effective mixing. The mixture was incubated at 56°C for 15 minutes in a heating block. The 1.5 ml tube was placed in a microcentrifuge (MSE Micro Centaur, UK) at 8000 rpm for 1 minute to remove drops from the inside of the lid. 250 μl of absolute ethanol (96 – 100%) were added to the sample, then pulse-vortexed for 15 seconds. The lysate with ethanol was incubated at room temperature for five minutes, and then placed in microcentrifuge (MSE Micro Centaur, UK) briefly to remove the drops from the inside of the lid. The lysate was carefully applied onto the QIAamp MinElute column without wetting the rim, and then placed in a microcentrifuge (MSE Micro Centaur, UK) at 8000 rpm for 1 minute. The collection tube containing the filtrate was discarded, and the QIAamp MinElute column was placed in a clean 2 ml collection tube and 500 μl of buffer AW1 was added without wetting the rim, and then placed in a microcentrifuge (MSE Micro Centaur, UK) at 8000 rpm for 1 minute. The collection tube containing the filtrate was again discarded, and the QIAamp MinElute column was placed in a clean 2 ml collection tube and 500 μl of buffer AW2 were added without wetting the rim, and then placed in the microcentrifuge (MSE Micro Centaur, UK) at 8000 rpm for 1 minute. The collection tube containing the filtrate was discarded and the QIAamp MinElute column was placed in a clean 2 ml collection tube and 500 μl of absolute ethanol (96-100%) was added without wetting the rim, and then placed in microcentrifuge (MSE Micro Centaur, UK) at 8000 rpm for 1 minute. The collection tube containing the filtrate was discarded and the QIAamp MinElute column was placed in a clean 2 ml collection tube and then placed in microcentrifuge (MSE Micro Centaur, UK) at 13, 000 rpm for 3 minutes to dry the membrane completely. The QIAamp MinElute column was placed in a clean 2 ml collection tube and incubated at 56°C for 3 minutes in a heating block to evaporate any remaining liquid. The QIAamp MinElute column was placed in a clean 2 ml collection tube and 150 μl of RNase-free water was added to the centre of the membrane to elute the

bound RNA and DNA, and then incubated at room temperature for 1 minute before placing it in the microcentrifuge (MSE Micro Centaur, UK) at 13, 000 rpm for 1 minute. The nucleic acid extracts were stored at -80°C until quantification.

3.7.2 Preparation of shellfish samples

Shellfish samples were prepared as described in section 3.5.1. The digestive glands were separated from the whole flesh and intravalvular fluid of the shellfish. The digestive glands appear to demonstrate the highest concentration of viruses as observed by many authors investigating viruses in shellfish (Jothikumar *et al*., 2005; Le Guyader *et al*., 2009; Pinto *et al*., 2009; Westrell *et al*., 2010; Iizuka *et al*., 2010; Baker *et al*., 2011; Bosch *et al*., 2011; Lowther *et al*., 2012; Trajano Gomes Da Silva, 2013). The glands were therefore chopped finely to expose the content and stored in centrifuge tubes at -80°C until nucleic acid extraction.

3.7.2.1 Nucleic acid extraction from digestive glands of shellfish

The frozen glands were equilibrated at room temperature and 200 µl of the finely chopped glands was placed in a 1.5 ml microcentrifuge tube. Viral nucleic acids were extracted using the QIAamp MinElute Virus Spin Kit (Qiagen GmbH) according to the procedure highlighted in section 3.7.1.2. The nucleic acid extracts were stored at -80°C until quantification.

3.7.3 Molecular detection

Norovirus (NoV) genogroups I and II (GI and GII) were enumerated by reverse transcription real-time polymerase chain reaction (RT-qPCR) on QIAGEN Rotor-Gene® Q thermocycler using PrimerDesign™ genesig NoV GI capsid protein gene and NoV GII

RNA dependent RNA polymerase gene Advanced kit (Primerdesign, UK) following manufacturer's protocol. The components of the kit were reconstituted in RNase/DNase-free water appropriately, and then vortexed to ensure complete resuspension. Thermocycling conditions included a 10 minutes RT step at 42°C, followed by a 2 minutes enzyme activation step at 95°C, and then 50 cycles of denaturation at 95°C for 10 seconds and fluorogenic data were collected (during the step through the FAM™ and VIC® channels) at 60°C for 60 seconds. Data were analysed using Rotor-Gene 2.1.0.9 software with a threshold fluorescence value of 1.000. Standards were prepared, serially diluted and quantified to make standard curves following manufacturer's protocol. The highest concentration of NoV GI and GII standard was 2×10^5 copies/µl. Standard curve was run in triplicate and the 'pooled' standard curve was then used to relate quantification cycles to copy numbers and quantity of NoV GI and GII in samples.

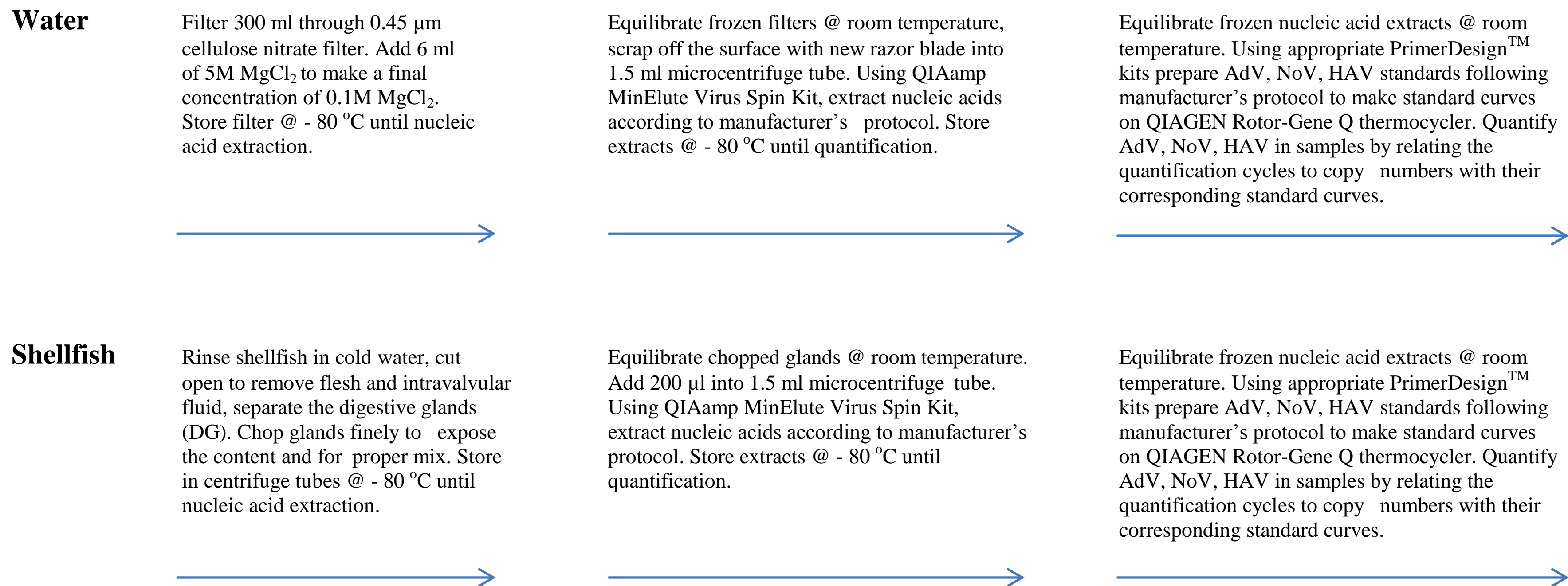
Hepatitis A virus (HAV) was enumerated by reverse transcription real-time polymerase chain reaction (RT-qPCR) on QIAGEN Rotor-Gene® Q thermocycler using PrimerDesign™ genesig HAV 5' Non-Coding Region (NCR) Advanced kit (Primerdesign, UK) following manufacturer's protocol. The components of the kit were reconstituted in RNase/DNase-free water appropriately, and then vortexed to ensure complete resuspension. Thermocycling conditions were RT step (42°C for 10 minutes), enzyme activation step (95°C for 2 minutes), 50 cycles of denaturation step (95°C for 10 seconds) and data collection (60°C for 60 seconds). Data were analysed using Rotor-Gene 2.1.0.9 software with a threshold fluorescence value of 1.000. Standards were prepared, serially diluted and quantified to make standard curves following manufacturer's protocol. The highest concentration of HAV standard was 2×10^5 copies/µl. Standard curve was run in

triplicate and the ‘pooled’ standard curve was then used to relate quantification cycles to copy numbers and quantity of HAV in samples.

Adenovirus Type F and G (AdV F and G) was enumerated by real-time polymerase chain reaction (qPCR) on QIAGEN Rotor-Gene® Q thermocycler using PrimerDesign™ genesig AdV F and G Hexon gene Advanced kit (Primerdesign, UK) following manufacturer’s protocol. The components of the kit were reconstituted in RNase/DNase-free water appropriately, and then vortexed to ensure complete resuspension. Thermocycling conditions were enzyme activation step (95°C for 15 minutes), 50 cycles of denaturation step (95°C for 10 seconds) and data collection (60°C for 60 seconds). Data were analysed using Rotor-Gene 2.1.0.9 software with a threshold fluorescence value of 1.000. Standards were prepared, serially diluted and quantified to make standard curves following manufacturer’s protocol. The highest concentration of AdV F and G standard was 2×10^5 copies/µl. Standard curve was run in triplicate and the ‘pooled’ standard curve was then used to relate quantification cycles to copy numbers and quantity of AdV F and G in samples.

The flow chart for molecular detection of viral pathogens by real time polymerase chain reaction is shown in Figure 3.11.

Figure 3.11 Flow chart for molecular detection of viral pathogens (adenovirus – AdV, norovirus – NoV and hepatitis A virus – HAV) by real-time polymerase chain reaction (Mendez *et al*., 2004; Harwood *et al*., 2013)



Key: AdV - Adenovirus, NoV - Norovirus, HAV - Hepatitis A virus, DG - Digestive glands

3.8 Physicochemical analysis of overlying waters

The physico-chemical properties of all overlying water samples from the R. Ouse shellfish bed were determined biweekly at the point of collection (i.e., on-site) using a handheld multi-parameter instrument (Aquaread AP-2000), calibrated according to the manufacturer's instruction before each use. The properties measured were temperature, dissolved oxygen, electrical conductivity, turbidity, salinity, pH and total dissolved solids. These physical and chemical properties were selected in order to evaluate their influence on microbial indicators and pathogens in the overlying waters.

3.9 Meteorological conditions prior to and during sampling

Meteorological conditions prior to sampling (24 and 48 hours before) and during sampling day were recorded during each event. High flow events were defined as a period during which the river flowed at greater than 5 m^3/s. Wet weather periods were defined as those with rainfall greater than 3 mm in 24 to 48 hours resulting in wet ground and elevated levels of water in the river. Dry weather periods were defined as those with less than 0.1 mm rainfall for two days prior to sampling leading to dry ground around the river. Intermittent rain showers were defined as a period of stopping and starting of rainfall at irregular intervals. A storm event was defined as a period of heavy rainfall that leads to increased river flow. Sunny weather was defined as a period of sunshine with temperatures above $10^{o}C$.

Air temperature and hourly rainfall (mm) data were obtained from a station operated by the Environment Agency, situated about 3.38 km from the sampling site, and hydrological data, such as daily mean river flow (m^3/s) were all obtained with kind permission from the Environment Agency – Southeast, United Kingdom.

3.10 Statistical analysis of dataset from environmental survey

Statistical Package for Social Sciences (SPSS) 20.0 was used to support the analysis of data on concentrations of microbial parameters in shellfish samples and their overlying waters. The mean concentrations were subjected to one-way analysis of variance and a Post-hoc test of significance was performed using Duncan's multiple range tests. Physicochemical, meteorological and hydrological data were also analysed using the same package. A non-parametric Spearman's rank correlation test was used to assess the relationship between all the analysed parameters.

3.11 Laboratory-based experiment on uptake and bioaccumulation in shellfish

The aim of the laboratory-based experimental phase of this study was to evaluate the bioaccumulation, uptake and retention of bacterial and viral indicators in shellfish under controlled conditions. The specific objectives of the experiments were: 1. to examine the rate of uptake of faecal indicator bacteria and bacteriophages in *Mytilus edulis* (Mussels) and *Crassostrea gigas* (Pacific oysters) exposed to faecal contamination to determine the rate of bioaccumulation of the various indicators in shellfish and; 2. to examine the persistence of these indicators in shellfish over a period of time.

Clean plastic fish tanks measuring 310 mm (length) by 210 mm (width) by 230 mm (height), with a working volume of 12 litres, were prepared and placed in the EPHReG laboratory at room temperature. Stainless test tube racks were placed at the bottom of each tank to act as a mesh and to avoid recontamination by voided faecal indicator bacteria and bacteriophages. During the trials, *Mytilus edulis* and shellfish-harvesting waters (10-20 litres) were obtained from the river estuary – River Ouse in southeast England. The physiochemical properties of the shellfish-harvesting waters at the time of collection,

including temperature and salinity were recorded. *Mytilus edulis* were collected by hand-picking from the shores of the river at low tide between 0.5-1.5 metres. Similarly, freshly collected *Crassostrea gigas* (harvested from Class A site) were obtained from a ready-to-sell outlet in Shoreham-by-sea, West Sussex, England. All samples were transported to the laboratory within one hour. *Mytilus edulis* and *Crassostrea gigas* were placed in two different experimental tanks containing laboratory-prepared artificial seawater (17.15 g NaCl, 4.18 g $MgSO_4$, 3.37 g $MgCl_2$, 0.87 g $CaCl_2$, 0.44 g KCl) (Woods and Ayres, 1977). Bubble stones connected to aquarium airlines and fixed to a peristaltic pump were placed in the experimental tanks for aeration. The setups were left for a period of two days to allow the shellfish to acclimatise to their new environment.

3.11.1 Isolation, purification and propagation of faecal indicator bacteria

Colonies of bacteria on Tryptone bile glucuronide (TBX) agar, Membrane faecal coliform '*m*-FC' agar and Membrane *Enterococcus* '*m-Enterococcus*' agar (all from Difco, BDMS, UK), representing *E. coli*, faecal coliforms and intestinal enterococci respectively, were subcultured up to three times to obtain pure culture of each bacteria. Thereafter, each pure isolate was picked aseptically with sterile inoculating loop into freshly prepared single-strength Modified Mineral Glutamate Broth (ssMMGB) and incubated at 37^oC for 24 hours.

The concentration of the bacterial cultures was determined by serial dilution in ¼ strength Ringer's solution and plated using TBX agar, *m*-FC agar and *m-Enterococcus* agar incubated appropriately (see section 3.4) to ensure that a cell density of approximately 1×10^8 colony forming unit per millilitre (cfu/ml) was achieved.

3.11.2 Isolation, purification and propagation of phages

Plaques on Modified Scholtens' agar (MSA), Tryptone-yeast extract glucose agar (TYGA) and *Bacteroides* phage recovery medium agar (BPRMA) plates representing phages of somatic coliphages, F-specific RNA coliphages and human specific *Bacteroides fragilis* bacteriophages GB124 respectively, were picked using sterile glass Pasteur pipettes for isolation, propagation and purification using methods previously described by Fard *et al*. (2011) and Trajano Gomes da Silva (2013). In brief, the cores of the agar, containing distinct single plaques were suspended in 400 µl of phage buffer (19.5 mM Na_2HPO_4, 22 mM KH_2PO_4, 85.5 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) in microcentrifuge tubes (Fisher Scientific, UK). The phage suspensions were incubated at $4^{o}C$ for at least four hours, to allow complete diffusion of phage into the buffer, mixed gently and then filtered through a 0.22 µm membrane cellulose nitrate filter (Millipore). The phage suspensions were diluted serially in phage buffer up to ten-fold dilutions and were retested using the double-agar layer method (see section 3.6). The process of phage isolation and analysis was repeated three times by selecting phages on plates with well-spaced zones of lysis to finally obtain purified phage without contaminant.

To concentrate the purified phages, 5 ml of phage buffer was added to plates showing near complete-lysis of host bacterium, and left for at least one hour at room temperature with occasional swirling to allow diffusion of phage into the buffer. Thereafter, the top agar-layer and the liquid were scrapped into a 50 ml centrifuge tube, mixed using a vortex mixer (Fisherbrand®) and left at room temperature for 30 minutes. The top agar-layer was removed from the suspension by centrifugation at 2000 g for 15 minutes. The supernatant was filtered through a 0.22 µm membrane cellulose nitrate filter (Millipore), labelled as 'stock' and stored in light-tight glass tubes in the dark at $4^{o}C$ for not longer than 6 months.

The phage 'stock' was diluted serially in phage buffer and retested using the double-agar layer method, to determine the titre of the stock and to ensure that a titre of 1×10^8 plaque forming unit per millilitre (pfu/ml) was achieved.

3.11.3 Experimental design of uptake and bioaccumulation study

As stated in Table 3.4, 'low temperature' (approximately 8°C using a LMS Cooled Incubator, UK) and 'high temperature' (approximately 24°C using a 200W submersible aquarium fish tank heater) were used to simulate the extremes of river water temperature commonly observed during winter and summer months respectively, in southern England, 'low salinity' (approximately 5 ppt) and 'high salinity' (approximately 25 ppt) were selected, based on results from routine environmental monitoring of the river water at the Piddinghoe sampling site (see chapter 4), that demonstrated a significant positive correlation between water temperature and salinity, and negative correlations between water temperature and river flow as well as between salinity and river flow. Rainfall correlated positively and strongly with river flow. Furthermore, low salinities were observed when rainfall and river flow were high (i.e. during winter months when temperatures were low), this scenario was simulated in experimental tank C, whereas in some exceptional cases, salinities may be high during winter months, and this was simulated in experimental tank D. High salinities were observed when rainfall and river flow were low (i.e. during summer months when temperatures were high), and this scenario was simulated in tank B, whereas in some exceptional cases, salinities may be low during summer months, and this was simulated in tank A. Clearly, these selected variables (temperature and salinity) influenced the bioaccumulation of faecal indicator organisms in shellfish during the four seasons investigated during the two-year field study.

The time increments and duration selected for analysis (i.e. 0, 6, 12, 24, 48 and 96 hours) to determine uptake and bioaccumulation of faecal indicator bacteria and phages in shellfish matrices were based on the findings of Grodzki *et al*. (2014) who observed variation in the bioaccumulation efficiency of viral pathogens in different shellfish species using a maximal theoretical bioaccumulation (MTB) calculation. The authors found that the highest bioaccumulation efficiencies were detected after 1 hour in mussels and, after 24 hours in oysters and flat oysters.

Table 3.4: Experimental design of uptake and bioaccumulation study

Tank	Temperature (^{o}C)	Salinity (ppt)	Time (hours)
A	24	5	0, 6, 12, 24, 48, 96
B	24	25	0, 6, 12, 24, 48, 96
C	8	5	0, 6, 12, 24, 48, 96
D	8	25	0, 6, 12, 24, 48, 96
E (Positive control)	20	16	0, 6, 12, 24, 48, 96
F (Negative control)	20	16	0, 6, 12, 24, 48, 96

Positive control – Spiked; Negative control – Not spiked

In this experiment, a slight modification was made to the time selected for analysis. Shellfish and artificial seawater in tanks were examined prior to commencement of the spiking experiments (representing zero hour analysis) to ensure the absence of background faecal contamination, which could have affected the results. All values for microbial counts in shellfish and artificial seawater were found to be below detection limits at zero hours (for faecal indicator bacteria in shellfish the minimum detection limit is 20 MPN/100 g and in water it is 1 CFU/100 ml; for phages in shellfish, detection limit is 1 PFU/g and in water it is 1 PFU/ml). Analyses at 6, 12 and 24 hours were carried out to observe the rate of uptake and bioaccumulation of the indicators in the shellfish while analyses at 48 and 96 hours were to examine patterns of persistence (if it existed) of the indicators in the shellfish.

The positive and negative control tanks were run in parallel and both were maintained at optimum salinities (approximately 16 ppt) and temperature (approximately 20°C). These optimum conditions were based on the findings of Walne (1972) who observed variation in the filtration rates of shellfish when the temperature was reduced below optimum (20°C) to 10°C. In fact, *Crassostrea gigas*, *Ostrea edulis* and *Mytilus edulis* demonstrated 25%, 45% and 25% reduction in filtration rates, respectively. Similarly, Solic and Krstulovic (1992) demonstrated that increasing salinities above 15 ppt or reducing it below 15 ppt was detrimental to the survival of faecal coliforms in seawater. The positive control tank contained artificial seawater and shellfish spiked with faecal indicator bacteria and phages, whereas the negative control tank contained artificial seawater and shellfish, which were not spiked with faecal indicator bacteria and phages.

3.11.4 Dosing experimental tanks with faecal indicator bacteria and phages

A volume of 2.5 ml each of known concentrations of six microorganisms (see sections 3.11.1 and 3.11.2) commonly observed in faecally impacted waters (*E. coli*, faecal coliforms, intestinal enterococci, somatic coliphages, F-RNA coliphages and bacteriophages infecting *Bacteroides fragilis* GB124) were added to experimental tanks (Figure 3.12 and 3.13) containing *C. gigas* and *M. edulis* placed in artificial seawater at different salinities and temperatures.

3.11.5 Enumeration of spiked faecal indicator bacteria and phages in shellfish

Mussels (n = 5) and oysters (n = 3) were removed from contaminated experimental tanks at intervals (6, 12, 24, 48, 96 hours) and assayed for *E. coli*, faecal coliforms, intestinal enterococci, somatic coliphages, F-specific RNA coliphages and human specific *Bacteroides fragilis* phages GB124 using most probable number and double-agar layer

methods (see sections 3.5 and 3.6 respectively). A schematic diagram describing the entire bioaccumulation experiment is shown in Figure 3.14.

3.11.6 Statistical analysis of dataset from laboratory-based experiment

The mean values of faecal indicator bacteria and bacteriophages observed in shellfish and overlying waters were subjected to descriptive statistics using SPSS Statistics Version 20.0. The relationship between temperature, salinity and mean values of bioaccumulated microbial indicators in mussels and oysters in the laboratory-based bioaccumulation experiment were analysed using the Pearson’s correlation matrix.

Figure 3.12: Experimental tanks containing *Crassostrea gigas* and *Mytilus edulis* dosed with faecal indicator bacteria and phages at low temperature (8°C) and varying salinities.

Figure 3.13: Experimental tanks containing *Crassostrea gigas* and *Mytilus edulis* dosed with faecal indicator bacteria and phages at high temperature (24°C) and varying salinities.

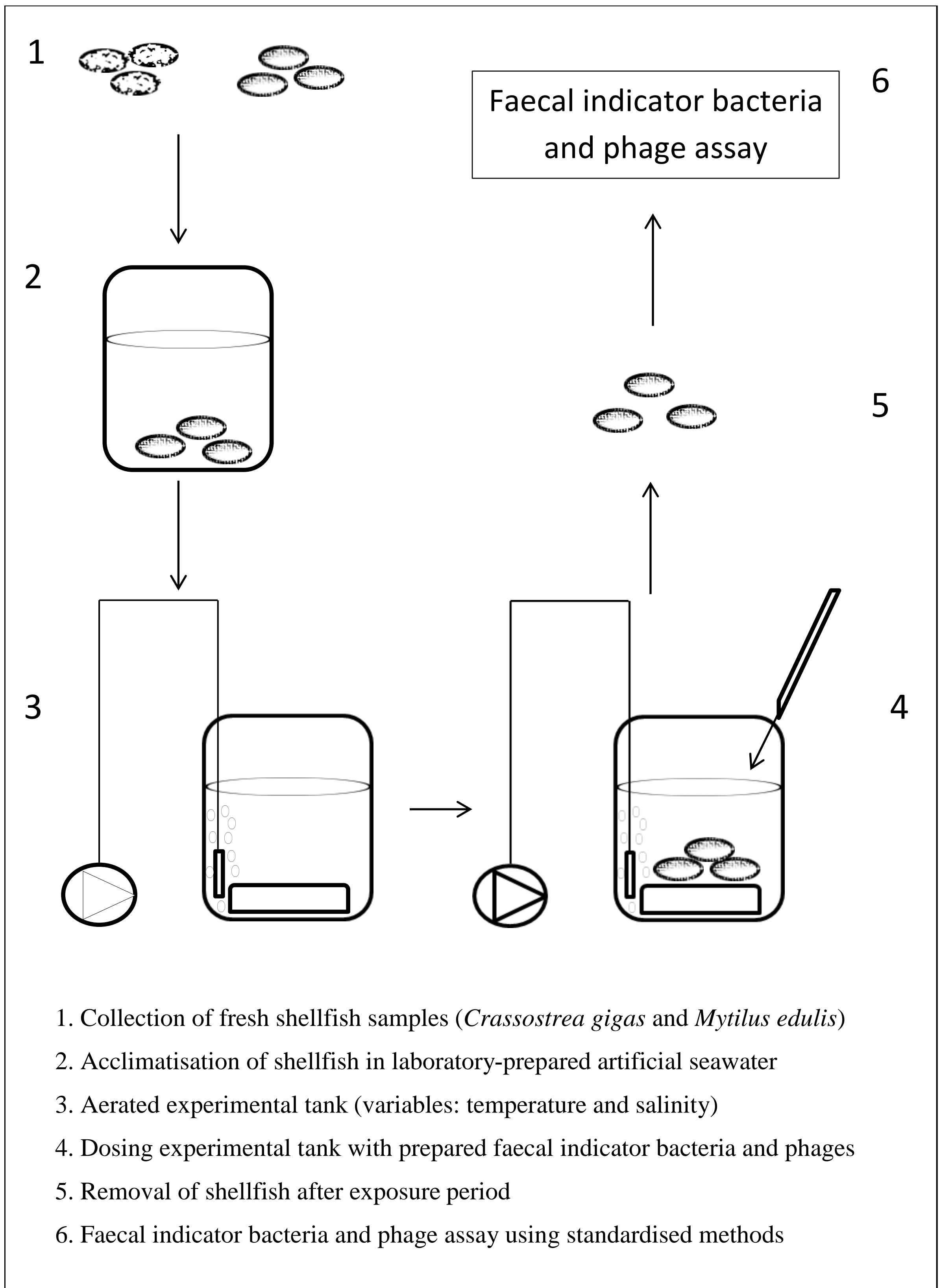


Figure 3.14: Schematic diagram of bioaccumulation experiment

Chapter Four: Results of Environmental Survey

4.1 Microbial examination of shellfish and their overlying waters

Forty five sampling trips were undertaken (approximately every two weeks) over a 24 month period (May 2013 to April 2015) to the Piddinghoe sampling site on the estuary of the River Ouse in southeast England, during which over 1801 mussels (*Mytilus edulis*) and 45 litres of overlying waters were analysed for levels of faecal indicator bacteria, viral indicators, and enteric viral pathogens (see Chapter 3 for methodologies used).

4.1.1 Detection of faecal indicator bacteria

The faecal indicator bacteria used to measure the sanitary quality of shellfish and their overlying waters in this study were *E. coli*, faecal coliforms and intestinal enterococci. The range of concentration of *E. coli* in *M. edulis* was 2.43 to 4.27 log_{10} most probable number (MPN) per 100 g of shellfish flesh and intravalvular fluid and in overlying waters the range was 1.55 to 4.00 log_{10} colony-forming units (CFU) per 100 ml. The range of concentration of faecal coliforms in *M. edulis* was 2.52 to 4.30 log_{10} MPN per 100 g of shellfish flesh and intravalvular fluid and in overlying waters the range was 1.56 to 4.16 log_{10} CFU per 100 ml. Similarly, the range of concentrations of intestinal enterococci in *M. edulis* was 2.23 to 3.97 log_{10} MPN per 100 g of shellfish flesh and intravalvular fluid and in overlying waters the range was 0.99 to 3.50 log_{10} CFU per 100 ml (Appendix 2A).

In Figure 4.1, the mean concentrations of *E. coli* (3493 MPN/100 g) over a 12 month period (July 2013 to June 2014) in shellfish suggest that the site would fail to meet the EU shellfish Class A category for which the standard is that shellfish must contain $\leq$ 230 *E. coli* per 100 g of flesh and intravalvular fluid.

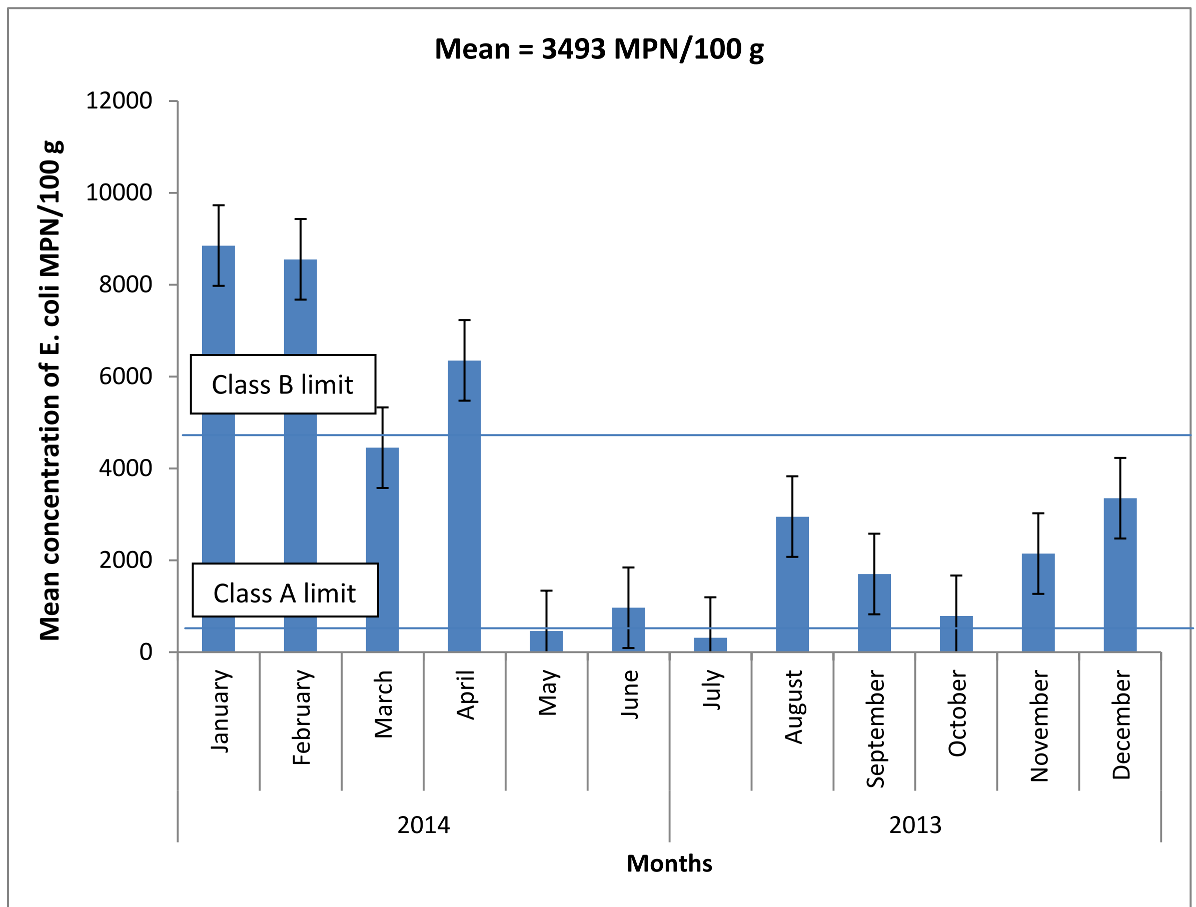


Figure 4.1: Comparison of monthly mean (number 'n' of replicates per month = 2, standard error) concentration of *E. coli* in shellfish with European Union microbiological criteria for shellfish.

This site would be classified as a Class B harvesting area in accordance with Regulation (EC) No 854/2004, which states that 90% of the shellfish samples must not exceed 4600 *E. coli* MPN/100 g and 10% must not exceed 46,000 *E. coli* MPN/100 g. This observation also agrees with the findings of Trajano Gomes Da Silva (2013) who demonstrated that 92% of mussels harvested from the River Ouse at Piddinghoe were within the Class B limits during a study undertaken between 2010 and 2012.

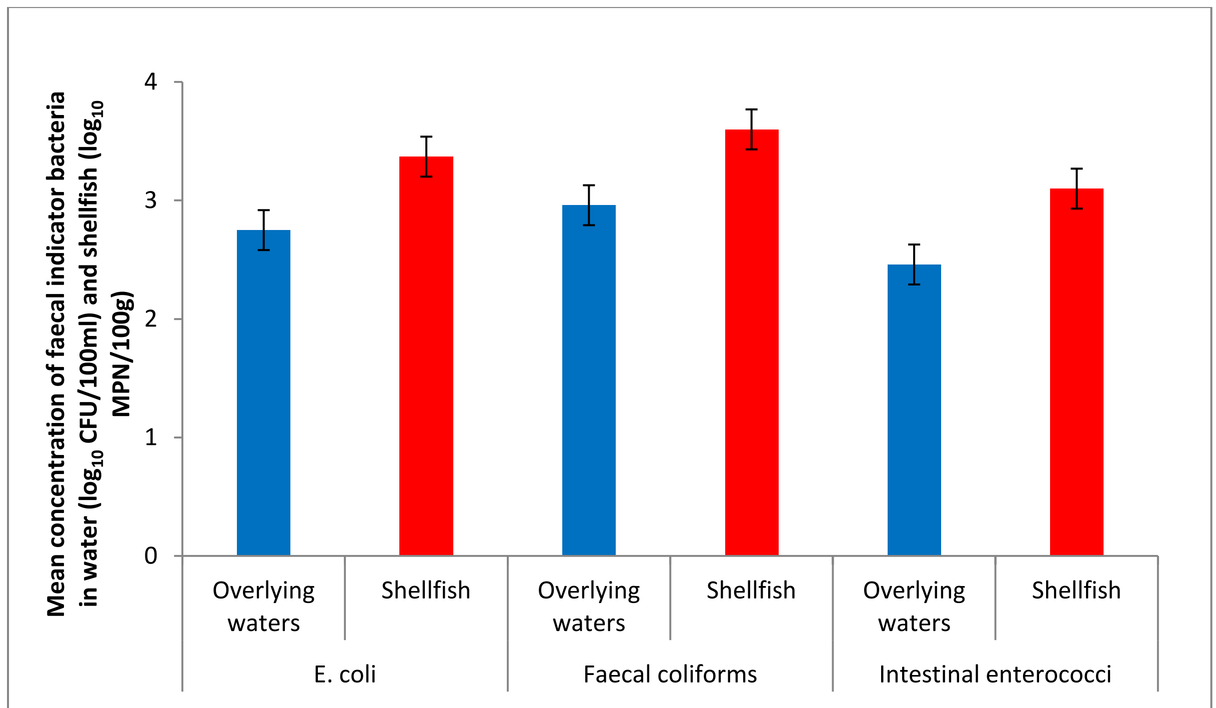


Figure 4.2: Mean (number 'n' of replicates for overlying waters/shellfish = 45, standard error) concentration of *E. coli*, faecal coliforms and intestinal enterococci in *M. edulis* and their overlying waters (2013 – 2015).

In shellfish samples, the mean concentration of faecal coliforms (3.60 ± 0.50 $\log_{10}$ MPN/100 g) was higher compared with that of *E. coli* (3.37 ± 0.51 $\log_{10}$ MPN/100 g) and intestinal enterococci (3.10 ± 0.47 MPN/100 g) (Figure 4.2).

In overlying water samples, the mean concentration of faecal coliforms (2.96 ± 0.78 $\log_{10}$ CFU/100 ml) was higher than *E. coli* (2.75 ± 0.76 $\log_{10}$ CFU/100 ml) and intestinal enterococci (2.46 ± 0.74 $\log_{10}$ CFU/100 ml) (Figure 4.2). The acceptable levels of faecal coliforms per 100 ml of water, as stated in the EU Shellfish Water Directive 2006/113/EC is ≤ 300 (i.e., 2.48 $\log_{10}$ CFU/100 ml) in shellfish flesh and intravalvular fluid for 75% of samples taken at the minimum frequency over a period of 12 months. Significant elevated levels of *E. coli*, faecal coliforms and intestinal enterococci were observed in shellfish

samples compared with their overlying waters (Figure 4.3). This suggests the uptake and bioaccumulation of bacteria in shellfish during their filter-feeding process.

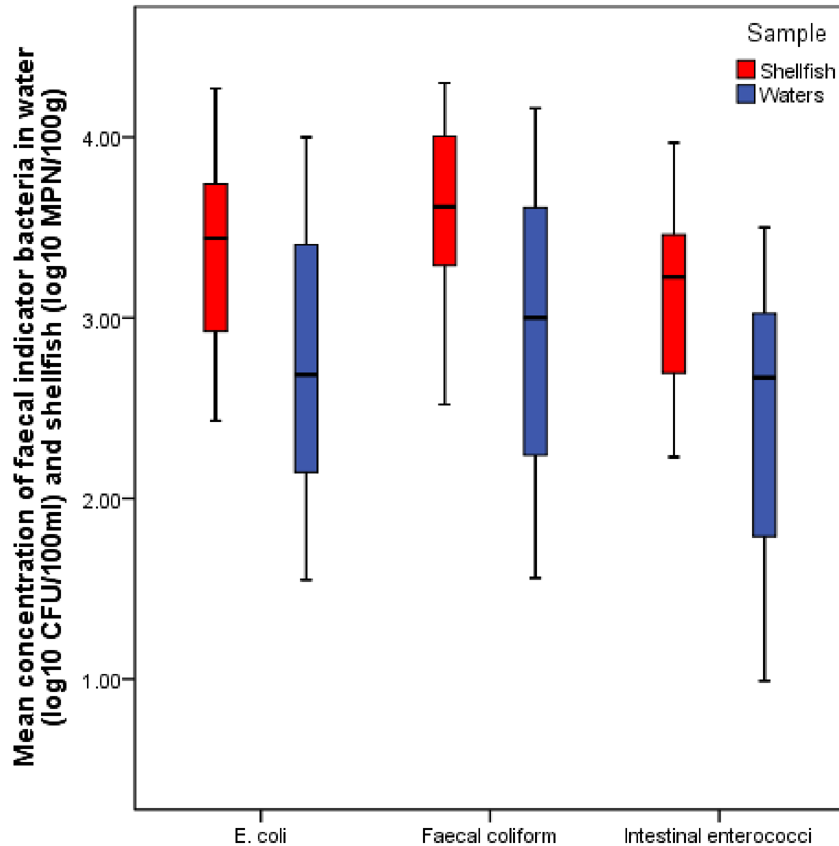


Figure 4.3: Boxplot of concentration of faecal indicator bacteria in shellfish and their overlying waters (number 'n' of replicates for overlying waters/shellfish = 45, standard error). The median value is represented by a line inside the box, 95% confidence intervals (bars).

4.1.2 Detection of viral indicators (bacteriophages)

The bacteriophages used in this study as viral indicators were somatic coliphages, F-RNA coliphages and bacteriophages infecting *Bacteroides fragilis* GB124. For statistical purposes, $\log_{10}$ values were recorded as 'zero' on sampling occasions where phages were below the limit of detection. The range of concentrations of somatic coliphages in *M. edulis* was 3.43 to 5.36 $\log_{10}$ plaque-forming units (PFU) per 100 g of shellfish digestive

gland and in overlying waters the range was 2.00 to 4.02 log_{10} PFU per 100 ml. The range of concentrations of F-RNA coliphages in *M. edulis* was zero to 3.82 log_{10} PFU per 100 g of shellfish digestive gland and in overlying waters the range was zero to 2.30 log_{10} PFU per 100 ml. The range of concentrations of bacteriophages infecting *Bacteroides fragilis* GB124 in *M. edulis* was zero to 5.29 log_{10} PFU per 100 g of shellfish digestive gland and in overlying waters the range was zero to 3.35 log_{10} PFU per 100 ml (Appendix 2B).

Seven of the 45 overlying water samples (16%) and 14 of the 45 shellfish batch samples (31%) presented positive for bacteriophages infecting *Bacteroides fragilis* GB124 with a mean concentration of 1.79 ± 1.64 log_{10} PFU/100 g in *M. edulis* and 0.67 ± 1.13 log_{10} PFU/100 ml in overlying waters, of which one of the positive samples (taken in July 2013) was excessively high. This is thought to have been associated with a discharge from a combined sewer overflow (CSO). Similarly, seven of the 45 overlying water samples (16%) and 14 of the 45 shellfish batch samples (31%) presented positive for F-RNA coliphages with mean concentration of 1.84 ± 1.62 log_{10} PFU/100 g in *M. edulis* and 0.60 ± 0.95 log_{10} PFU/100 ml in overlying waters. However, all the 45 samples (100%) of shellfish and overlying water presented positive for somatic coliphages with mean concentrations of 4.60 ± 0.52 log_{10} PFU/100 g in *M. edulis* and 3.01 ± 0.54 log_{10} PFU/100 ml in overlying waters (Figure 4.4).

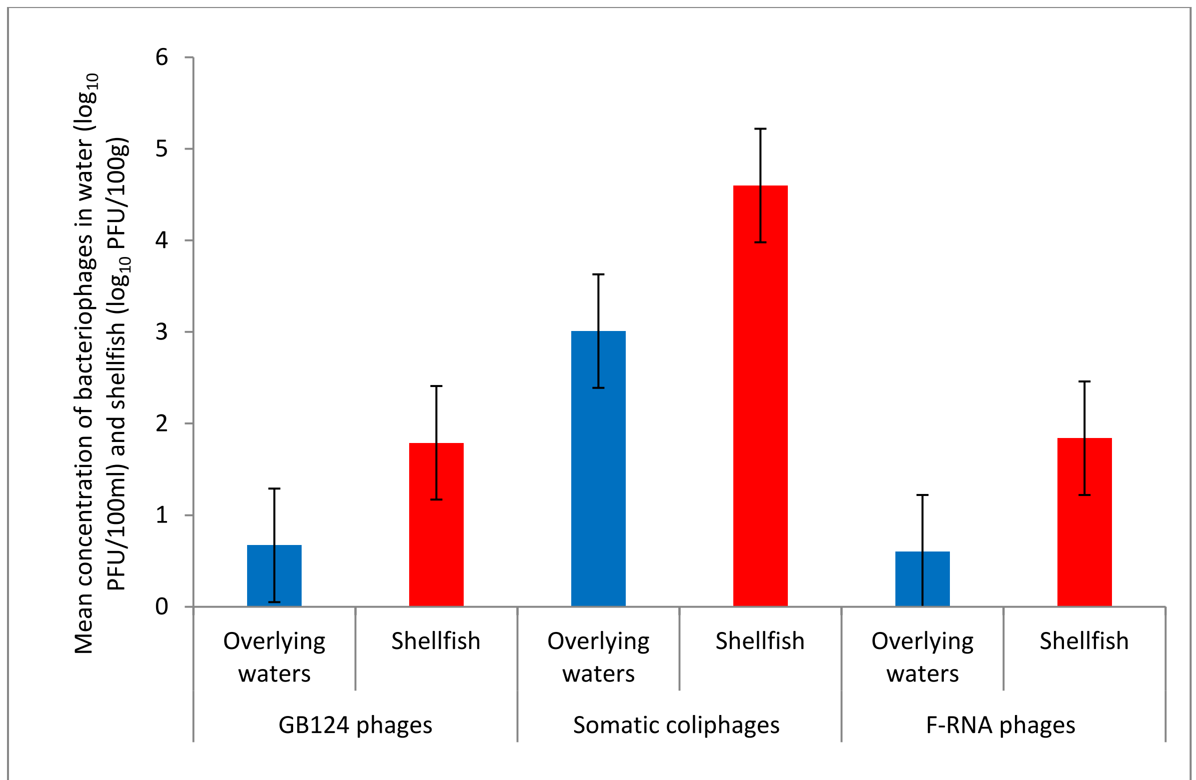


Figure 4.4: Mean (number 'n' of replicates for overlying waters/shellfish = 45, standard error) concentration of bacteriophages infecting *Bacteroides fragilis* GB124, somatic coliphages, F-RNA coliphages in shellfish and overlying water (2013 – 2015).

Elevated levels of somatic coliphages, F-RNA coliphages and bacteriophages infecting *Bacteroides fragilis* GB124 were recorded in shellfish samples compared with their overlying waters (Figure 4.5). This also suggests the uptake and bioaccumulation of viruses in shellfish during their filter-feeding process.

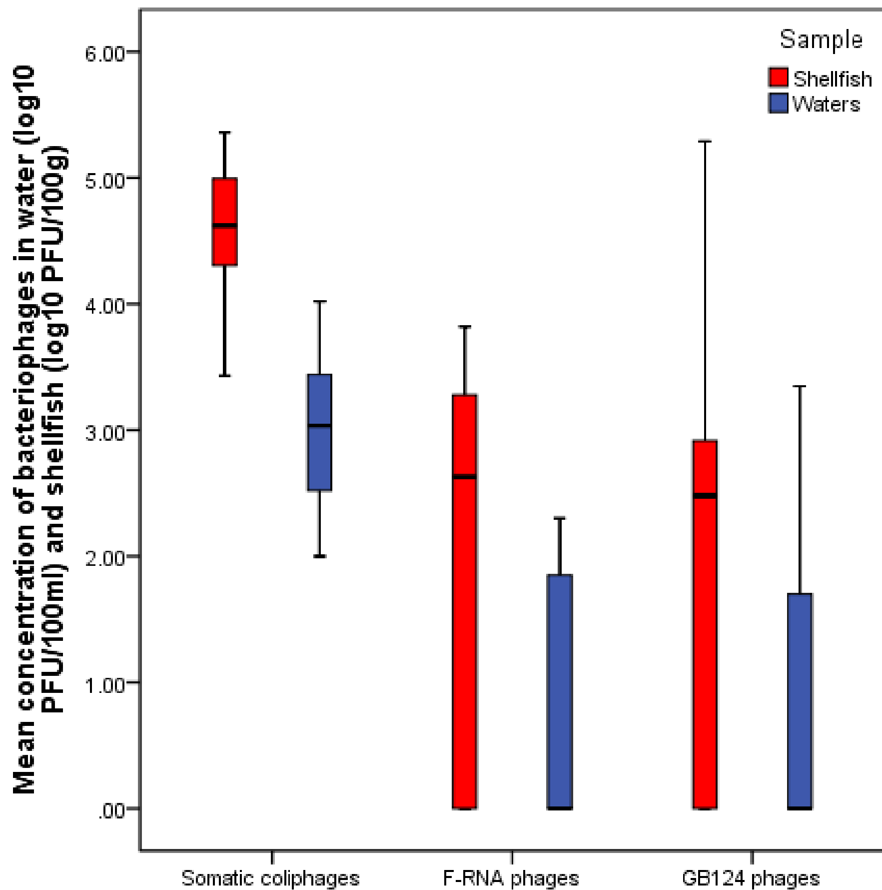


Figure 4.5: Boxplot of concentration of bacteriophages in shellfish and their overlying waters (number 'n' of replicates for overlying waters/shellfish = 45, standard error). The median value is represented by a line inside the box, 95% confidence intervals (bars).

4.2 Physicochemical parameters of shellfish-overlying waters

The physicochemical characteristics of the overlying waters obtained in this study were temperature, salinity, pH level, turbidity, dissolved oxygen, total dissolved solids and conductivity. These were determined on every sampling occasion to assess their role in the occurrence and survival of faecal indicator bacteria, bacteriophages and enteric viruses in shellfish.

The mean recorded water temperature was at its lowest (8.2°C) in December 2013 and at its highest (21.6°C) in July 2014 (Figure 4.6). Studies have shown that metabolic activities of shellfish are highly dependent on temperature. For example, Solic and Krstulovic (1992) highlighted a 25% reduction in the filtration rate of *Crassostrea gigas* and *Mytilus edulis*; and a 45% reduction in *Ostrea edulis* when temperature decreased from 20°C to 10°C.

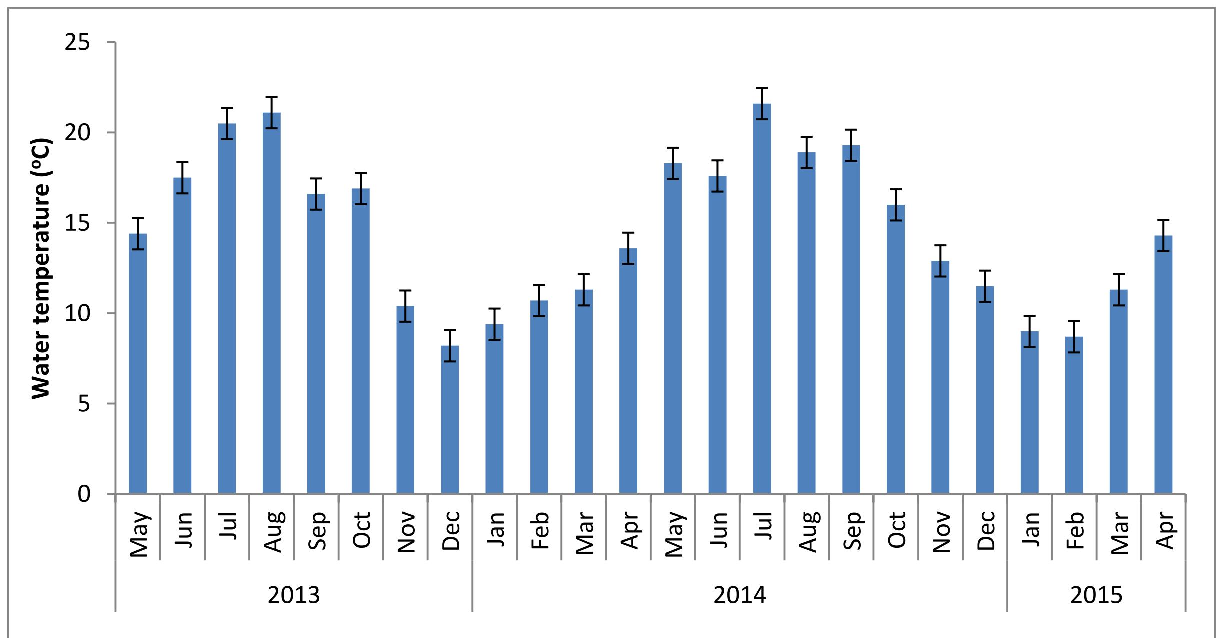


Figure 4.6: Monthly (number 'n' of replicates per month = 2, standard error) water temperature (°C) of river water at the Piddinghoe sampling site during sampling period

The range of recorded salinity levels was 1.21 ppt (February 2014) to 16.50 ppt (June 2014). Average salinity levels were at their lowest (November 2013 to March 2014 and November 2014 to March 2015) when the rainfall values were high. This is most likely due to the increase in the rate of chemical dilution in rivers when precipitation rates are high. Figure 4.7 shows inverse proportional pattern of salinity with tide.

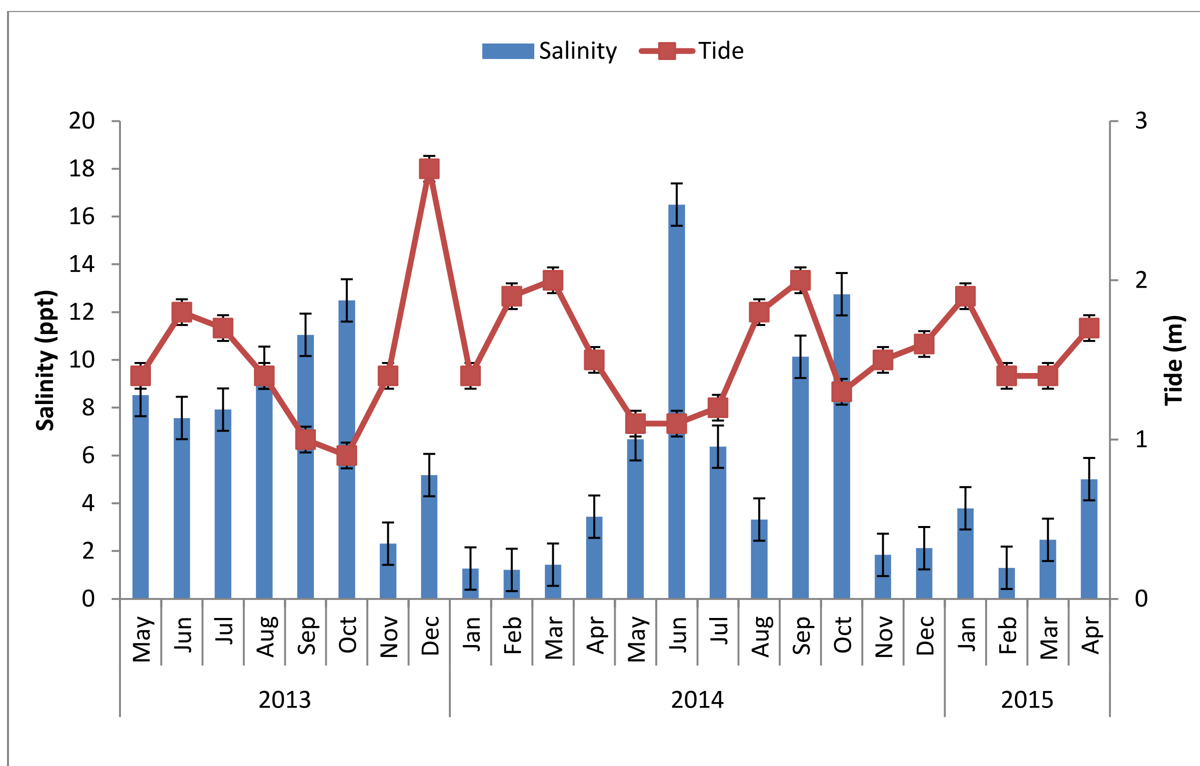


Figure 4.7: Monthly (number 'n' of replicates per month = 2, standard error) salinity (ppt) and tide (m) of river water at the Piddinghoe sampling site during sampling period

The maximum pH value observed was 8.82 (May 2013) and the minimum value was 6.83 (November 2013) (Appendix 3A). Generally, pH values lower than 7.0 were observed when water temperature and salinity were low (i.e., November 2013, December 2013 and January 2014).

Recorded turbidity level ranged from 5.40 NTU (September 2014) to 133.10 NTU (December 2013). The recorded levels of turbidity were relatively high from November 2013 to February 2014 and November 2014 to February 2015 (Figure 4.8). Similarly, these were periods of relatively low salinity and high input of surface water.

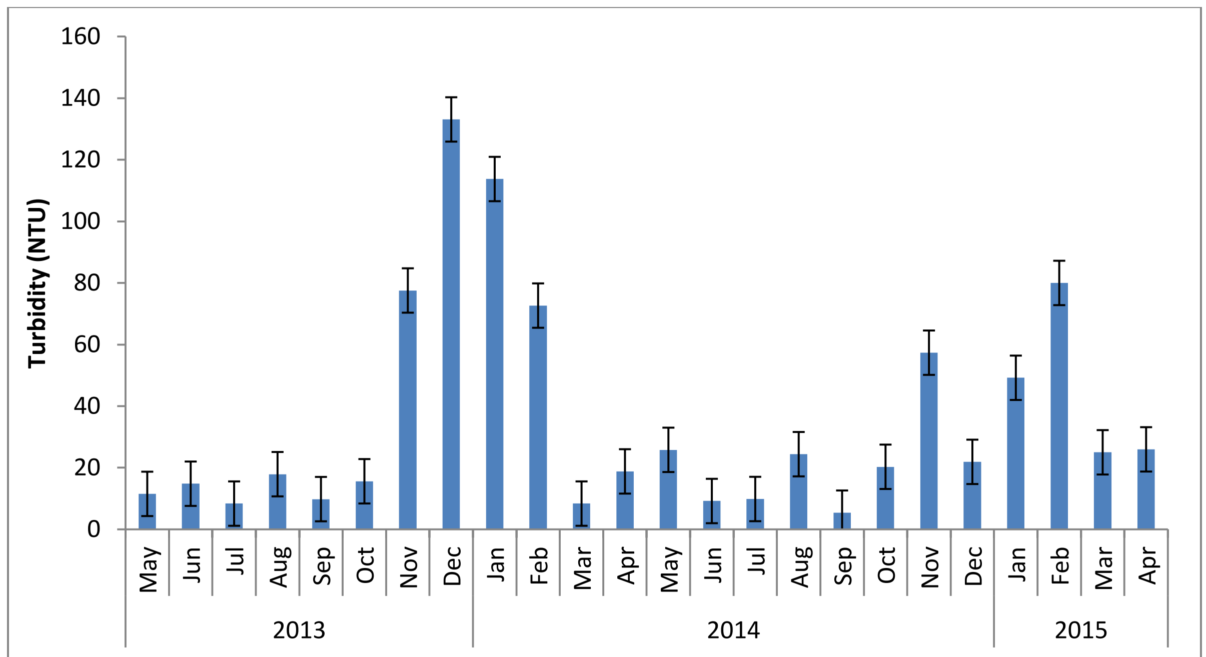


Figure 4.8: Monthly (number 'n' of replicates per month = 2, standard error) turbidity (NTU) level of river water at the Piddinghoe sampling site during sampling period

The minimum recorded value of dissolved oxygen was 5.69 mg/l (March 2014) and maximum value was 17.19 mg/l (August 2014) (Appendix 3B).

The range of total dissolved solids recorded values was 762 mg/l (May 2014) to 9757 mg/l (May 2013), similarly, the range of recorded conductivity values was 1171 µS/cm (May 2014) to 12913 µS/cm (May 2013) (Figure 4.9).

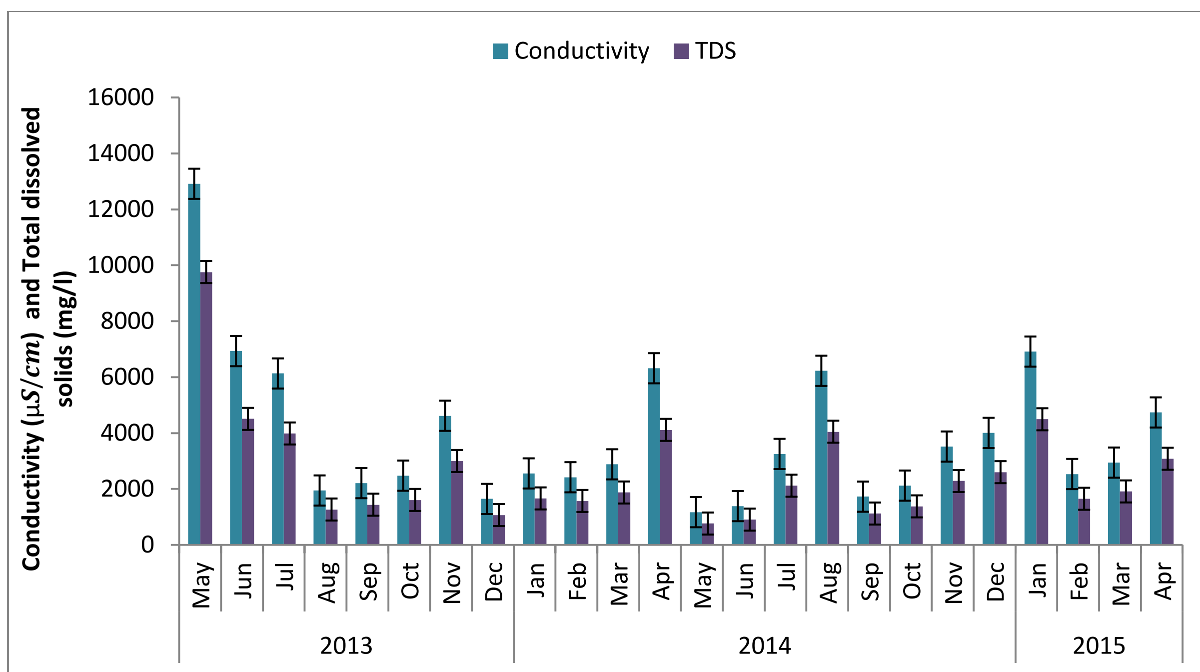


Figure 4.9: Monthly (number 'n' of replicates per month = 2, standard error) total dissolved solids (mg/l) and conductivity (µS/cm) of river water at the Piddinghoe sampling site during sampling period

4.3 Meteorological and hydrological data for The River Ouse catchment

The meteorological conditions prior to sampling and during sampling are presented in Table 4.1. (Recorded parameters are described in section 3.9).

Rainfall (mm) values were obtained from a weather station operated by the Environment Agency and located approximately 3.38 km from the sampling site. Maximum rainfall value of 11.74 mm was recorded in January 2014 and minimum rainfall value of 0.27 mm was recorded in April 2015. In general, rainfall values were highest when the lowest temperatures were recorded (October 2013 to February 2014, October 2014 to February 2015) (Appendix 3C).

Table 4.1: Meteorological conditions prior to sampling (24 and 48 hour earlier) and during sampling

Year	Month	Average monthly weather condition		
		During sampling	24 hours earlier	48 hours earlier
2013	May	Dry	Dry	Dry
	June	Warm	Dry	Intermittent showers
	July	Warm	Dry	Wet
	August	Dry, warm and sunny	Dry	Intermittent showers
	September	Dry	Storm event	Wet
	October	Slightly cold	Wet	Dry
	November	Intermittent showers	Storm event	Wet
	December	Wet	Storm event	Wet
2014	January	Wet	Wet	Wet
	February	Wet	Wet	Intermittent showers
	March	Wet	Dry and sunny	Dry and sunny
	April	Dry and sunny	Dry and sunny	Dry and sunny
	May	Dry	Dry and sunny	Dry and sunny
	June	Dry	Dry and sunny	Dry and sunny
	July	Dry and sunny	Dry and sunny	Dry and sunny
	August	Wet	Wet	Dry
	September	Dry and sunny	Dry and sunny	Dry and sunny
	October	Wet	Dry	Wet and cold
	November	Wet, storm event	Wet	Wet
	December	Cold	Intermittent showers	Cold
2015	January	Cold	Cold, snow	Cold
	February	Cold	Intermittent showers	Dry and cold
	March	Cold and sunny	Dry and sunny	Cold and sunny
	April	Dry and sunny	Dry and foggy	Dry and sunny

Daily mean river flow (m^3/s) data were obtained from the Environment Agency – Southeast Region. River flow rates greater than 2.0 m^3/s were observed from November 2013 to March 2014 and October 2014 to February 2015. On the other hand, river flow rates less than 2.0 m^3/s were observed from May to October 2013 and April to September 2014, periods characterised by higher temperatures and relatively low rainfall. River flow rate ranged from 0.62 m^3/s (August 2013 and September 2014) to 12.01 m^3/s (February 2014) (Figure 4.10). The maximum recorded air temperature observed was 19.5^{o}C (July 2013) and the minimum recorded value was 4.0^{o}C (February 2015). Generally, variations in ambient air temperature values corresponded to variations in water temperature (Appendix 3D).

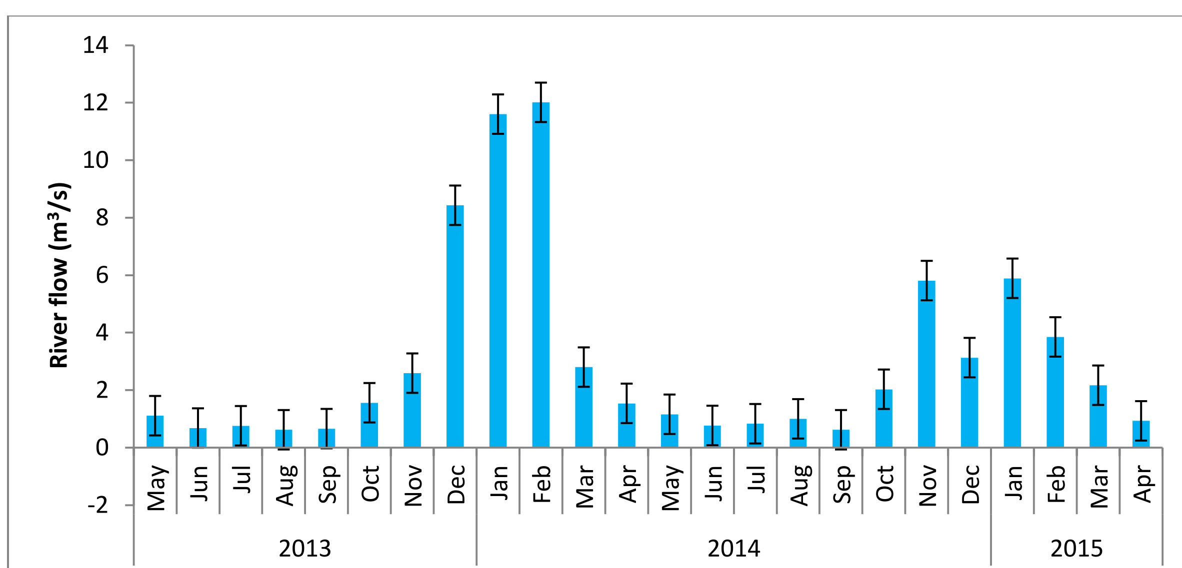


Figure 4.10: Monthly (number 'n' of replicates per month = 2, standard error) river flow (m^3/s) of water at the Piddinghoe sampling site during sampling period (Source: Environment Agency, Southeast UK)

4.4 Seasonal stability of microbial parameters

In this study, four seasons were considered, i.e., spring (March to May), summer (June to August), autumn (September to November), and winter (December to February). Spring

was characterised by average rainfall, river flow, and water and air temperature. In the summer, water and air temperature reached their maximum levels, while rainfall and river flow rates were at their minimum. However, in autumn the rate of precipitation began to increase and there was a corresponding decrease in water and air temperature. The winter period was characterised by the lowest water and air temperature as well as the highest rainfall and river flow rates, especially during storm events (Table 4.2).

Table 4.2: Average values of some environmental parameters during different seasons

Seasons	Rainfall (mm)	River flow (m^3/s)	Water temperature(^oC)	Airtemperature(^oC)
Spring (n = 6)	1.64 ± 1.19	1.65 ± 0.79	13.87 ± 2.58	8.83 ± 2.48
Summer (n = 6)	1.39 ± 1.02	0.78 ± 0.13	19.53 ± 1.79	17.00 ± 2.07
Autumn (n = 6)	4.18 ± 2.11	2.21 ± 1.92	15.35 ± 3.18	12.00 ± 3.39
Winter (n = 6)	6.83 ± 3.67	8.21 ± 3.78	9.58 ± 1.26	5.67 ± 1.03

Key: Mean values ± standard deviation 'n' = number of replicates per season

4.4.1 Seasonal variations in levels of faecal indicator bacteria

The mean concentrations of *E. coli*, faecal coliforms and intestinal enterococci in spring, summer and autumn were consistently higher in shellfish than in their overlying waters (Figure 4.11), though the differences in their concentrations were not statistically significant ($P > 0.05$). Highest bioaccumulation levels of *E. coli*, faecal coliforms and intestinal enterococci in shellfish were observed in the summer. This is most likely due to the increase in water temperature, which results in higher metabolic activity of the shellfish. In the winter, the mean concentration of *E. coli* was lower in the shellfish compared with their overlying waters, with the exception of faecal coliforms and intestinal enterococci which remained present at higher concentrations in shellfish.

In general, between seasons there were significant differences in the concentrations of *E. coli*, faecal coliforms and intestinal enterococci (Duncan, $P < 0.05$).

4.4.2 Seasonal variations in levels of bacteriophages

Somatic coliphages showed the highest prevalence (100%) and maintained higher concentrations in shellfish than in overlying waters during all seasons (Figure 4.12). The differences in these concentrations were statistically significant ($P < 0.05$). F-RNA coliphages were observed in all seasons except during summer, when they were undetected in the overlying waters and this may likely be due to inactivation by high temperatures, fewer run-offs as well as less pollution. Their mean concentration was higher in the shellfish than in overlying waters during spring, autumn and winter. The bacteriophages infecting *Bacteroides fragilis* GB124 were detected in spring, summer and winter with higher concentrations in shellfish than in overlying waters. Although the GB124 phages were below detection limit in overlying waters during autumn, they were detected in shellfish samples during this period.

In general, there were significant differences in the concentrations of F-RNA coliphages between seasons ($P > 0.05$), but no significant differences in the concentrations of somatic coliphages and the bacteriophages infecting *Bacteroides fragilis* GB124 between seasons ($P < 0.05$).

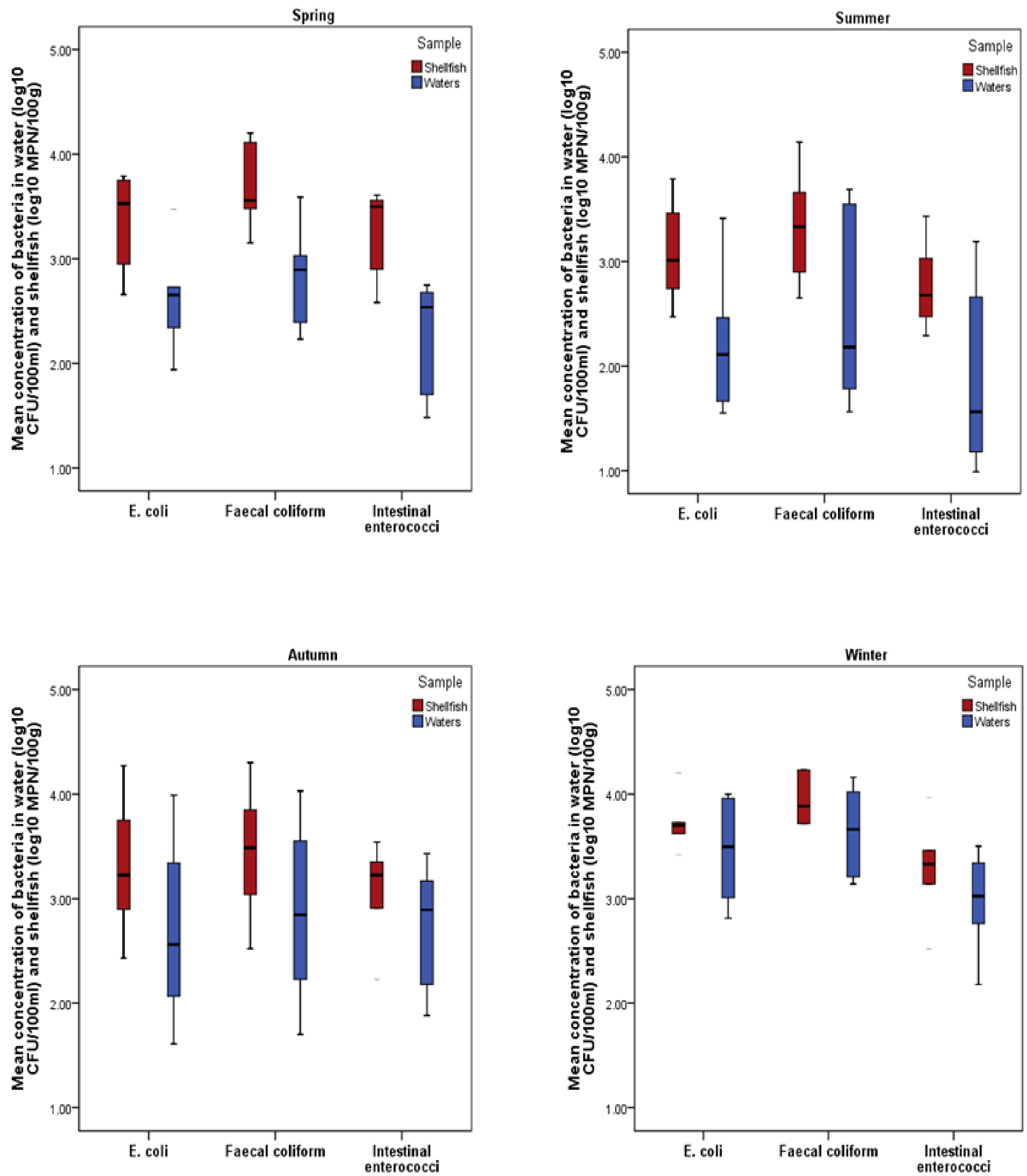


Figure 4.11: Boxplots of concentration of faecal indicator bacteria in shellfish and their overlying waters (number 'n' of replicates for overlying waters/shellfish = 6, standard error) during spring, summer, autumn and winter. The median value (represented by a line inside the box, 95% confidence intervals) varies in all seasons.

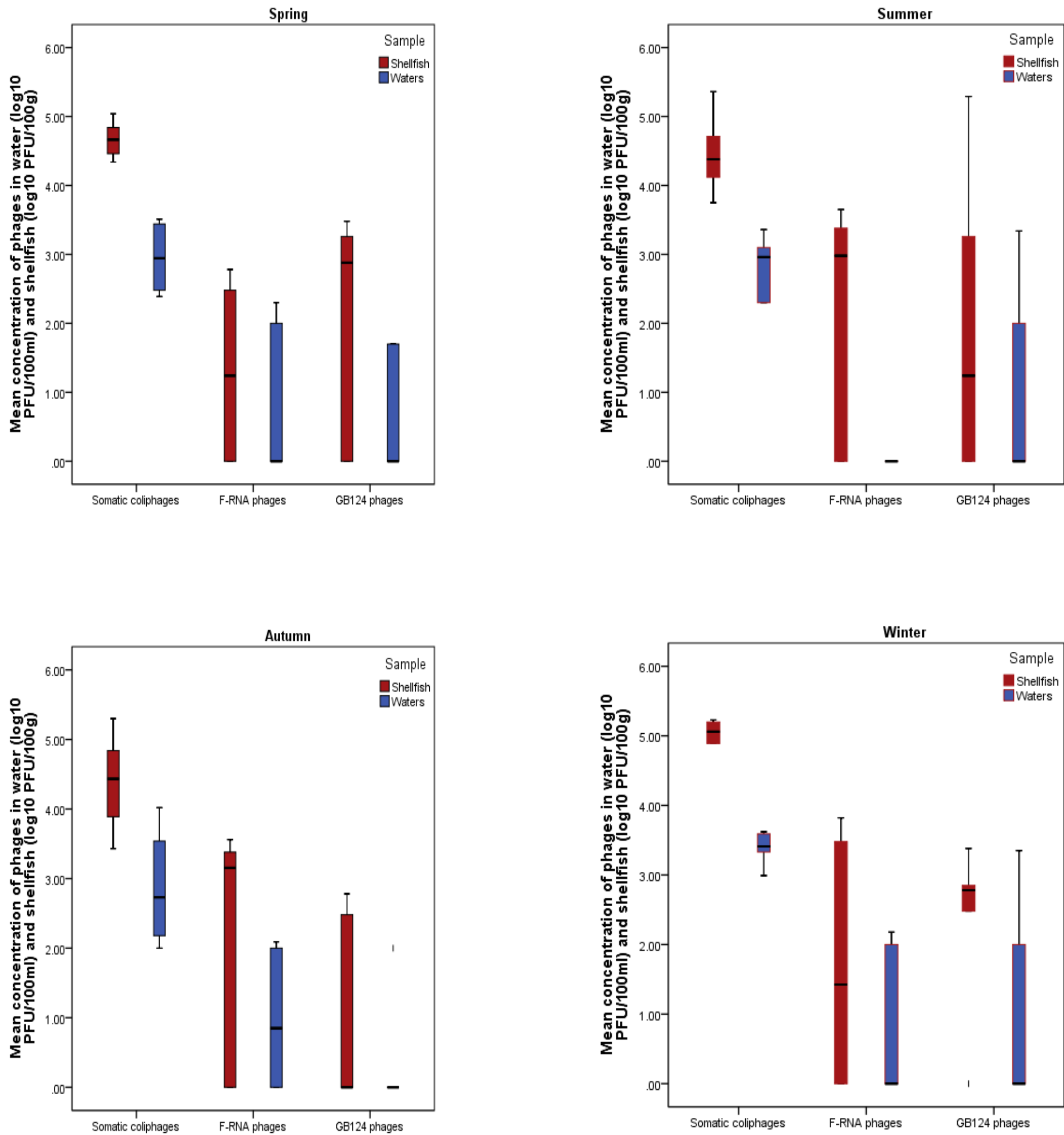


Figure 4.12: Boxplots of concentration of bacteriophages in shellfish and their overlying waters (number 'n' of replicates for overlying waters/shellfish = 6, standard error) during spring, summer, autumn and winter. The median value (represented by a line inside the box, 95% confidence intervals) varies in all seasons.

4.5 The relationship between bacterial and viral indicators, physicochemical, meteorological and hydrological data

Concentrations of faecal indicator bacteria and bacteriophages in shellfish and their overlying waters, physicochemical characteristics, meteorological and hydrological data at the Piddinghoe sampling site were analysed using non-parametric two-tailed Spearman's rank correlation at two levels of significance ($P < 0.01$ and $P < 0.05$) representing 99% and 95% confidence interval respectively (Table 4.3). The levels of GB124 phages in overlying waters showed a positive correlation with those in mussels (rho = 0.42, $P < 0.05$). The levels of GB124 phages in mussels showed a positive correlation with levels of somatic coliphages in mussels (rho = 0.51, $P < 0.05$). The levels of somatic coliphages in overlying waters showed a positive correlation with those in mussels (rho = 0.64, $P < 0.01$). The levels of F-RNA coliphages in overlying waters showed a positive correlation with those in mussels (rho = 0.52, $P < 0.01$).

Water temperature showed a positive correlation with pH level (rho = 0.61, $P < 0.01$), salinity (rho = 0.66, $P < 0.01$), and air temperature (rho = 0.95, $P < 0.01$), whereas negative correlations were observed between water temperature and turbidity, rainfall, river flow, levels of *E. coli*, faecal coliforms, intestinal enterococci and somatic coliphages. Similarly, turbidity showed positive correlations with rainfall (rho = 0.63, $P < 0.01$), river flow (rho = 0.75, $P < 0.01$), levels of *E. coli* in overlying waters (rho = 0.63, $P < 0.01$), levels of faecal coliforms in overlying waters (rho = 0.61, $P < 0.01$), levels of intestinal enterococci in overlying waters (rho = 0.50, $P < 0.05$), and levels of somatic coliphages in mussels (rho = 0.61, $P < 0.01$). Negative correlations were observed between turbidity and pH levels, salinity and air temperature. Also, salinity showed a positive correlation with air temperature (rho = 0.71, $P < 0.01$), and negative correlations were observed between

salinity and river flow, levels of *E. coli*, faecal coliforms, intestinal enterococci, GB124 phages, and somatic coliphages.

Interestingly, electrical conductivity showed positive correlations with total dissolved solids (rho = 1.00), levels of GB124 phages in overlying waters (rho = 0.54, P < 0.01), and levels of GB124 phages in mussels (rho = 0.43, P < 0.05). Similarly, total dissolved solids showed positive correlations with the levels of GB124 phages in overlying waters (rho = 0.54, P < 0.01), and levels of GB124 phages in mussels (rho = 0.43, P < 0.05). However, dissolved oxygen showed negative correlations with rainfall (rho = -0.41, P < 0.05) and levels of F-RNA coliphages in mussels (rho = -0.52, P < 0.01).

Air temperature showed negative correlations with river flow (rho = -0.86, P < 0.01), levels of *E. coli* in overlying waters (rho = -0.69, P < 0.01), levels of *E. coli* in mussels (rho = -0.46, P < 0.05), levels of faecal coliforms in overlying waters (rho = -0.60, P < 0.01), levels of faecal coliforms in mussels (rho = -0.57, P < 0.01), levels of intestinal enterococci in overlying waters (rho = -0.48, P < 0.05), levels of intestinal enterococci in mussels (rho = -0.44, P < 0.05), levels of somatic coliphages in overlying waters (rho = -0.53, P < 0.01), and levels of somatic coliphages in mussels (rho = -0.50, P < 0.05). River flow showed positive correlations with the levels of *E. coli* in overlying waters (rho = 0.81, P < 0.01), levels of *E. coli* in mussels (rho = 0.51, P < 0.05), levels of faecal coliforms in overlying waters (rho = 0.77, P < 0.01), levels of faecal coliforms in mussels (rho = 0.65, P < 0.01), levels of intestinal enterococci in overlying waters (rho = 0.55, P < 0.01), levels of somatic coliphages in overlying waters (rho = 0.66, P < 0.01), and levels of F-RNA coliphages in overlying waters (rho = 0.44, P < 0.05).

The levels of *E. coli* in overlying waters showed positive correlations with those in mussels (rho = 0.75, $P < 0.01$), levels of faecal coliforms in overlying waters (rho = 0.97, $P < 0.01$), levels of faecal coliforms in mussels (rho = 0.84, $P < 0.01$), levels of intestinal enterococci in overlying waters (rho = 0.71, $P < 0.01$), levels of GB124 phages in mussels (rho = 0.43, $P < 0.05$), levels of somatic coliphages in overlying waters (rho = 0.69, $P < 0.01$), and levels of somatic coliphages in mussels (rho = 0.54, $P < 0.01$). The levels of *E. coli* in mussels showed positive correlations with levels of faecal coliforms in overlying waters (rho = 0.72, $P < 0.01$), levels of faecal coliforms in mussels (rho = 0.90, $P < 0.01$), levels of intestinal enterococci in overlying waters (rho = 0.55, $P < 0.01$), levels of intestinal enterococci in mussels (rho = 0.60, $P < 0.01$), and levels of F-RNA coliphages in overlying waters (rho = 0.45, $P < 0.05$). The levels of faecal coliforms in overlying waters showed positive correlations with those in mussels (rho = 0.84, $P < 0.01$), levels of intestinal enterococci in overlying waters (rho = 0.73, $P < 0.01$), levels of somatic coliphages in overlying waters (rho = 0.69, $P < 0.01$), and levels of somatic coliphages in mussels (rho = 0.51, $P < 0.05$).

The levels of faecal coliforms in mussels showed positive correlations with the levels of intestinal enterococci in overlying waters (rho = 0.65, $P < 0.01$), levels of intestinal enterococci in mussels (rho = 0.59, $P < 0.01$), levels of GB124 phages in mussels (rho = 0.47, $P < 0.05$), and levels of somatic coliphages in overlying waters (rho = 0.48, $P < 0.05$). The levels of intestinal enterococci in overlying waters showed positive correlations with the levels of somatic coliphages in overlying waters (rho = 0.55, $P < 0.01$), and levels of somatic coliphages in mussels (rho = 0.52, $P < 0.01$). The levels of intestinal enterococci in mussels showed a negative correlation with the levels of F-RNA coliphages in mussels (rho = -0.42, $P < 0.05$).

Table 4.3: Spearman's rank correlation between microbial, physicochemical, meteorological and hydrological data (24 months dataset)

	Water Temp.	Turb-idity	pH	Salinity	Condu-ctivity	TDS	DO	Rain-fall	Air Temp	River flow	Water *E. coli*	Mussel *E. coli*	Water FC	Mussel FC	Water Ent.	Mussel Ent.	Water GB124	Mussel GB124	Water SomC	Mussel SomC	Water F-RNA	Mussel F-RNA
Water Temp.	1.00																					
Turb-idity	-0.72**	1.00																				
pH	0.61**	-0.58**	1.00																			
Sali-nity	0.66**	-0.62**	0.65**	1.00																		
Condu ctivity	-0.01	-0.01	0.12	-0.28	1.00																	
TDS	-0.01	-0.01	0.12	-0.28	1.00**	1.00																
DO	0.19	0.01	0.33	0.06	0.19	0.91	1.00															
Rain-fall	-0.53**	0.63**	-0.60**	-0.34	-0.81	-0.18	-0.41*	1.00														
Air Temp	0.95**	-0.68**	0.56**	0.71**	-0.11	-0.11	0.07	-0.46*	1.00													
River flow	-0.86**	0.75**	-0.73**	-0.70**	0.07	0.07	-0.38	0.83**	-0.86**	1.00												
Water *E. coli*	-0.70**	0.63**	-0.80**	-0.83**	0.17	0.17	-0.22	0.61**	-0.69**	0.81**	1.00											
Mussel *E. coli*	-0.44*	0.37	-0.38	-0.47*	0.34	0.34	-0.00	0.46*	-0.46*	0.51*	0.75**	1.00										
Water FC	-0.60**	0.61**	-0.80**	-0.81**	0.20	0.20	-0.18	0.62**	-0.60**	0.77**	0.97**	0.72**	1.00									
Mussel FC	-0.52**	0.45*	-0.54**	-0.61**	0.28	0.28	0.00	0.50*	-0.57**	0.65**	0.84**	0.90**	0.84**	1.00								
Water Ent.	-0.47*	0.50*	-0.61**	-0.57**	0.09	0.09	0.15	0.50*	-0.48*	0.55**	0.71**	0.55**	0.73**	0.65**	1.00							
Mussel Ent.	-0.42*	0.09	-0.07	-0.27	0.29	0.29	0.14	-0.13	-0.44*	0.21	0.38	0.60**	0.38	0.59**	0.39	1.00						
Water GB124	-0.18	0.17	0.04	-0.18	0.54**	0.54**	0.04	-0.04	-0.12	0.12	-0.01	-0.16	-0.00	-0.06	0.05	-0.13	1.00					
Mussel GB124	-0.34	0.22	-0.25	-0.47*	0.43*	0.43*	0.06	0.01	-0.36	0.33	0.43*	0.30	0.39	0.47*	0.23	0.36	0.42*	1.00				
Water SomC	-0.57**	0.71**	-0.68**	-0.63**	0.16	0.16	0.10	0.36	-0.53**	0.66**	0.69**	0.36	0.69**	0.48*	0.55**	0.13	0.25	0.40	1.00			
Mussel SomC	-0.60**	0.61**	-0.49*	-0.58**	0.19	0.19	0.20	0.31	-0.50*	0.43	0.54**	0.23	0.51*	0.31	0.52**	0.33	0.32	0.51*	0.64**	1.00		
Water FRNA	-0.32	0.31	-0.20	-0.27	0.21	0.21	-0.31	0.44*	-0.21	0.44*	0.39	0.45*	0.36	0.27	0.26	0.11	0.18	-0.18	0.19	0.20	1.00	
Mussel FRNA	-0.08	0.09	-0.21	-0.03	0.02	0.02	-0.52**	0.41*	0.09	0.35	0.15	0.05	0.10	-0.04	-0.04	-0.42*	0.27	-0.04	0.03	0.09	0.52**	1.00

Key: Temp.-Temperature; TDS-Total dissolve solids; DO-Dissolved oxygen; FC-Faecal coliforms; Ent-Enterococci; SomC-Somatic coliphages; F-RNA-F-RNA coliphages; GB124-GB124 phages; **-Correlation is significant at the 0.01 level (2-tailed); *-Correlation is significant at the 0.05 level (2-tailed)

Chapter Five: Results of Uptake and Bioaccumulation Studies

5.1 Environmental assessment of bioaccumulation in shellfish

This chapter describes an investigation undertaken to study the influence of water temperature on bioaccumulation of bacteriophages (somatic coliphages, F-RNA coliphages and human-specific phages of *Bacteroides fragilis* GB124) and faecal indicator bacteria (*E. coli,* faecal coliforms and intestinal enterococci) in mussels (*Mytilus edulis*) harvested from a site in southeast England. The study aimed to gain a better understanding of how microorganisms, including pathogens, are bioaccumulated in shellfish in their natural environment. The sampling site was situated at Piddinghoe in the tidal stretch of the River Ouse in southern England with a depth of approximately three to four metres (from the lowest point at normal tide), about eight metres at high tide and less than one metre at low tide and a fluctuating salinity between 0.6 to 16 ppt (recorded minimum and maximum values from 45 sampling occasions over 24 months). In addition, this estuarine site is affected by fluctuating faecal inputs from partially-treated municipal wastewater discharges and diffuse and point source agricultural inputs. Although not a designated shellfish harvesting site, if the site were to be designated a shellfish/harvesting water under the EU classification categories of shellfish growing/harvesting waters (EC No. 854/2004), according to the data obtained during this study and during a previous study (Trajano Gomes da Silva, 2013) it would most probably be classified as a ‘Class B area’.

Freshly-collected mussels (*Mytilus edulis*) and samples of overlying waters were analysed using standard microbiological methods over a twenty-four month period. The concentrations of faecal coliforms, intestinal enterococci and *E. coli* were determined by membrane filtration or most probable number methods (MPN) according to standard methods (see section 3.4 and 3.5), while the concentrations of somatic coliphages, F-RNA

coliphages and GB124 phages were determined by direct plaque assay using the standardised double-agar layer method (see section 3.6).

5.1.1 The application of 'accumulation factor' (AF) to the microbial dataset

Canzonier (1971) and Richards (1988) defined bioaccumulation as the ratio of the concentration of coliphage S-13 in clams to those in seawater. In 2013, Kershaw *et al*. described bioaccumulation as a measure of the intensity of the accumulation of faecal indicator organisms in bivalve shellfish, and that the measure is given by the ratio of the concentrations of faecal indicator organisms in shellfish relative to the concentration of faecal indicator organisms in the overlying water. In this study, the accumulation factor (AF) of each parameter was obtained by dividing the log concentration of each organism in *M. edulis* (PFU or MPN/100 g) by the corresponding log concentration of organisms in the overlying water (PFU or CFU/100 ml) at the same point in time. Table 5.1 shows the mean accumulation factors (AF) of faecal indicator bacteria and bacteriophages in *M. edulis* over the entire study period and values highlighted showed indicators with highest bioaccumulation per month.

Table 5.1: Accumulation factors (AF) of faecal indicator bacteria and bacteriophages in *M. edulis* over the entire study period

Year	Month	Faecal indicator bacteria			Bacteriophages		
		E. coli	Faecal coliforms	Intestinal enterococci	*B. fragilis* GB124	Somatic coliphages	F-RNA coliphages
2013	May	1.55	2.32	**3.17**	0.00	1.93	1.08
	June	1.86	1.89	**3.57**	0.00	1.89	0.00
	July	1.51	1.56	**2.29**	2.12	1.61	0.00
	August	1.56	1.58	**1.95**	0.00	1.55	0.00
	September	1.32	1.28	**1.62**	0.00	1.50	0.00
	October	1.40	1.36	1.19	0.00	**1.57**	0.00
	November	0.99	0.99	1.00	1.24	1.32	**1.51**
	December	1.34	1.22	**1.57**	0.00	1.54	0.00
2014	January	**1.88**	0.98	0.90	0.83	1.44	1.74
	February	0.90	0.89	0.92	0.00	1.47	**1.75**
	March	1.05	1.12	1.45	0.00	**1.84**	0.00
	April	1.44	1.48	1.29	1.46	**1.70**	1.39
	May	1.37	1.41	**1.71**	0.00	1.47	0.00
	June	1.40	1.49	1.36	0.00	**1.55**	0.00
	July	**1.34**	1.22	1.15	0.00	1.22	0.00
	August	1.12	1.15	1.08	0.00	**1.61**	0.00
	September	1.51	1.48	1.02	0.00	**2.27**	0.00
	October	1.46	1.34	1.14	0.00	1.58	**1.78**
	November	1.08	1.08	1.01	0.00	1.37	**1.99**
	December	**1.17**	**1.17**	1.08	0.00	1.10	0.00
2015	January	1.10	1.15	1.19	**1.69**	1.53	0.00
	February	1.23	1.21	1.14	0.00	**1.66**	0.00
	March	1.28	1.18	1.31	0.00	**1.44**	0.00
	April	1.13	1.26	0.99	**1.92**	1.47	0.00

AF - log concentration of organism in mussels divided by the log concentration of organism in the waters; 0.00 = Phage is below detection limit. Figures in **bold** denote the highest AF per month.

5.1.2 Bioaccumulation of faecal indicator bacteria in *M. edulis*

Levels of *E. coli* were observed to bioaccumulate to densities averaging 1.39 ± 0.26 greater than the levels recorded in overlying waters. The mean accumulation factor of *E. coli* ranged from 0.90 to 1.88, and was less than one (AF < 1) in November 2013 and February 2014, but greater than one (AF > 1) during the summer months, suggesting higher rates of metabolic activities and filtration in *M. edulis* during summer months (Figure 5.1).

Levels of faecal coliforms were observed to bioaccumulate to densities averaging 1.38 ± 0.34 greater than the levels recorded in overlying waters. The mean recorded accumulation factor of faecal coliforms ranged from 0.89 to 2.32, and was observed to be less than one (AF < 1) during the coldest months (i.e. November 2013, January 2014 and February 2014), but greater than one (AF > 1) in all other months (Figure 5.1).

Levels of intestinal enterococci were observed to bioaccumulate to densities averaging 1.58 ± 0.75 greater than the levels recorded in overlying waters. The mean accumulation factor of intestinal enterococci ranged from 0.90 to 3.57, and was less than one (AF < 1) in January 2014 and February 2014, but greater than one (AF > 1) in all other months (Figure 5.1).

5.1.3 Bioaccumulation of viral indicators (bacteriophages) in *M. edulis*

Bacteroides fragilis GB124 phages were isolated on only 7 sampling occasions in overlying waters and 14 sampling occasions in *M. edulis*. Given the relatively high levels of these phages recorded in wastewater treatment effluents in the catchment (Ebdon *et al*., 2007), this suggests that human faecal pollution is a relatively minor component of the

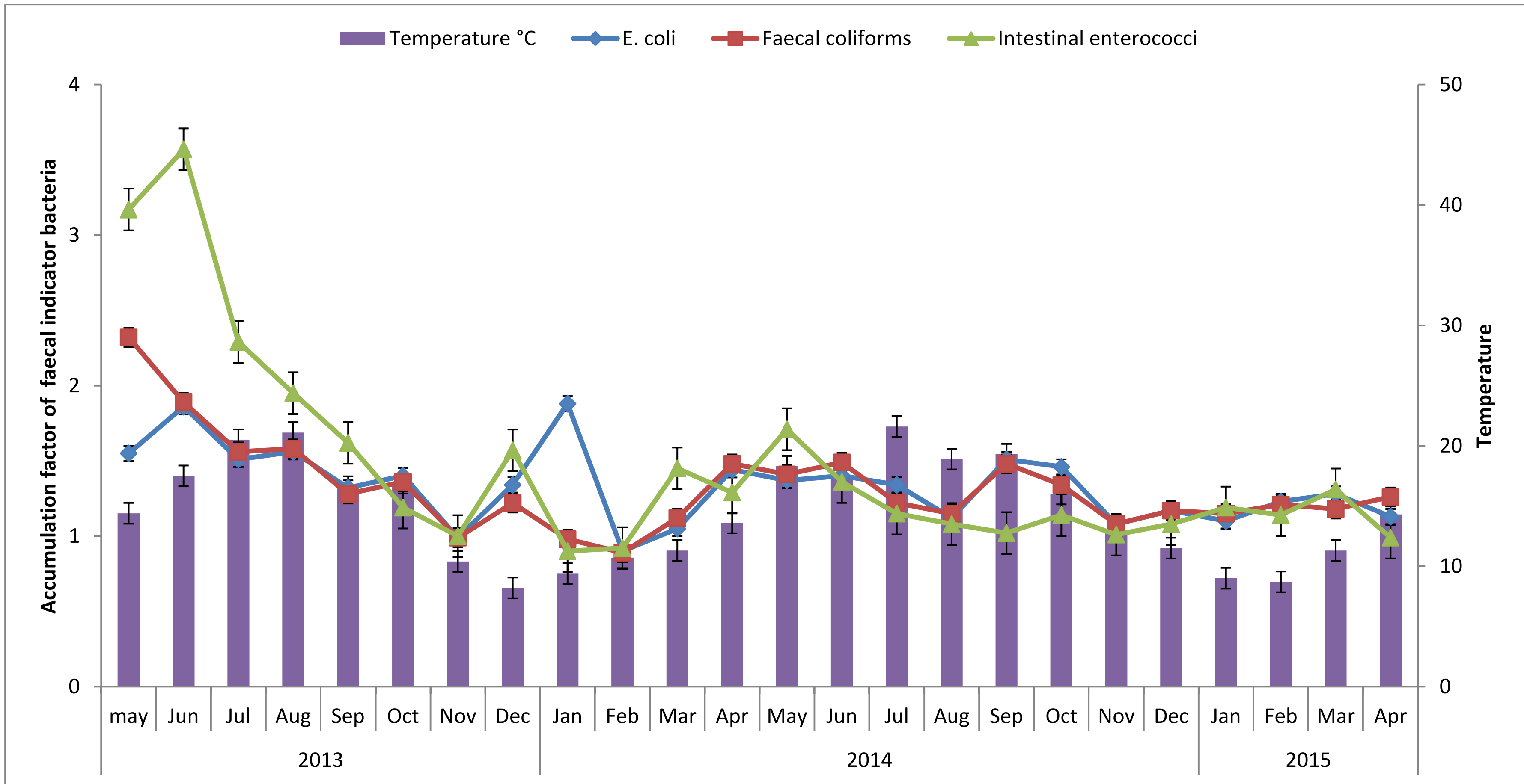


Figure 5.1: Monthly (number 'n' of replicates per month = 2, standard error) bioaccumulation of faecal indicator bacteria in *M. edulis* and water temperature over the study period.

faecal load entering the river body under most circumstances and that in the catchment of the River Ouse non-human faecal sources (probably of predominantly agricultural origin) are likely to be themain source of traditional faecal indicator bacteria (Ebdon *et al*., 2007). The percentages of positive samples recorded were 13% in overlying waters and 34% in *M. edulis*. Recorded levels of *Bacteroides fragilis* GB124 phages in *M. edulis* averaged 1.41 ± 0.54 greater than the levels recorded in overlying waters and their mean accumulation factor ranged from 0.83 to 2.12 (Figure 5.2).

Somatic coliphages are one of the most abundant groups of bacteriophages shed by all warm-blooded mammals and so tend to be detected in faecally-impacted environments (Grabow, 2004) at higher levels and with greater frequency than other phage groups. Somatic coliphages were detected in 94% of overlying water samples and 100% of *M. edulis* samples. Recorded levels of somatic coliphages in *M. edulis* averaged 1.60 ± 0.25 greater than the levels in overlying waters and their mean accumulation factor ranged from 1.10 to 2.27 (Figure 5.2).

F-RNA coliphages were not isolated in all 45 sampling occasions. The percentages of positive samples were 24% in overlying waters and 50% in *M. edulis*. Recorded levels of F-RNA coliphages in *M. edulis* averaged 1.49 ± 0.28 greater than the levels in overlying waters and their mean accumulation factor ranged from 1.08 to 1.99. F-RNA coliphages showed a positive relationship with rainfall (Figure 5.2).

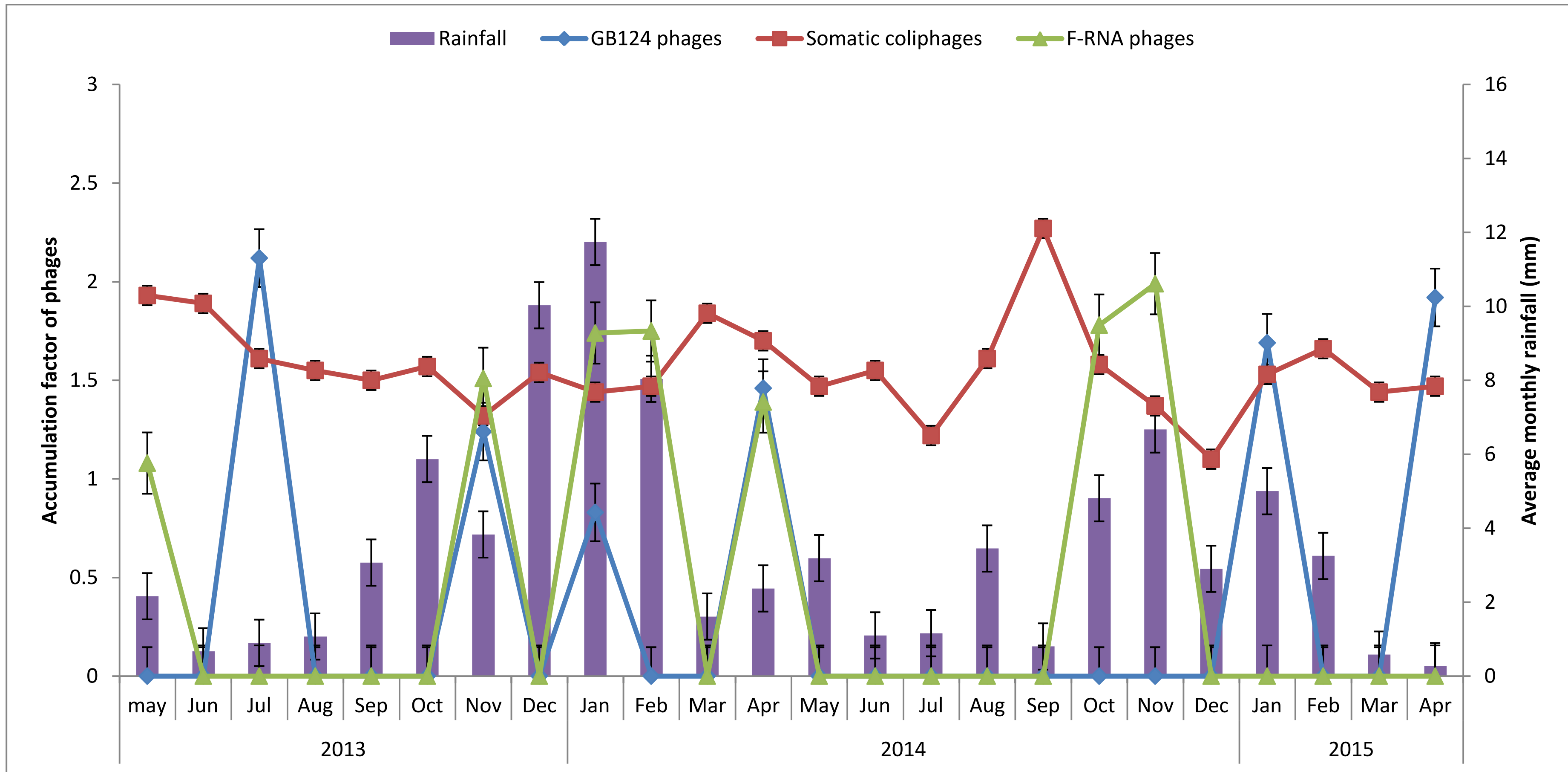


Figure 5.2: Monthly (number 'n' of replicates per month = 2, standard error) bioaccumulation of bacteriophages in *M. edulis* over the study period

5.1.4 Seasonal variation in the bioaccumulation of bacterial and viral indicators in mussels

For the purpose of this study, undertaken in a temperate climate, four seasons were considered, i.e., spring, summer, autumn and winter (see section 4.4). Higher bioaccumulation levels of *E. coli*, faecal coliforms and intestinal enterococci in mussels were observed during the summer (Figure 5.3). This is most likely due to the elevated ambient and water temperature during this season, which results in higher metabolic activity of the shellfish. The winter season demonstrated the lowest recorded bioaccumulation values in mussels. This may likely be due to relatively low temperatures, which are associated with a reduction in the metabolic rate of the mussels, thereby accumulating lower levels of faecal indicator bacteria compared with other seasons.

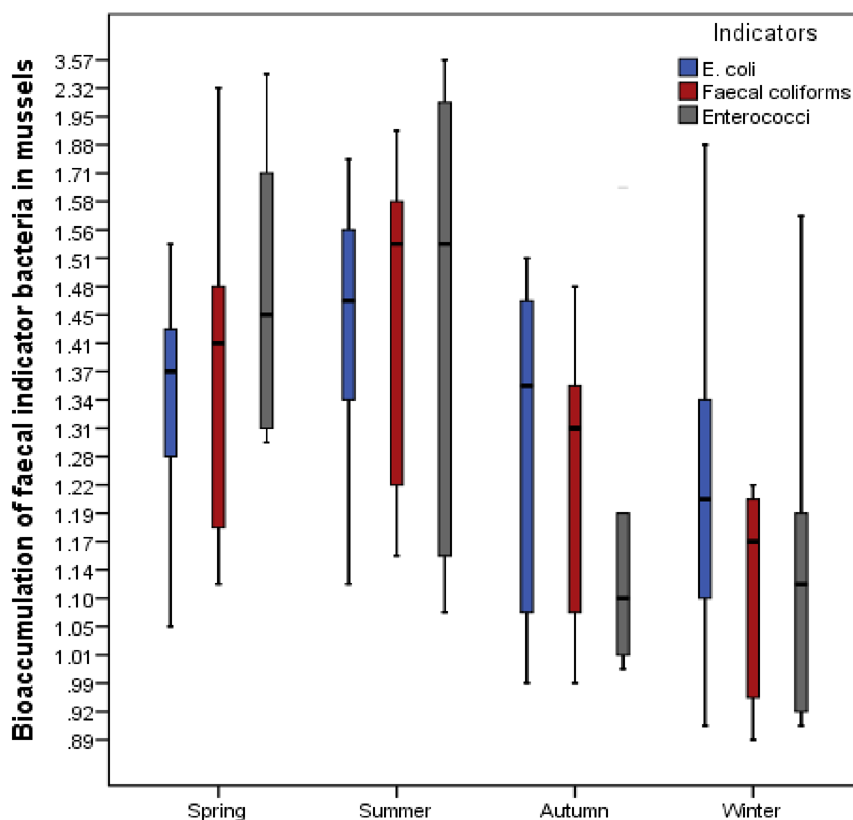


Figure 5.3: Boxplot of bioaccumulation of faecal indicator bacteria in mussels (number 'n' of replicates per season = 6, standard error) during spring, summer, autumn and winter. The median value is represented by a line inside the box, 95% confidence intervals (bars).

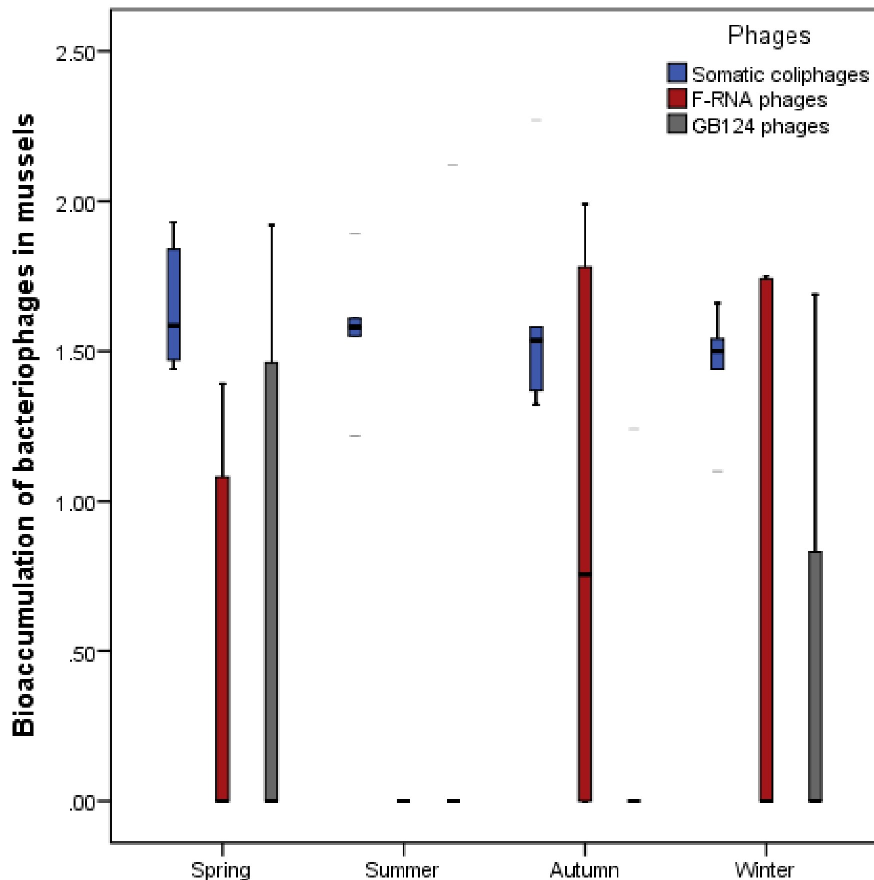


Figure 5.4: Boxplot of bioaccumulation of bacteriophages in mussels (number 'n' of replicates per season = 6, standard error) during spring, summer, autumn and winter. The median value is represented by a line inside the box, 95% confidence intervals (bars).

In general, between seasons there were significant differences in the concentrations of *E. coli*, faecal coliforms and intestinal enterococci (Duncan, $P < 0.05$). Recorded levels of bioaccumulation of intestinal enterococci were higher during spring and summer and lower during autumn and winter. Somatic coliphages showed the highest prevalence (100%) and maintained significantly high bioaccumulation values in mussels in all seasons (Figure 5.4). F-RNA coliphages were observed during all seasons other than summer and, this may be due to inactivation by high temperatures in the summer. Bioaccumulation of GB124 phages was shown to occur during the spring and winter seasons, as was bioaccumulation of norovirus (see chapter 6). In general, there were significant differences in the recorded bioaccumulation of F-RNA coliphages and GB124 phages in mussels between seasons (P

> 0.05), but no significant differences in the bioaccumulation of somatic coliphages between seasons ($P < 0.05$).

5.1.5 The relationship between environmental bioaccumulation in *M. edulis* and physicochemical, meteorological and hydrological factors

Spearman's rank non-parametric two-tailed correlation analysis with test of significance at 0.01 and 0.05 levels (representing 99% and 95% confidence intervals, respectively), using SPSS Statistics Version 20.0 was used to determine how environmental factors such as physicochemical, hydrological, and meteorological parameters influenced the bioaccumulation kinetics of *E. coli,* faecal coliforms, intestinal enterococci, somatic coliphages, F-RNA coliphages and human-specific *Bacteroides fragilis* phages – GB124 in *Mytilus edulis* (Table 5.2).

Water temperature showed positive correlations with the bioaccumulation of *E. coli* (rho = 0.46, $P < 0.05$) and faecal coliforms (rho = 0.64, $P < 0.01$) in *M. edulis*. Turbidity showed negative correlations with the bioaccumulation of faecal coliforms (rho = -0.62, $P < 0.01$), intestinal enterococci (rho = -0.50, $P < 0.05$), and somatic coliphages (rho = -0.49, $P < 0.05$) in *M. edulis*. Positive correlations were observed between pH and the bioaccumulation of *E. coli* (rho = 0.58, $P < 0.01$), faecal coliforms (rho = 0.85, $P < 0.01$), intestinal enterococci (rho = 0.66, $P < 0.01$) and somatic coliphages (rho = 0.52, $P < 0.01$) in *M. edulis.* Salinity showed positive correlations with the bioaccumulation of *E. coli* (rho = 0.57, $P < 0.01$), faecal coliforms (rho = 0.80, $P < 0.01$), and intestinal enterococci (rho = 0.53, $P < 0.01$) in *M. edulis.*

Electrical conductivity showed a positive correlation with the bioaccumulation of GB124 phages (rho = 0.48, $P < 0.05$) in *M. edulis.* Similarly, total dissolved solids showed a positive correlation with the bioaccumulation of GB124 phages (rho = 0.48, $P < 0.05$) in *M. edulis.* Rainfall showed a positive correlation with the bioaccumulation of F-RNA coliphages (rho = 0.52, $P < 0.01$), but showed a negative a correlation with the bioaccumulation of faecal coliforms (rho = -0.58, $P < 0.01$) in *M. edulis.* Air temperature showed positive correlations with the bioaccumulation of *E. coli* (rho = 0.47, $P < 0.05$), and faecal coliforms (rho = 0.62, $P < 0.01$) in *M. edulis.* The rate of river flow showed a positive correlation with the bioaccumulation of F-RNA coliphages (rho = 0.51, $P < 0.05$), but showed negative correlations with the bioaccumulation of *E. coli* (rho = -0.49, $P < 0.05$), faecal coliforms (rho = -0.76, $P < 0.01$), intestinal enterococci (rho = -0.54, $P < 0.05$) and somatic coliphages (rho = -0.46, $P < 0.05$) in *M. edulis.*

The bioaccumulation of *E. coli* correlated positively with the bioaccumulation of faecal coliforms (rho = 0.74, $P < 0.01$), intestinal enterococci (rho = 0.46, $P < 0.05$), and somatic coliphages (rho = 0.42, $P < 0.05$) in *M. edulis.* The bioaccumulation of faecal coliforms showed positive correlations with the bioaccumulation of intestinal enterococci (rho = 0.72, $P < 0.01$), and somatic coliphages (rho = 0.58, $P < 0.01$) in *M. edulis.* The bioaccumulation of intestinal enterococci showed a positive correlation with the bioaccumulation of somatic coliphages (rho = 0.45, $P < 0.05$) and a negative correlation with the bioaccumulation of F-RNA coliphages (rho = -0.45, $P < 0.05$) in *M. edulis.*

Table 5.2: Spearman's rank correlation between environmental bioaccumulation of bacterial and viral indicators in *Mytilus edulis*, physicochemical, meteorological and hydrological data (24 months dataset)

	Water temperature	Turbidity	pH	Salinity	Conductivity	TDS	Dissolved oxygen	Rainfall	Air temperature	River flow	*E. coli*	Faecal coliforms	Intestinal enterococci	GB124 phages	Somatic coliphages	F-RNA coliphages
Water temperature	1.00															
Turbidity	-0.72**	1.00														
pH	0.61**	-0.58**	1.00													
Salinity	0.66**	-0.62**	0.65**	1.00												
Conductivity	-0.01	-0.01	0.12	-0.28	1.00											
TDS	-0.01	-0.01	0.12	-0.28	1.00**	1.00										
Dissolved oxygen	0.19	0.10	0.33	0.06	0.19	0.19	1.00									
Rainfall	-0.53**	0.63**	-0.60**	-0.34	-0.18	-0.18	-0.41*	1.00								
Air temperature	0.95**	-0.68**	0.56**	0.71**	-0.11	-0.11	0.07	-0.46*	1.00							
River flow	-0.86**	0.75**	-0.73**	-0.70**	0.07	0.07	-0.38	0.83**	-0.86**	1.00						
E. coli	0.46*	-0.37	0.58**	0.57**	-0.10	-0.10	0.08	-0.28	0.47*	-0.49*	1.00					
Faecal coliforms	0.64**	-0.62**	0.85**	0.80**	-0.03	-0.03	0.23	-0.58**	0.62**	-0.76**	0.74**	1.00				
Intestinal enterococci	0.36	-0.50*	0.66**	0.53**	0.02	0.02	0.02	-0.40	0.34	-0.54*	0.46*	0.72**	1.00			
GB124 phages	-0.19	0.18	0.03	-0.17	0.48*	0.48*	0.06	-0.05	-0.14	0.19	-0.02	-0.09	-0.20	1.00		
Somatic coliphages	0.23	-0.49*	0.52**	0.36	0.11	0.11	0.09	-0.28	0.23	-0.46*	0.42*	0.58**	0.45*	-0.10	1.00	
F-RNA phages	-0.32	0.37	-0.29	-0.30	0.12	0.12	-0.32	0.52**	-0.21	0.51*	-0.06	-0.34	-0.45*	0.13	-0.17	1.00

Key: TDS-Total dissolve solids; **-Correlation is significant at the 0.01 level (2-tailed); *-Correlation is significant at the 0.05 level (2-tailed)

5.2 Laboratory-based experiment on uptake and bioaccumulation in shellfish

Several research studies have previously been undertaken in a variety of locations, to evaluate presence of microorganisms in shellfish (Table 5.3). However, the research described here evaluates for the first time the bioaccumulation of human-specific *Bacteroides fragilis* phages GB124 in *Crassostrea gigas*. Among other parameters investigated were; the bioaccumulation of *E. coli*, faecal coliforms, intestinal enterococci, somatic coliphages and F-specific RNA coliphages in *Crassostrea gigas* and *Mytilus edulis* under controlled laboratory conditions. The variables examined included time, salinity and temperature. The results of these experiments were then compared with those obtained during the routine environmental assessment of bioaccumulation of *E. coli*, faecal coliforms, intestinal enterococci, somatic coliphages, F-specific RNA coliphages and human specific *Bacteroides fragilis* phages GB124 in *Mytilus edulis* at the estuarine field site in southeast England.

After two to three hours of bioaccumulation, *M. edulis* and *C. gigas* in tanks B, D, E and F were open with all their gills out and were filtering more water than those in tanks A and C (i.e. the filtering activities exhibited by shellfish in tanks with water of higher salinity were greater compared with those with lower salinity). This is likely to account in part for the variation observed in the bioaccumulation efficiency of the shellfish. After 48 hours, the artificial seawater in experimental tanks with high temperatures, including positive control tanks containing *C. gigas* became turbid with the oysters showing signs of poor health (i.e., the valves were not tightly closed) and reduction in filtration rate, whereas the artificial seawater in experimental tanks at low temperatures remained clear. On the other hand, artificial seawater in experimental tanks at both low and high temperatures remained clear

and the *M. edulis* appeared to be in good health (i.e., valves were tightly closed) throughout the duration of the experiment.

Table 5.3: Previously published shellfish bioaccumulation studies

Shellfish	Microorganisms	Location	Reference
Oysters (*Crassotrea gigas*) Flat oysters (*Ostrea edulis*) Mussels (*Mytilus edulis*) Clams (*Ruditapes philippinarum*)	Hepatitis E virus	France	Grodzki *et al*., 2014
Oysters (*Crassotrea virginica*)	*Cryptosporidium parvum*	Canada	Willis *et al.,* 2014
Mussels (*Mytilus galloprovincialis*)	*Arcobacter butzleri*	Italy	Ottaviani *et al*., 2013
Mussels (*Mytilus edulis*)	*Enterococcus faecalis* *Enterococcus faecium*	Denmark	Roslev *et al*., 2009
Oysters (*Crassotrea virginica*) Oysters (*Crassotrea ariakensis*)	Hepatitis A virus Mouse norovirus 1 Human norovirus	United States	Nappier *et al*., 2009
Oysters (*Crassotrea virginica*) Oysters (*Crassotrea ariakensis*)	Hepatitis A virus Poliovirus MS2 bacteriophage Murine norovirus 1 Human norovirus	United States	Nappier *et al*., 2008
Oysters (*Crassotrea virginica*)	Faecal coliforms *E. coli* *Clostridium perfringens* F^+ coliphage	United States	Burkhardt and Calci, 2000

Table 5.4: Bioaccumulation of indicator bacteria and phages by mussels (*M. edulis*)

	Experimental tanks									
Parameters	**A (5 ppt, 24 °C)**		**B (25 ppt, 24 °C)**		**C (5 ppt, 8 °C)**		**D (25 ppt, 8 °C)**		**E (16 ppt, 20 °C)**	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
GB124 phages	2.05±1.60	3.26-0	1.93±1.50	3.08-0	ND		ND		**2.45±1.21**	3.08-0
Somatic coliphages	3.68±1.81	4.72-0	3.77±1.86	4.77-0	**4.03±1.98**	5.03-0	3.97±1.94	4.83-0	3.86±1.89	4.83-0
F-RNA coliphages	2.82±1.41	3.62-0	2.91±1.45	3.78-0	0.98±1.52	3.08-0	1.60±1.77	3.56-0	**2.99±1.52**	3.82-0
E. coli	3.10±1.81	4.26-0	3.23±1.82	4.26-0	4.37±2.28	6.20-0	**4.92±2.51**	6.26-0	2.69±1.59	4.26-0
Faecal coliforms	3.17±1.84	4.26-0	3.28±1.85	4.26-0	4.59±2.42	6.26-0	**4.92±2.51**	6.26-0	2.81±1.63	4.26-0
Intestinal enterococci	2.81±1.66	4.26-0	2.29±1.33	3.38-0	3.23±1.59	4.11-0	**3.46±1.70**	4.36-0	2.50±1.56	4.26-0

Key: N (number of **mussels** tested at each point) = 5; Mean ± Standard Deviation (over whole experiment time) Log_{10} PFU/100 g or Log_{10} MPN/100 g; Range (Maximum-minimum); ppt – parts per thousand; ND – Non detects (i.e. below detection limit); Figures in **bold** denote the highest bioaccumulation mean value at each point.

Table 5.5: Bioaccumulation of indicator bacteria and phages by oysters (*C. gigas*)

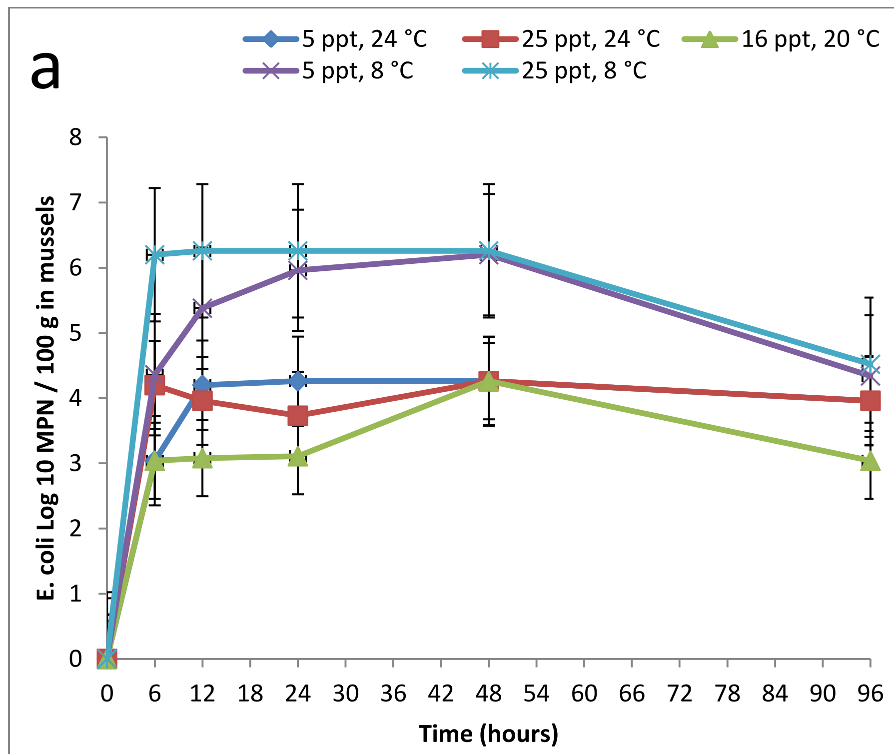
	Experimental tanks									
Parameters	**A (5 ppt, 24 °C)**		**B (25 ppt, 24 °C)**		**C (5 ppt, 8 °C)**		**D (25 ppt, 8 °C)**		**E (16 ppt, 20 °C)**	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
GB124 phages	0.51±1.26	3.08-0	**0.54±1.33**	3.26-0	ND		0.46±1.14	2.78-0	ND	
Somatic coliphages	0.98±1.52	3.08-0	1.02±1.57	3.26-0	**1.39±1.52**	2.78-0	0.51±1.26	3.08-0	1.03±1.59	3.08-0
F-RNA coliphages	ND		ND		ND		ND		ND	
E. coli	4.31±2.22	5.96-0	4.25±2.15	5.96-0	3.92±1.98	5.38-0	**4.52±2.34**	6.20-0	4.01±1.99	5.38-0
Faecal coliforms	4.29±2.13	5.45-0	4.25±2.14	5.96-0	3.88±1.98	5.38-0	**4.35±2.19**	5.96-0	3.84±1.89	4.96-0
Intestinal enterococci	2.38±1.20	3.23-0	2.25±1.14	3.11-0	**2.55±1.32**	3.60-0	2.54±1.32	3.60-0	2.14±1.07	2.90-0

Key: N (number of **oysters** tested at each point) = 3; Mean ± Standard Deviation (over whole experiment time) Log_{10} PFU/100 g or Log_{10} MPN/100 g; Range (Maximum-minimum); ppt – parts per thousand; ND – Non detects (i.e. below detection limit); Figures in **bold** denote the highest bioaccumulation mean value at each point.

5.2.1 Bioaccumulation of *E. coli* under experimental conditions

E. coli were bioaccumulated to the highest concentration in *M. edulis* after 48 hours of contamination (Figure 5.5), but the highest mean concentration (4.92 ± 2.51 Log_{10} CFU/100 g) was observed in tank D (high salinity, low temperature), containing artificial seawater at 25 ppt and the lowest (2.69 ± 1.59 Log_{10} CFU/100 g) in the positive control tank E. The maximum concentration of *E. coli* in *M. edulis* (6.26 Log_{10} CFU/100 g) was again observed in tank D (Table 5.4). Lower levels of *E. coli* in *M. edulis* in tanks A, B and E may have been due to the higher temperatures in the experimental tanks, which may be responsible for inactivation of *E. coli*, thus leading to partially reduced survival of these such organisms in *M. edulis.*

In *C. gigas, E. coli* were bioaccumulated to the highest concentration after 6 hours of contamination (Figure 5.5). The values in *C. gigas* are larger compared with those in *M. edulis* and this might be responsible for the observed faster bioaccumulation of *E. coli*. The highest mean concentration (4.52 ± 2.34 Log_{10} CFU/100 g) was observed in tank D (high salinity, low temperature) and the lowest (3.92 ± 1.98 Log_{10} CFU/100 g) in tank C (low salinity, low temperature). The maximum concentration of *E. coli* in *C. gigas* (6.26 Log_{10} CFU/100 g) was once again observed in tank D (high salinity, low temperature) and the minimum concentration was observed in tanks C and E (Table 5.5). The pattern of persistence of *E. coli* in *M. edulis* between 48 and 96 hours showed a mean reduction from 5.05 to 3.96 Log_{10} CFU/100 g, but increased in *C. gigas* from 4.42 to 4.65 Log_{10} CFU/100 g (Figure 5.5). *C. gigas* bioaccumulated *E. coli* to slightly higher densities compared with *M. edulis.*



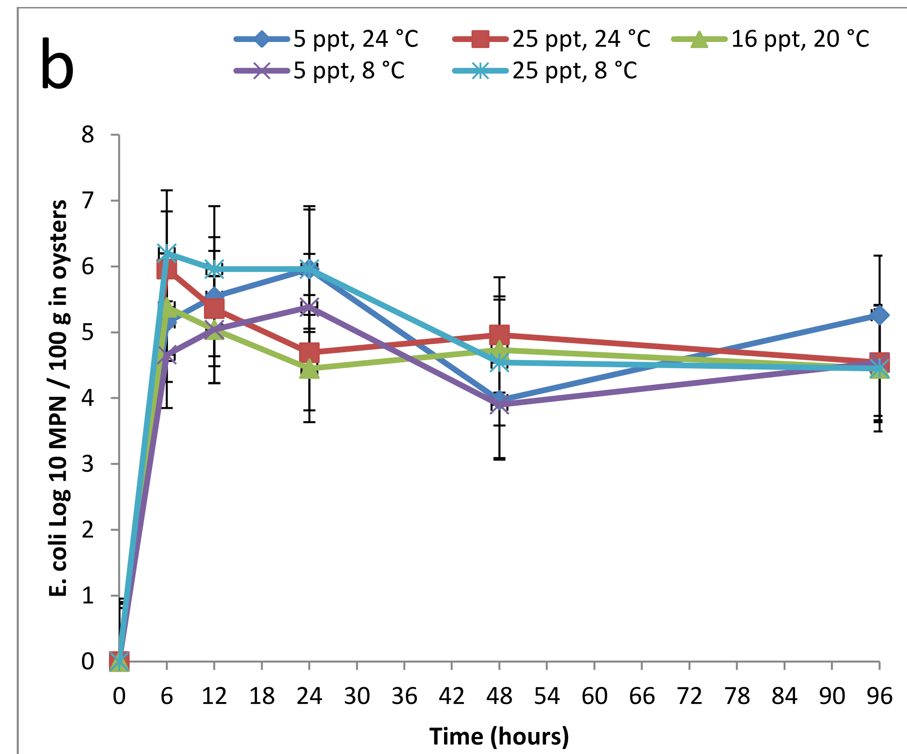


Figure 5.5: Bioaccumulation of *E. coli* in *M. edulis* (a) and *C. gigas* (b) in artificial seawater at low and high temperatures and salinities (Number of mussels (5) and oysters (3) tested at each point; Mean ± Standard Deviation Log_{10} MPN/100 g).

5.2.2 Bioaccumulation of faecal coliforms under experimental conditions

Given that *E. coli* are normally predominant member of the faecal coliform group, it is perhaps not surprising that faecal coliforms were also bioaccumulated to the highest concentration in *M. edulis* after 48 hours of contamination (Figure 5.6), but the highest mean concentration (4.92 ± 2.51 Log_{10} CFU/100 g) was observed in tank D (high salinity, low temperature) containing artificial seawater at 25 ppt and the lowest (2.81 ± 1.63 Log_{10} CFU/100 g) in the positive control tank E. The maximum concentration of faecal coliforms observed in *M. edulis* was 6.26 Log_{10} CFU/100 g in tanks C and D; and minimum was observed in tanks A, B and E (Table 5.4). Lower levels of faecal coliforms in *M. edulis* in tanks A, B and E (Figure 5.6) may be due to the higher temperatures in these experimental tanks, which may be responsible for greater inactivation of faecal coliforms, thus leading to reduced survival of the organisms in *M. edulis.*

In *C. gigas,* faecal coliforms were bioaccumulated to the highest concentration after 12 hours of contamination (Figure 5.6). The highest mean concentration (4.35 ± 2.19 Log_{10} CFU/100 g) was observed in tank D (high salinity, low temperature) and the lowest mean concentration (3.84 ± 1.89 Log_{10} CFU/100 g) was observed in the positive control tank E. The maximum concentration of faecal coliforms in *C. gigas* (5.96 Log_{10} CFU/100 g) was observed in tanks B (high salinity, high temperature) and D (high salinity and low temperature); and minimum concentration (4.96 Log_{10} CFU/100 g) was observed in the positive control tank E (Table 5.5). The pattern of persistence of faecal coliforms in *M. edulis* between 48 and 96 hours showed a mean reduction from 5.06 to 4.09 Log_{10} CFU/100 g, but increased in *C. gigas* from 4.53 to 4.83 Log_{10} CFU/100 g (Figure 5.6). *C. gigas* bioaccumulated faecal coliforms to slightly higher densities compared with *M. edulis.*

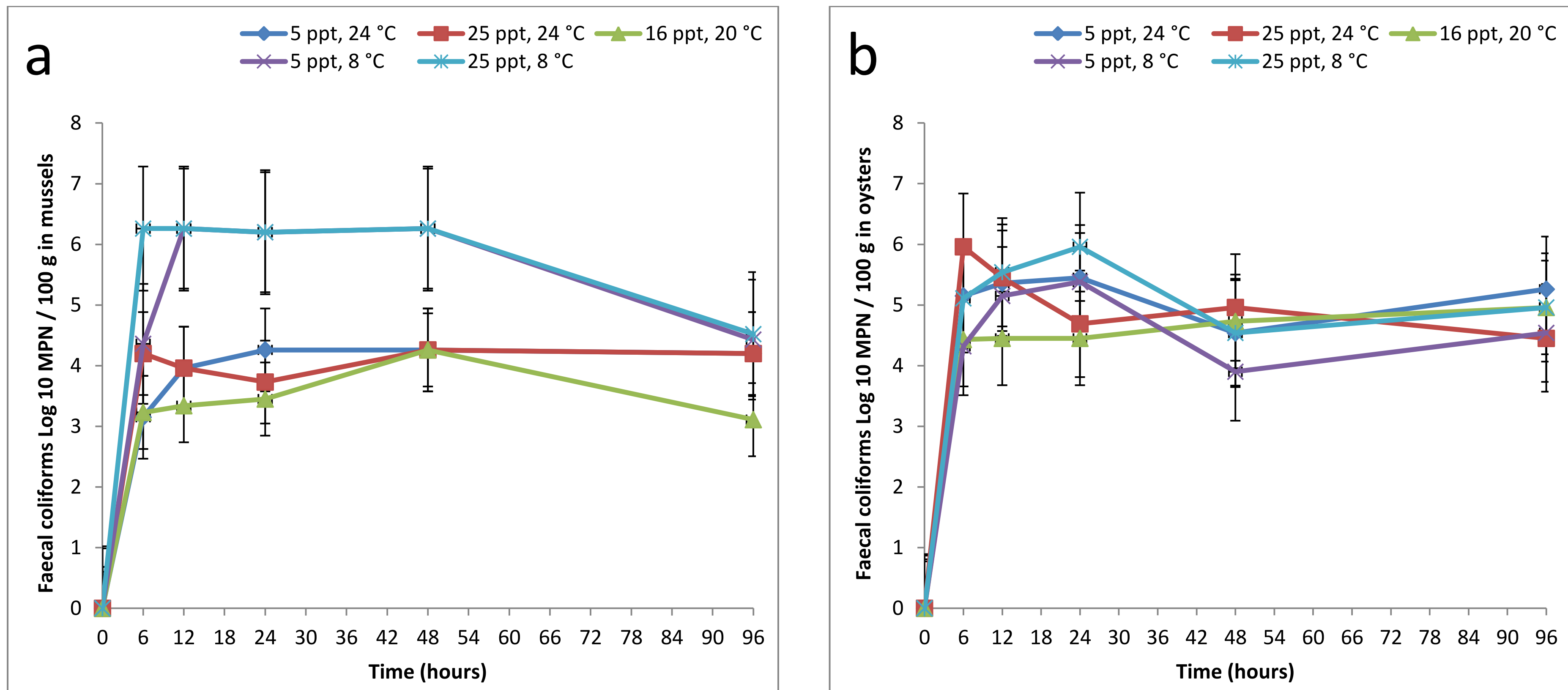
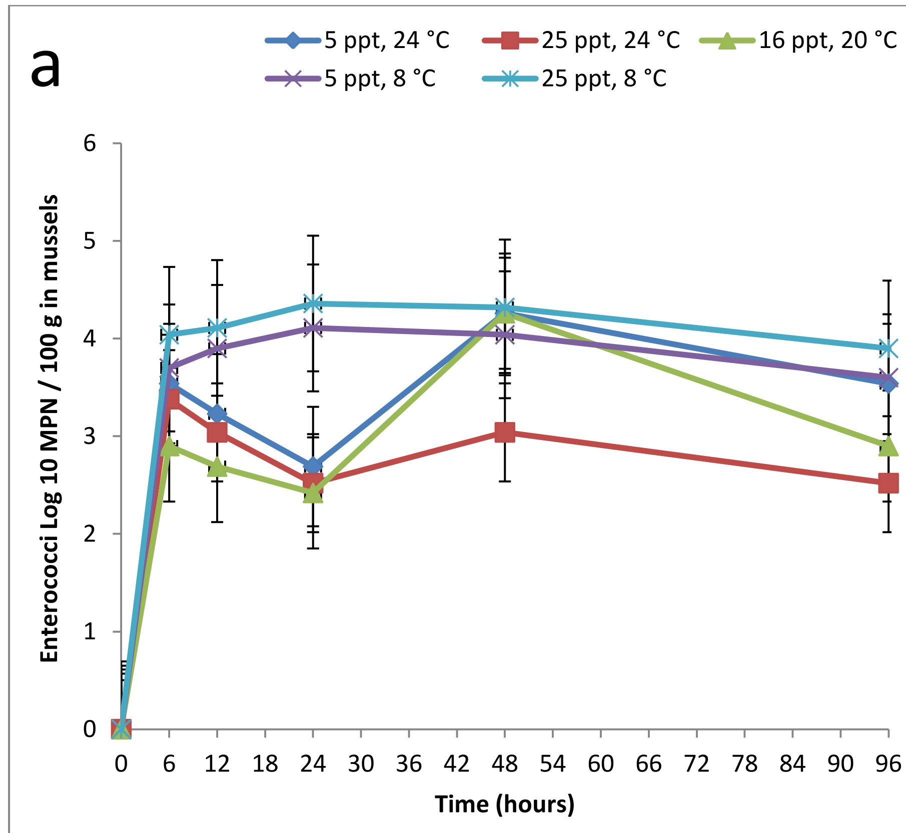


Figure 5.6: Bioaccumulation of faecal coliforms in *M. edulis* (a) and *C. gigas* (b) in artificial seawater at low and high temperatures and salinities. (Number of mussels (5) and oysters (3) tested at each point; Mean ± Standard Deviation Log_{10} MPN/100 g).

5.2.3 Bioaccumulation of intestinal enterococci under experimental conditions

Intestinal enterococci were bioaccumulated to the highest concentration in *M. edulis* after 48 hours of contamination (Figure 5.7), but the highest mean concentration (3.46 ± 1.70 Log_{10} CFU/100 g) was observed in tank D (high salinity, low temperature) and the lowest (2.29 ± 1.33 Log_{10} CFU/100 g) in tank B (high salinity, high temperature). The maximum concentration of intestinal enterococci in *M. edulis* (4.36 Log_{10} CFU/100 g) was also observed in tank D and the minimum concentration (3.38 Log_{10} CFU/100 g) was observed in tank B (Table 5.4).

In *C. gigas,* intestinal enterococci were bioaccumulated to the highest concentration after 24 hours of contamination (Figure 5.7). The highest mean concentration (2.55 ± 1.32 Log_{10} CFU/100 g) was observed in tank C (low salinity, low temperature) and the lowest (2.14 ± 1.07 Log_{10} CFU/100 g) in the positive control tank E. The maximum concentration of intestinal enterococci in *C. gigas* was highest (3.60 Log_{10} CFU/100 g) was observed in tanks C (low salinity, low temperature) and D (high salinity, low temperature); and minimum concentration (2.90 Log_{10} CFU/100 g) was observed in the positive control tank E (Table 5.5). The pattern of persistence of intestinal enterococci in *M. edulis* between 48 and 96 hours showed a mean reduction from 3.98 to 3.29 Log_{10} CFU/100 g, and in *C. gigas* from 3.00 to 2.91 Log_{10} CFU/100 g (Figure 5.7). *M. edulis* bioaccumulated intestinal enterococci to slightly higher densities compared with *C. gigas*.



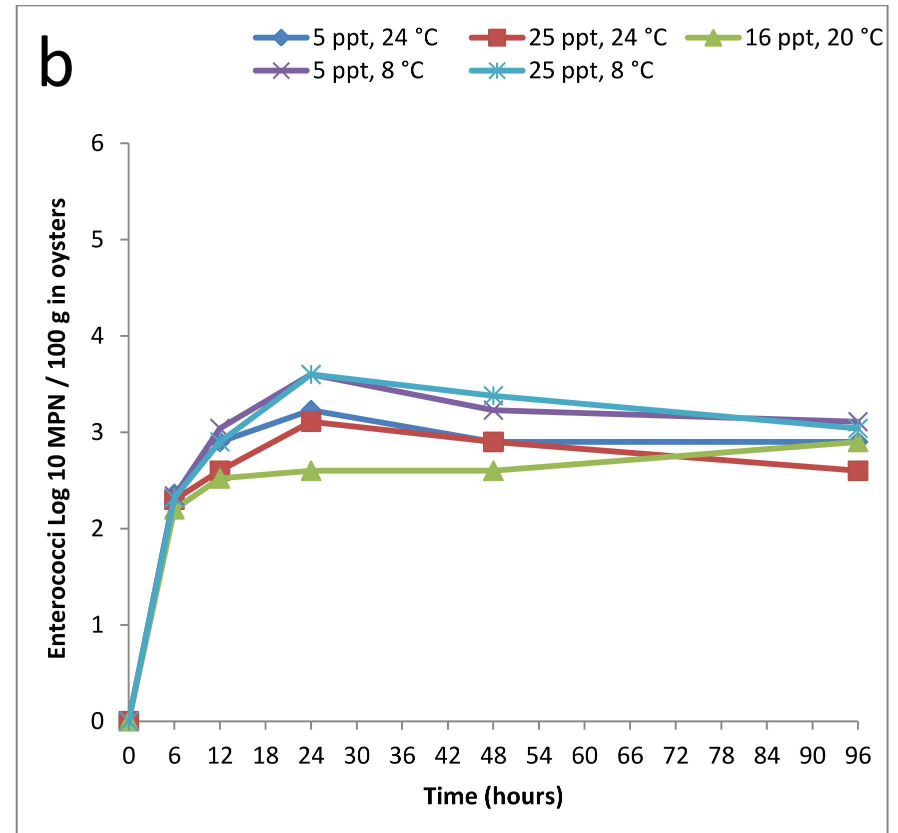


Figure 5.7: Bioaccumulation of intestinal enterococci in *M. edulis* (a) and *C. gigas* (b) in artificial seawater at low and high temperatures and salinities. (Number of mussels (5) and oysters (3) tested at each point; Mean ± Standard Deviation Log_{10} MPN/100 g).

5.2.4 Bioaccumulation of GB124 phages under experimental conditions

Phages of human-specific *Bacteroides fragilis* GB124 were bioaccumulated to the highest concentration in *M. edulis* after 24 hours of contamination (Figure 5.8), but the highest mean concentration of 2.45 ± 1.21 was observed in the positive control tank containing artificial seawater at 16 ppt and the lowest (1.93 ± 1.50) in tank B (high salinity, high temperature). However, GB124 phages were below their detection limit in *M. edulis* in tanks C and D throughout the experiment (Table 5.4). The maximum concentration of GB124 phages in *M. edulis* (3.26 Log_{10} PFU/100g) was observed in tank A (low salinity, high temperature). This suggests that lower salinities may be favourable to the bioaccumulation of GB124 phages in *M. edulis.* After 96 hours of contamination, the *Bacteroides fragilis* GB124 phages were not isolated in *M. edulis* in tanks A and B, whereas those in tanks E still contained the phages (Figure 5.8) suggesting that extreme salinities may decrease the ability of these phages to survive in shellfish.

In *C. gigas,* GB124 phages were also bioaccumulated to the highest concentration after 24 hours of contamination (Figure 5.8). However, this time the highest mean concentration of 0.54 ± 1.33 was observed in tank B (high salinity, high temperature) and the lowest concentration (0.46 ± 1.14) was observed in tank D (high salinity, low temperature). GB124 phages were undetected (below their detection limit) in *C. gigas* in tanks C and E throughout the experiment. The maximum concentration of GB124 phages in *C. gigas* (3.26 Log_{10} PFU/100 g) was also observed in tank B (high salinity, high temperature) (Table 5.5). The pattern of persistence of GB124 phages in *M. edulis* between 48 and 96 hours showed a mean reduction from 1.73 to 0.56 Log_{10} PFU/100 g, and these phages were undetected (below their detection limit) in *C. gigas* during this period (Figure 5.8).

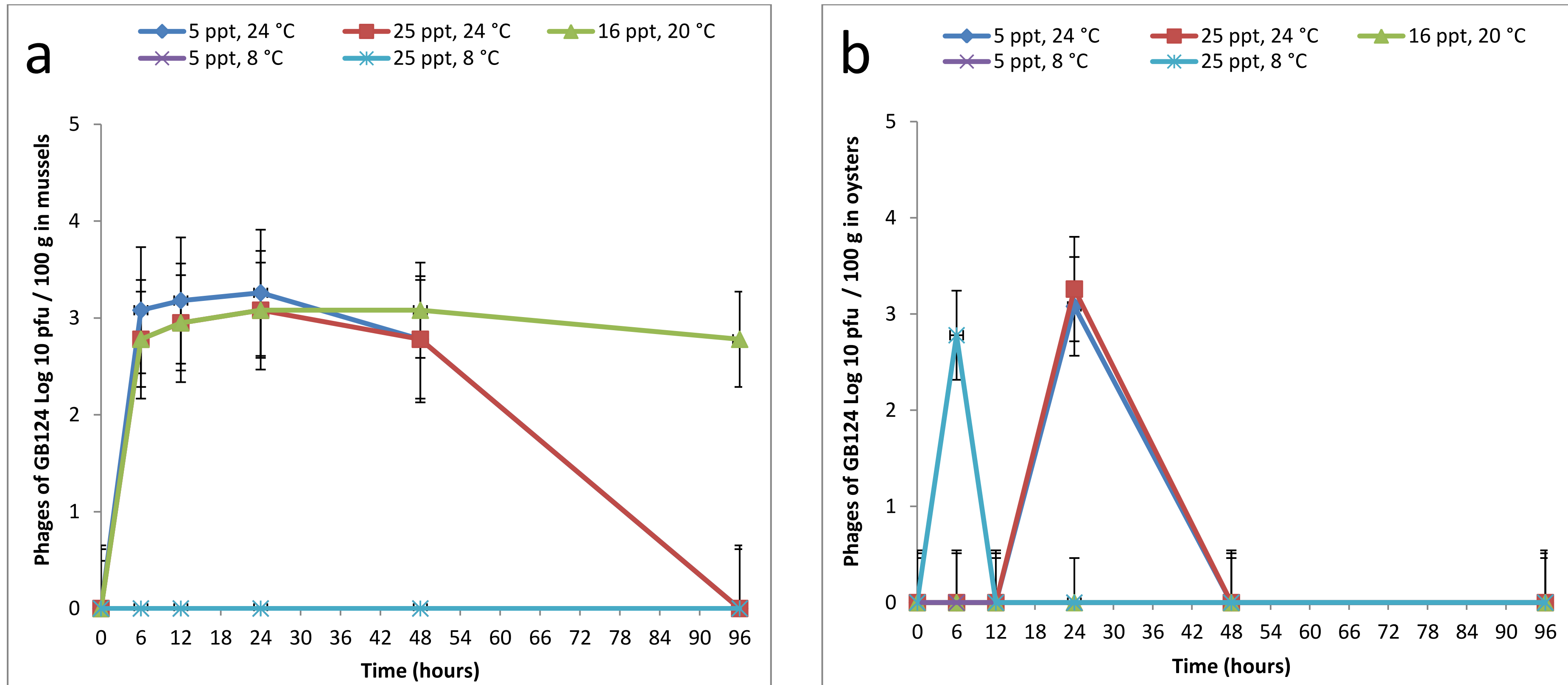


Figure 5.8: Bioaccumulation of phages of human-specific *Bacteroides fragilis* GB124 in *M. edulis* (a) and *C. gigas* (b) in artificial seawater at low and high temperatures and salinities. (Number of mussels (5) and oysters (3) tested at each point; Mean ± Standard Deviation Log_{10} PFU/100 g).

5.2.5 Bioaccumulation of somatic coliphages under experimental conditions

Somatic coliphages were bioaccumulated to their highest concentration in *M. edulis* after 48 hours of contamination (Figure 5.9), but the highest mean concentration (4.03 ± 1.98 Log_{10} PFU/100 g) was observed in tank C (low salinity, low temperature) and the lowest concentration (3.68 ± 1.81 Log_{10} PFU/100 g) was observed in tank A (low salinity, high temperature). The highest maximum concentration of somatic coliphages (5.03 Log_{10} PFU/100 g) was also observed in mussels from tank C (low salinity, low temperature) (Table 5.4). This suggests that low salinities may be favourable to the bioaccumulation of somatic coliphages in *M. edulis.*

In *C. gigas,* somatic coliphages were bioaccumulated to the highest concentration after 48 hours of contamination (Figure 5.9). The highest mean concentration (1.39 ± 1.52 Log_{10} PFU/100 g) was observed in tank C (low salinity, low temperature) and the lowest concentration (0.51 ± 1.26 Log_{10} PFU/100 g) was observed in tank D (high salinity, low temperature). The maximum concentration of somatic coliphages in *C. gigas* was observed in tank B (high salinity, high temperature) (Table 5.5). The pattern of persistence of somatic coliphages in *M. edulis* between 48 and 96 hours showed a mean reduction from 4.84 to 4.61 Log_{10} PFU/100 g, and in *C. gigas* from 1.73 to 1.21 Log_{10} PFU/100 g (Figure 5.9). *M. edulis* bioaccumulated somatic coliphages to higher densities compared with *C. gigas*.

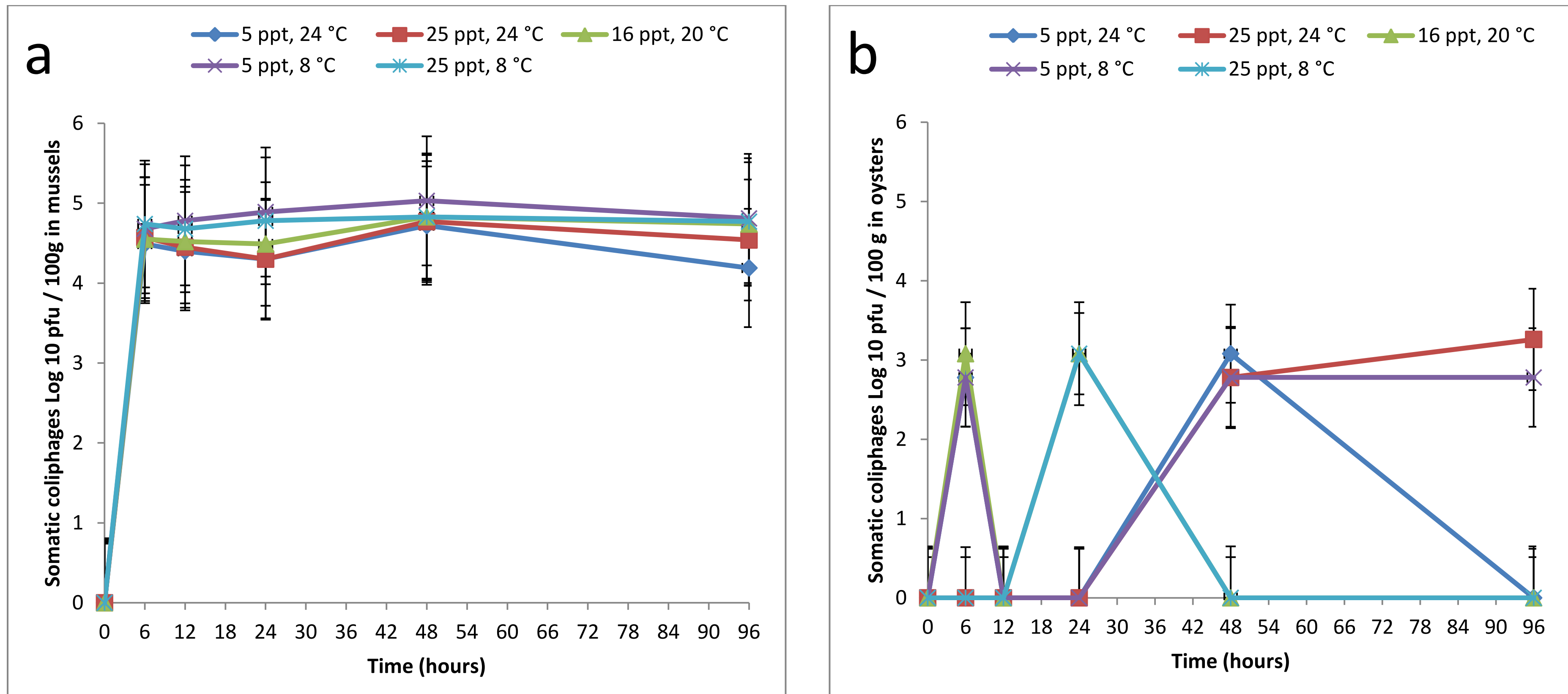


Figure 5.9: Bioaccumulation of somatic coliphages in *M. edulis* (a) and *C. gigas* (b) in artificial seawater at low and high temperatures and salinities. (Number of mussels (5) and oysters (3) tested at each point; Mean ± Standard Deviation Log_{10} PFU/100 g).

5.2.6 Bioaccumulation of F-RNA coliphages under experimental conditions

F-RNA coliphages were bioaccumulated to their highest concentration in *M. edulis* after 6 hours of contamination (Figure 5.10), but the highest mean concentration (2.99 ± 1.52 Log_{10} PFU/100 g) was observed in the positive control tank E containing artificial seawater at 16 ppt and 20°C and the lowest concentration (0.98 ± 1.52 Log_{10} PFU/100 g) was observed in tank C (low salinity, low temperature). The maximum concentration of F-RNA coliphages (3.82 Log_{10} PFU/100 g) was also observed in tank E (Table 5.4). This suggests that optimum salinities may be more favourable for the bioaccumulation of F-RNA coliphages in *M. edulis.* The pattern of persistence of F-RNA coliphages in *M. edulis* between 48 and 96 hours showed a mean reduction from 3.47 to 1.73 Log_{10} PFU/100 g (Figure 5.10). F-RNA coliphages were observed to be below their detection limit in *C. gigas* in tanks A, B, C, D and E throughout the experiment (Figure 5.10). *M. edulis* bioaccumulated F-RNA coliphages to higher densities compared with *C. gigas*. In general, *M. edulis* bioaccumulated all phages to higher densities compared with *C. gigas*, whereas *C. gigas* tended to bioaccumulate bacterial indicators more effectively.

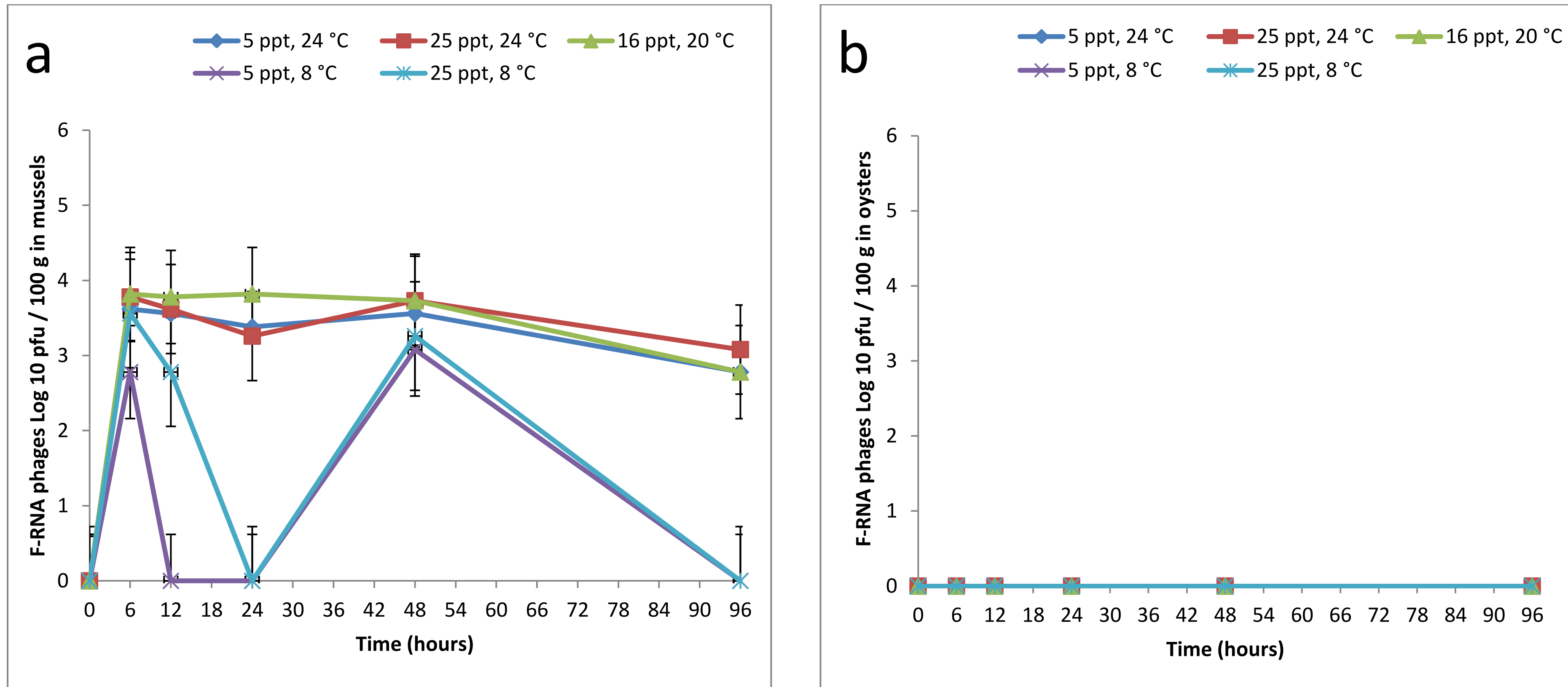


Figure 5.10: Bioaccumulation of F-RNA coliphages in *M. edulis* (a) and *C. gigas* (b) in artificial seawater at low and high temperatures and salinities. (Number of mussels (5) and oysters (3) tested at each point; Mean ± Standard Deviation Log_{10} PFU/100 g).

In general, and understandably given the overlap in the definition of these two groups, *E. coli* and faecal coliforms were observed to be similar in terms of their uptake pattern in both *M. edulis* and *C. gigas*. This observation is in agreement with Burkhardt and Calci (2000) who found significant correlation between faecal coliforms and *E. coli* accumulation by Gulf Coast oysters throughout all seasons. Faecal indicator bacteria were bioaccumulated to greater concentrations than phages in *M. edulis* and *C. gigas*. This is most likely because of the differences in the organisms' size, structure, morphology, surface charge of and/or possibly the physiological properties of the shellfish.

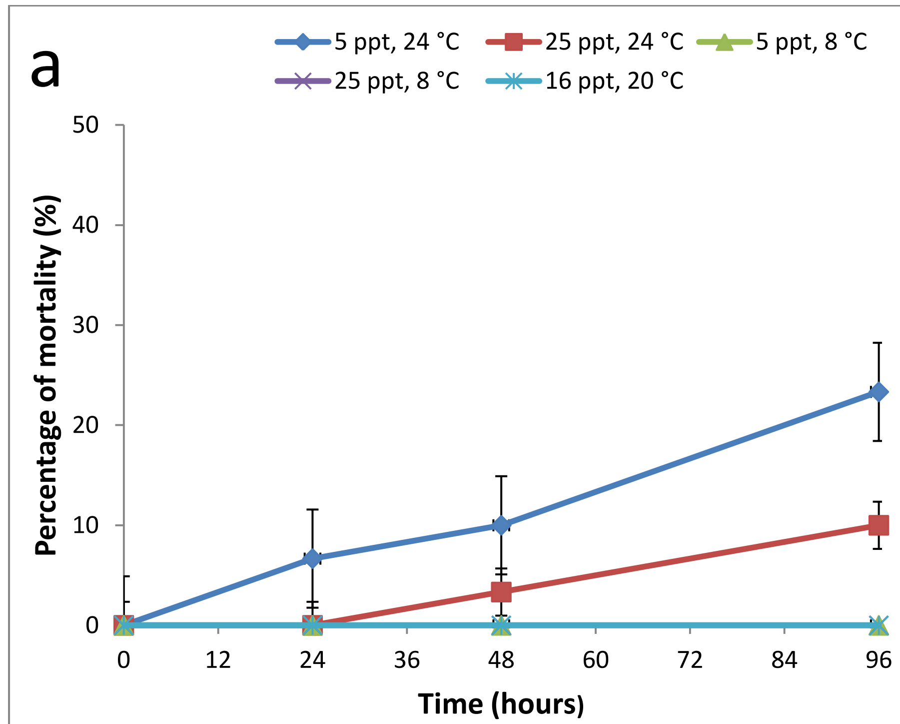
M. edulis filter fed at a faster rate than *C. gigas* and consequently accumulated more phages than *C. gigas* during the course of the experiment. Factors such as uptake kinetics, particle size sorting, ability of the labial palp and gills to reject or filter certain particles may be responsible for this observed phenomenon (Willis *et al*., 2014). This research suggests that mussels may be more sensitive to sporadic viral contamination events than oysters. This is similar to results obtained by Grodzki *et al*. (2014) in bioaccumulation experiments involving hepatitis E virus in different shellfish species (oysters, flat oysters, mussels and clams). These authors' results indicated that mussels and clams more readily accumulated viruses than the other shellfish species, as after 1 hour of contamination, they already detected significantly higher levels of virus.

5.2.7 Mortality rate of *M. edulis* and *C. gigas*

In experimental tanks containing *M. edulis*, the total mortalities recorded after 96 hrs of exposure were 12 in tanks A (low salinity, high temperature) and 4 in B (high salinity, high temperature) representing percentage mortality rates of 40% and 13% respectively (Figure 5.11). A common feature of tanks A and B was the high temperature (approximately 24°C), and this may be one of the factors responsible for the mortalities recorded in these tanks. No mortality was recorded in tanks C and D, or in the positive and negative control tanks.

In experimental tanks containing *C. gigas*, the total mortalities recorded after 96 hrs of exposure were 5, 5 and 5 (representing percentage mortality rates of 33 %) in tanks A, B and E respectively (Figure 5.11). Similarly, a common feature of tanks A, B and E was the high temperature (approximately 24°C in tanks A and B, and approximately 20°C in tank E), and this may be one of the factors responsible for the mortalities recorded in these tanks. No mortality was recorded in tanks C and D, or in the negative control tank.

In general, *M. edulis* and *C. gigas* in experimental tanks set at low temperature (approximately 8°C) survived the exposure period (approximately 5 days). This is most likely the result of adaptation of the shellfish species to low temperatures as they were obtained from communities growing in a temperate climate.



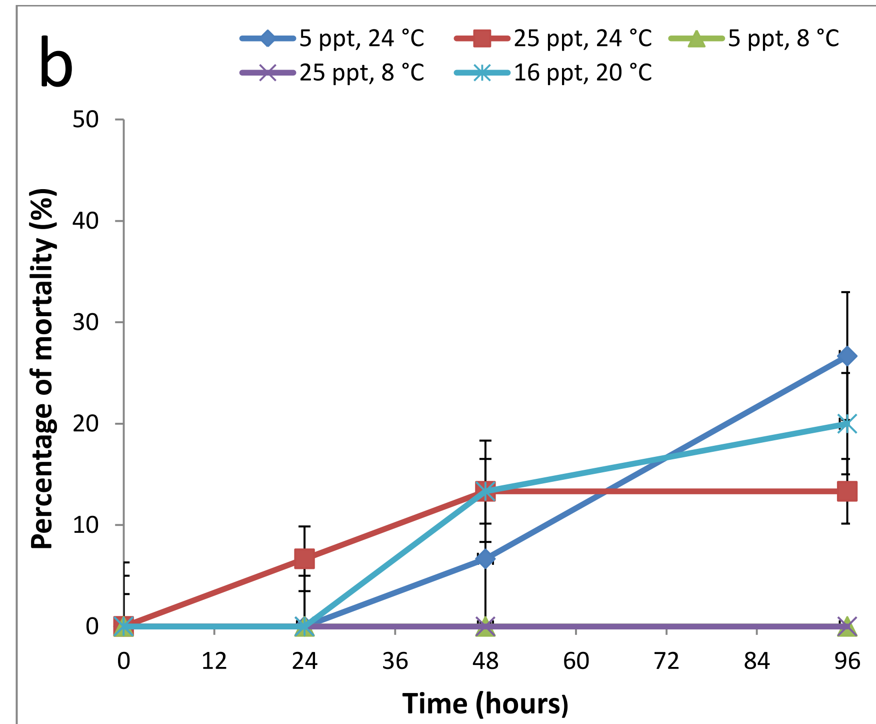


Figure 5.11: Percentage of mortality of *M. edulis* (a) and *C. gigas* (b) in artificial seawater at low and high temperatures and salinities.

(Total number of mussels (30) and oysters (15) in each experimental tank from which percentage of mortality was calculated)

5.3 Comparison of laboratory-based and *in-situ* field-based bioaccumulation in shellfish

Direct comparison of microbial contamination levels in shellfish tissue with those observed in their overlying waters is the most straight forward means of determining bioaccumulation (Lee, 1992). Although, there are other methods for predictive purposes, such as regression methods, simple kinetic models and physiologically-based pharmacokinetic models (Landrum *et al.*, 1992), bioaccumulation assessment remains the most widely used method for predicting the concentration of microbial contaminants in shellfish tissues. For instance, shellfish harvesting areas are classified, in part, according to the densities of faecal coliforms present in their surface waters (Burkhardt and Calci, 2000).

The applicability of bioaccumulation factor is based on equilibrium i.e., the assumption that the microbial contaminant of concern is in equilibrium in all matrices (overlying waters and shellfish), i.e. the level of exposure to contamination is constant and the period of shellfish exposure is extended. These conditions are not generally maintained in field and laboratory experiments, so, observed bioaccumulation values may vary greatly (Lee, 1992; Burkhardt and Calci, 2000).

Results from field analysis and laboratory-based experiments demonstrate that bioaccumulation of microbial indicators (*E. coli,* faecal coliforms, intestinal enterococci, somatic coliphages, F-RNA coliphages and GB124 phages) in mussels were significantly influenced by physiochemical conditions (temperature and salinity), but temperature has the strongest relationship in laboratory-based experiment (Table 5.6). In addition, their relationship can be further highlighted using a Pearson's correlation matrix deduced from

linear regression analysis (Table 5.6). In the laboratory-based study, bioaccumulation of microbial indicators in oysters showed a relatively weak relationship with temperature and salinity (Table 5.7). Other inherent factors such as, the ability of the labial palp and gills in oysters to control their feeding, filtering and bioaccumulation of pathogens, may likely be responsible for the weak relationship between temperature, salinity and the bioaccumulated bacterial and viral indicators.

Table 5.6: Comparison of the relationship between temperature, salinity and bioaccumulation of microbial indicators in mussels observed in field-based and laboratory-based bioaccumulation experiment using Pearson's correlation matrix

	Field-based		**Laboratory-based**	
	Temperature	Salinity	Temperature	Salinity
Temperature	1.000		1.000	
Salinity	0.602	1.000	0.010	1.000
GB124 phages	0.193	**0.254**	**0.936**	0.001
Somatic coliphages	0.216	**0.295**	**0.942**	0.052
F-RNA coliphages	**0.439**	0.346	**0.938**	0.214
E. coli	0.352	**0.417**	**0.895**	0.155
Faecal coliforms	0.445	**0.523**	**0.919**	0.092
Intestinal enterococci	**0.285**	0.267	**0.884**	0.167

Key: Figures (R) in **bold** denote a better predictor of bioaccumulation of microbial indicator in mussels in field study and laboratory experiment.

Table 5.7: The relationship between temperature, salinity and bioaccumulation of microbial indicators in oysters observed in laboratory-based bioaccumulation experiment using Pearson's correlation matrix

	Laboratory-based	
	Temperature	**Salinity**
Temperature	1.000	
Salinity	0.010	1.000
GB124 phages	0.117	**0.125**
Somatic coliphages	0.018	**0.156**
F-RNA coliphages	-	-
E. coli	0.012	**0.219**
Faecal coliforms	0.086	**0.209**
Intestinal enterococci	**0.389**	0.115

Key: Figures (R) in **bold** denote the better predictor of bioaccumulation of microbial indicator in oysters in laboratory experiment.

Chapter Six: Results of comparative studies of viral pathogens and bacteriophage surrogates

6.1 Results of an investigation into the use of bacteriophages as surrogates of viral pathogens in indigenous *Mytilus edulis* from an estuarine site

Molecular detection methods, involving qPCR and RT-qPCR were used to study the occurrence and distribution of adenovirus F and G (AdV F and G), norovirus genogroups I and II (NoV GI and GII) and hepatitis A viruses (HAV) in mussels (*Mytilus edulis*) and their overlying waters obtained from the estuary of the river Ouse in southeast England, United Kingdom. All samples were also tested for bacteriophages (somatic coliphages, F-RNA coliphages and GB124 phages) using standardised double-agar layer methods. Results from the enumeration of infective bacteriophages (by phage lysis) and gene copies of enteric viral pathogens (by qPCR) were compared using correlation analysis to evaluate the effectiveness of using bacteriophages as surrogates of enteric viral pathogens in shellfish hygiene monitoring.

6.1.1 Detection of enteric viruses in *M. edulis* and their overlying waters

The enteric viruses assayed in this study were norovirus genogroups I and II, adenovirus F and G and hepatitis A virus. The range of recorded concentrations of norovirus genogroups I in *M. edulis* was zero to 1.15 $\log_{10}$ detectable virus genome copies per 100 g of shellfish digestive gland and in overlying waters the recorded range was zero to 1.09 $\log_{10}$ detectable virus genome copies per 100 ml. The range of concentration of norovirus genogroups II in *M. edulis* was zero to 2.92 $\log_{10}$ detectable virus genome copies per 100 g of shellfish digestive gland and in overlying waters the range was zero to 2.77 $\log_{10}$ detectable virus genome copies per 100 ml. Norovirus genogroups II concentrations in both shellfish and water samples were significantly greater ($P < 0.05$) than concentrations

of norovirus genogroups I. The recorded range of concentration of adenovirus F and G in *M. edulis* was zero to 2.94 log_{10} detectable virus genome copies per 100 g of shellfish digestive gland and in overlying waters the range was zero to 1.34 log_{10} detectable virus genome copies per 100 ml. The level of hepatitis A virus was below the detection limit in both shellfish and overlying water samples (Table 6.1 and Appendix 7A). The limit of detection for norovirus genogroups I and II, adenovirus F and G and hepatitis A virus was 10 detectable virus genome copies per 100 ml and 100 g for overlying waters and shellfish samples, respectively.

Table 6.1: Mean concentration of enteric viral pathogens over the study period

Enteric virus	*M. edulis* (n = 45)		Overlying waters (n = 45)	
	Mean ± SD	Range	Mean ± SD	Range
Norovirus GI	0.05 ± 0.24	0.00-1.15	0.05 ± 0.23	0.00-1.09
Norovirus GII	0.77 ± 0.99	0.00-2.92	0.41 ± 0.70	0.00-2.77
Adenovirus F and G	0.43 ± 0.85	0.00-2.94	0.22 ± 0.35	0.00-1.34
Hepatitis A virus	ND		ND	

Mean (number 'n' of replicates for overlying waters/shellfish, standard deviation) Log_{10} detectable virus genome copies/100 ml overlying waters or 100 g digestive gland ± standard deviation; Range (Minimum-Maximum); ND – Non detect.

Two out of 45 overlying water samples (4%) and two out of the 45 shellfish batch samples (4%) were positive for norovirus genogroups I with mean concentrations of 0.05 ± 0.24 log_{10} detectable virus genome copies per 100 g of shellfish digestive gland in *M. edulis* and 0.05 ± 0.23 log_{10} detectable virus genome copies per 100 ml in overlying waters. Nine out of 45 overlying water samples (20%) and 12 out of the 45 shellfish batch samples (27%) were positive for norovirus genogroups II with mean concentrations of 0.77 ± 0.99 log_{10} detectable virus genome copies per 100 g of shellfish digestive gland in *M. edulis* and 0.41 ± 0.70 log_{10} detectable virus genome copies per 100 ml in overlying waters. Eleven out of

45 overlying water samples (24%) and nine out of the 45 shellfish batch samples (27%) were positive for adenovirus F and G with mean concentrations of 0.43 ± 0.85 $\log_{10}$ detectable virus genome copies per 100 g of shellfish digestive gland in *M. edulis* and 0.22 ± 0.35 $\log_{10}$ detectable virus genome copies per 100 ml in overlying waters (Figure 6.1). Elevated levels of norovirus genogroups II and adenovirus F and G were recorded in shellfish samples compared with their overlying waters (Figure 6.2).

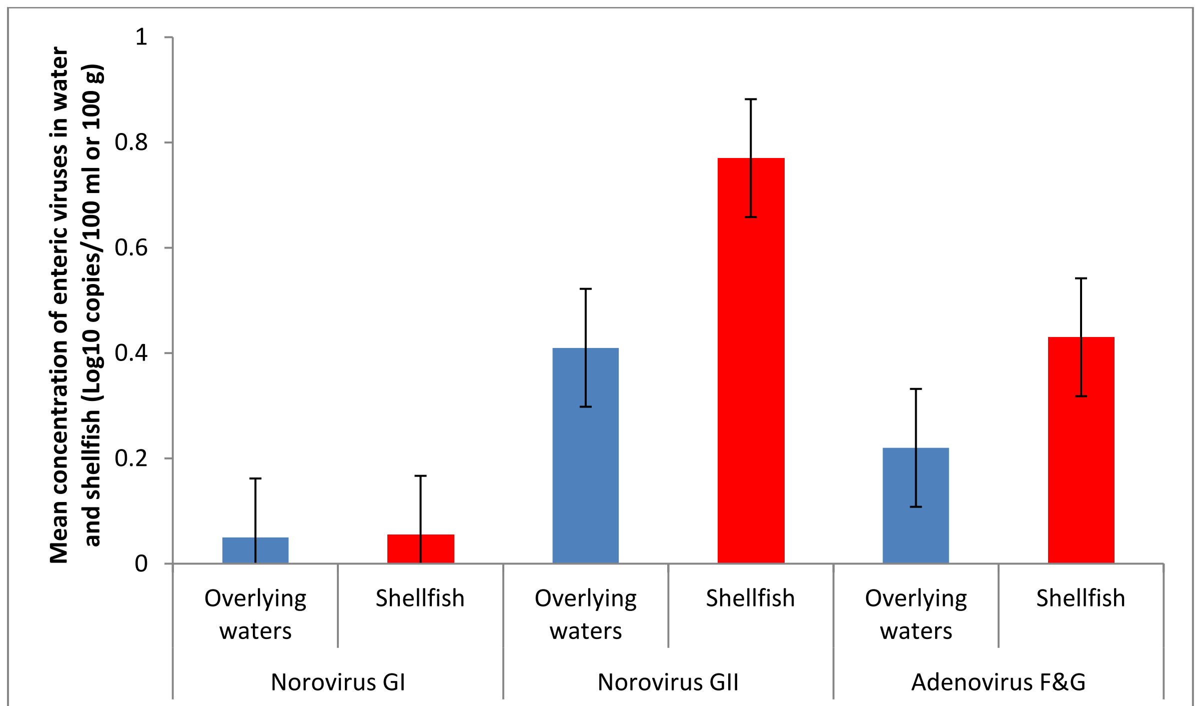


Figure 6.1: Mean (number 'n' of replicates for overlying waters/shellfish = 45, standard error) concentrations of norovirus genogroups I and II and adenovirus F and G in shellfish and overlying water.

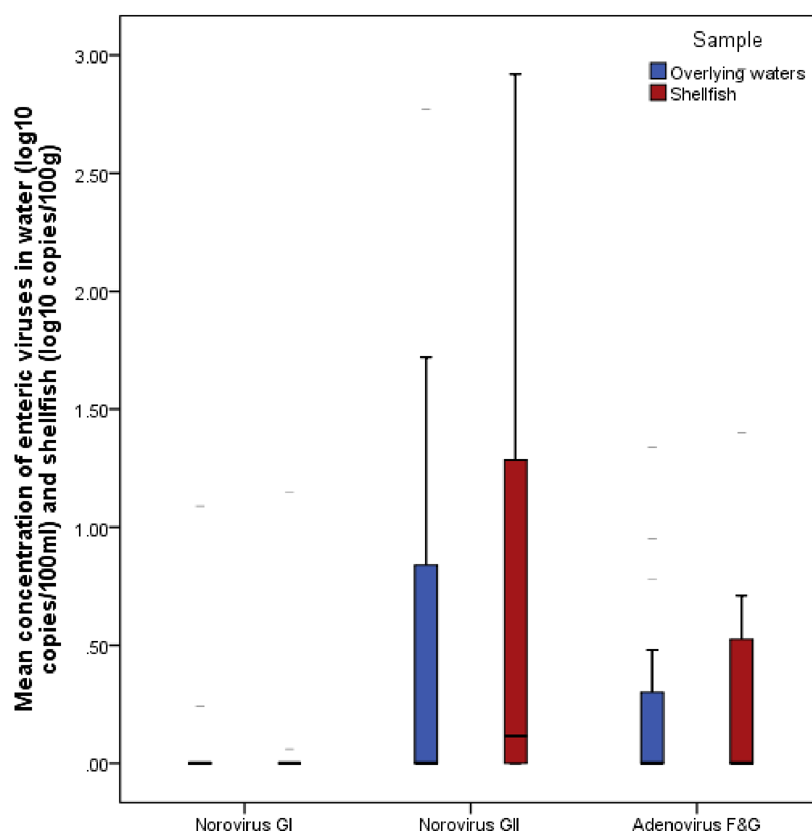


Figure 6.2: Boxplot of concentration of enteric viruses in shellfish and their overlying waters (number 'n' of replicates for overlying waters/shellfish = 45, standard error). The median value is represented by a line inside the box, 95% confidence intervals (bars).

6.1.2 Detection of enteric viral indicators in *M. edulis* and their overlying waters

The detection of viral indicators (bacteriophages) in *M. edulis* and their overlying waters in this study has been reported earlier (see section 4.1.2).

6.1.3 Seasonal variation of enteric viruses in *M. edulis* and overlying waters

For the purpose of this study, four seasons were considered, i.e., spring, summer, autumn and winter. The mean concentrations of norovirus genogroups I and II and adenovirus F and G in spring, autumn and winter months were higher in shellfish than in their overlying waters, although those of norovirus genogroups II were slightly higher in overlying waters than in shellfish during the summer period (Figure 6.3). Overall, norovirus genogroup II

showed the highest prevalence in shellfish and their overlying waters during all seasons, with the greatest concentrations observed during autumn and winter period. The recorded concentrations of norovirus genogroup II and adenovirus F and G were higher during the autumn and winter months compared with those observed during the spring and summer months.

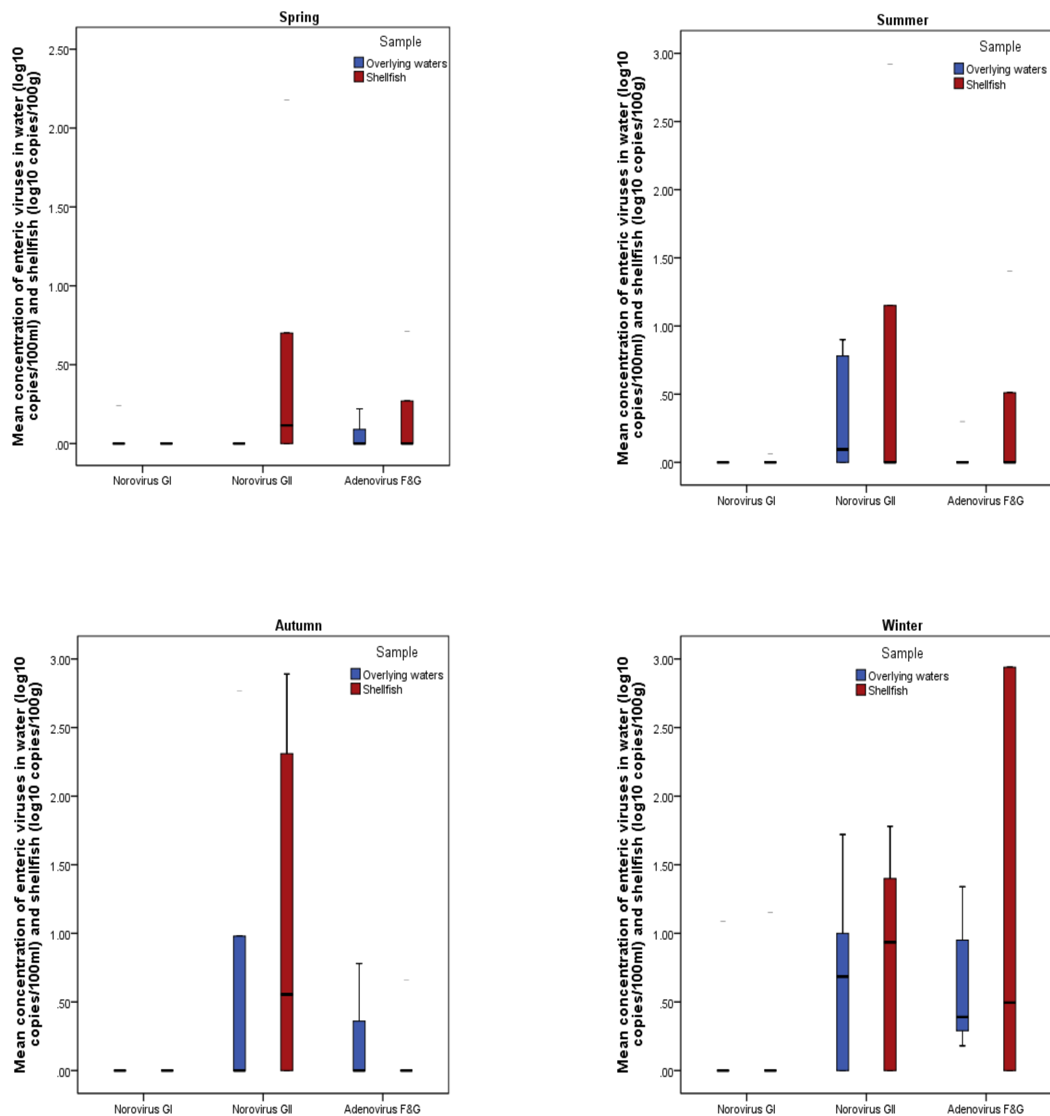


Figure 6.3: Boxplots of concentration of enteric viruses in shellfish and their overlying waters (number 'n' of replicates for overlying waters/shellfish = 6, standard error) during spring, summer, autumn and winter. The median value is represented by a line inside the box, 95% confidence intervals (bars).

6.1.4 The relationship between bacteriophages and enteric viruses in *M. edulis* and their overlying waters

Concentrations of viral faecal indicators – bacteriophages (somatic coliphages, F-RNA coliphages and GB124 phages) and those of enteric viral pathogens (total norovirus – norovirus genogroups I and II, and adenovirus F and G) in shellfish (*M. edulis*) and overlying waters were analysed using a two-tailed Spearman's rank correlation at two levels of significance ($P < 0.01$ and $P < 0.05$) representing 99% and 95% confidence interval respectively (Table 6.2).

Levels of norovirus genogroups I in overlying waters showed a positive correlation with those in *M. edulis* (rho = 0.50, $P < 0.05$). Positive correlations were observed between levels of norovirus genogroups II and levels of adenovirus F and G in overlying waters (rho = 0.52, $P < 0.01$). Similarly, positive correlations were observed between levels of norovirus genogroups II and levels of somatic coliphages in overlying waters (rho = 0.63, $P < 0.01$). Levels of norovirus genogroups II in *M. edulis* correlated significantly with levels of bacteriophages infecting *B. fragilis* GB124 in *M. edulis* (rho = 0.71, $P < 0.01$) (Figure 6.4). Similarly, levels of norovirus genogroups II in *M. edulis* showed a positive correlation with levels of somatic coliphages in *M. edulis* (rho = 0.49, $P < 0.05$).

Levels of adenovirus F and G in overlying waters showed a positive correlation with those in *M. edulis* (rho = 0.58, $P < 0.01$). Similarly, levels of adenovirus F and G in overlying waters showed positive correlations with levels of somatic coliphages in overlying waters (rho = 0.66, $P < 0.01$) and in *M. edulis* (rho = 0.48, $P < 0.05$). Interestingly, levels of adenovirus F and G in overlying waters showed slightly positive correlations with levels of bacteriophages infecting *Bacteroides fragilis* GB124 in overlying waters (rho = 0.43, P <

0.05) (Figure 6.5) and in *M. edulis* (rho = 0.49, $P < 0.05$). Levels of adenovirus F and G in *M. edulis* showed a positive correlation with levels of somatic coliphages in *M. edulis* (rho = 0.55, $P < 0.01$).

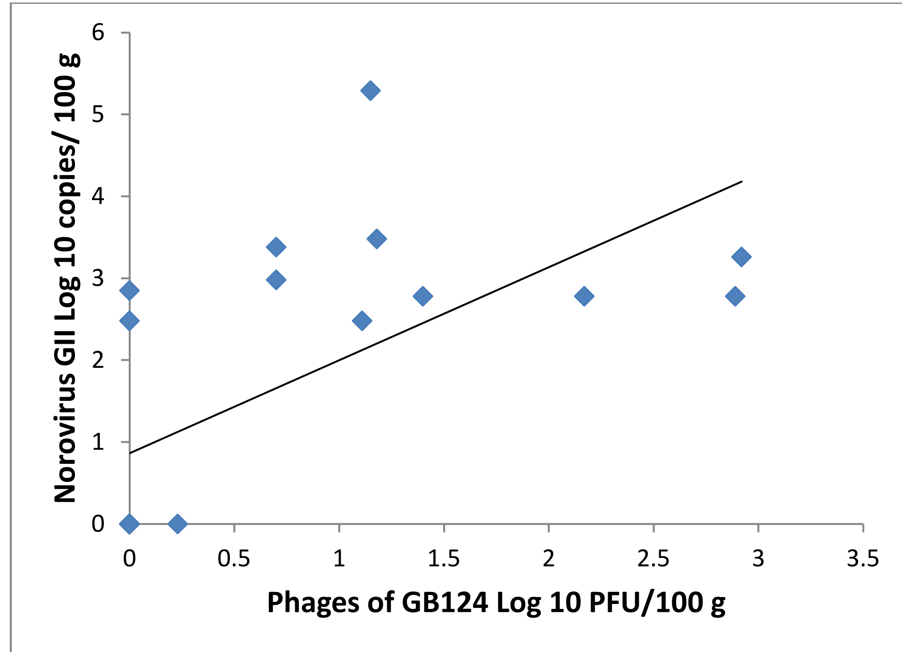


Figure 6.4: Monthly (number 'n' of replicates per month = 2, standard error) relationship between GB124 phages and norovirus genogroups II in *M. edulis.*

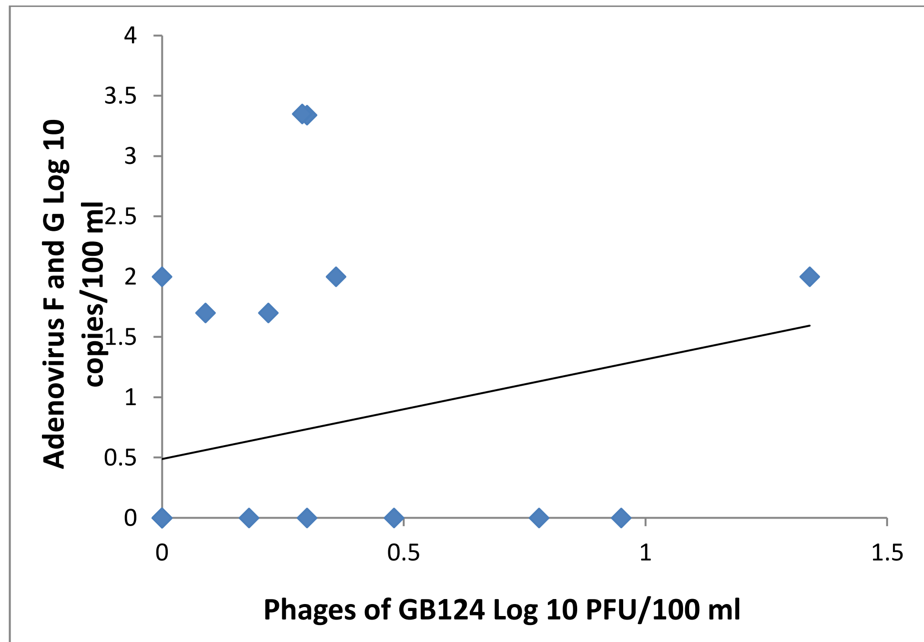


Figure 6.5: Monthly (number 'n' of replicates per month = 2, standard error) relationship between GB124 phages and adenovirus F and G in overlying waters.

Table 6.2: Spearman's rank correlation between bacteriophages and enteric viruses in *M. edulis* and overlying waters (24 months dataset)

	Water NoVI	Water NoVII	Water AdV	Water SomC	Water F-RNA	Water GB124	Mussel NoVI	Mussel NoVII	Mussel AdV	Mussel SomC	Mussel F-RNA	Mussel GB124
Water NoV I	1.00											
Water NoV II	-0.23	1.00										
Water AdV	0.24	0.52**	1.00									
Water SomC	0.05	0.63**	0.66**	1.00								
Water F-RNA	0.09	0.23	0.17	0.19	1.00							
Water GB124	0.06	0.27	0.43*	0.25	0.18	1.00						
Mussel NoVI	0.50*	-0.23	0.09	-0.09	-0.19	-0.19	1.00					
Mussel NoVII	-0.10	0.39	0.37	0.46*	0.09	0.12	-0.28	1.00				
Mussel AdV	0.25	-0.01	0.58**	0.40	-0.04	0.53**	0.06	0.08	1.00			
Mussel SomC	0.20	0.30	0.48*	0.64**	0.20	0.32	0.01	0.49*	0.55**	1.00		
Mussel F-RNA	0.08	0.14	0.11	0.03	0.52**	0.27	-0.09	0.01	0.05	0.09	1.00	
Mussel GB124	0.27	0.16	0.49*	0.41*	-0.18	0.36	0.12	0.71**	0.34	0.53**	-0.04	1.00

Key: SomC – Somatic coliphages; F-RNA – F-RNA coliphages; GB124 – GB124 phages; NoVI – Norovirus genogroups I; NoVII – Norovirus genogroups II; AdV – Adenovirus F and G; ** – Correlation is significant at the 0.01 level (2-tailed); * – Correlation is significant at the 0.05 level (2-tailed)

Levels of somatic coliphages in overlying waters showed a positive correlation with those in *M. edulis* (rho = 0.64, $P < 0.01$). Again, levels of somatic coliphages in overlying waters showed positive correlations with levels of bacteriophages infecting *B. fragilis* GB124 in *M. edulis* (rho = 0.41, $P < 0.05$), and levels of norovirus genogroups II in *M. edulis* (rho = 0.46, $P < 0.05$). Levels of somatic coliphages in *M. edulis* showed a positive correlation with levels of bacteriophages infecting *B. fragilis* GB124 in *M. edulis* (rho = 0.53, $P < 0.01$). Levels of F-RNA coliphages in overlying waters showed a positive correlation with those in *M. edulis* (rho = 0.52, $P < 0.01$). Levels of bacteriophages infecting *B. fragilis* GB124 in overlying waters showed a positive correlation with levels of adenovirus F and G in *M. edulis* (rho = 0.53, $P < 0.01$).

6.2 Results of an investigation into the use of phages as surrogates of viral pathogens in *Mytilus edulis* and *Crassostrea gigas* at selected coastal sites in southern England

Overlying river waters and shellfish (approximately 650 mussels (*Mytilus edulis*) and 1040 oysters (*Crassostrea gigas*) collected from Poole Harbour and Fleet lagoon, Weymouth, Dorset, southwest England by CEFAS (Centre for Environment, Fisheries and Aquaculture Science) (see section 3.2.1) from January 2013 to February 2015, were sent to the EPHReG (Environment and Public Health Research Group) laboratory at the University of Brighton for bacteriophage (phage) analysis using standardised methods (see section 3.6). These samples had been pretested for levels of traditional faecal indicator bacteria (*E. coli*) and norovirus genogroups I and II (NoV GI and NoV GII) at the Weymouth laboratories of CEFAS.

6.2.1 Detection of phages in *M. edulis* and *C. gigas* at selected site in southern England

The concentrations of phages (somatic coliphages, F-RNA coliphages and GB124 phages) detected in *M. edulis* and *C. gigas* from southern England are shown in Appendix 7B and Table 6.3. Somatic coliphages demonstrated mean concentrations of 2.53 log_{10} PFU/100 g mussels digestive gland, 3.01 log_{10} PFU/100 g oyster digestive gland from Poole and 1.96 log_{10} PFU/100 g oyster digestive gland from Fleet. F-RNA coliphages demonstrated mean concentrations of 0.39 log_{10} PFU/100 g mussel digestive gland, 0.23 log_{10} PFU/100 g oyster digestive gland from Poole and 0.61 log_{10} PFU/100 g oyster digestive gland from Fleet. Bacteroides GB124 phages demonstrated mean concentrations of 0.23 log_{10} PFU/100 g mussel digestive gland, 0.43 log_{10} PFU/100 g oyster digestive gland from Poole and 0.21 log_{10} PFU/100 g oyster digestive gland from Fleet. In general, somatic coliphages were the most abundant group of phages detected in both mussels and oysters.

Table 6.3: Mean concentrations of bacteriophages in *M. edulis* and *C. gigas*

Bacteriophages	***M. edulis* (Poole)** (n = 25)	***C. gigas* (Poole)** (n = 25)	***C. gigas* (Fleet)** (n = 25)
	Mean ± SD Range	Mean ± SD Range	Mean ± SD Range
Somatic coliphages	2.53 ± 1.84 0.00-4.71	3.01 ± 1.29 0.00-4.68	1.96 ± 1.62 0.00-3.92
F-RNA coliphages	0.39 ± 1.10 0.00-3.62	0.23 ± 0.81 0.00-3.38	0.61 ± 1.28 0.00-3.38
GB124 phages	0.23 ± 0.80 0.00-3.09	0.43 ± 1.03 0.00-3.08	0.21 ± 0.76 0.00-2.78

Mean log_{10} plaque-forming units per 100 g of shellfish digestive gland or per 100 ml of overlying waters ± standard deviation; n = number of replicates, SD = standard deviation Range (minimum - maximum).

6.2.2 Detection of norovirus in *M. edulis* and *C. gigas* at selected sites in southern England

The concentrations of norovirus genogroups I (NoV I) and norovirus genogroups II (NoV II) detected in *M. edulis* and *C. gigas* from southern England are shown in Appendix 7C and Table 6.4. Norovirus genogroups I demonstrated mean concentrations of 2.06 log_{10}

copies/100 g mussel digestive gland, 1.66 $\log_{10}$ copies/100 g oyster digestive gland from Poole, and 1.33 $\log_{10}$ copies/100 g oyster digestive gland from Fleet. Similarly, norovirus genogroups II demonstrated mean concentrations of 2.28 $\log_{10}$ copies/100 g mussel digestive gland, 1.98 $\log_{10}$ copies/100 g oyster digestive gland from Poole, and 1.56 $\log_{10}$ copies/100 g oyster digestive gland from Fleet. In general, norovirus genogroup II was the most abundant recorded viral pathogen in both mussels and oysters.

Table 6.4: Mean concentration of norovirus in *M. edulis* and *C. gigas*

Enteric virus	***M. edulis* (Poole)** (n = 13)	**_C. gigas_ (Poole)** (n = 13)	**_C. gigas_ (Fleet)** (n = 13)
	Mean ± SD Range	Mean ± SD Range	Mean ± SD Range
NoV I	2.06 ± 0.74 1.30-3.30	1.66 ± 0.42 1.30-2.52	1.33 ± 0.11 1.30-1.70
NoV II	2.28 ± 0.94 1.30-4.16	1.98 ± 0.78 1.30-3.56	1.56 ± 0.55 1.30-2.82

Mean $\log_{10}$ detectable virus genome copies/100g digestive gland ± standard deviation; n = number of replicates; SD = standard deviation; Range (minimum - maximum).

6.2.2 The relationship between bacteriophages and norovirus in *M. edulis* and *C. gigas* at selected coastal sites in southern England

Concentrations of phages (somatic coliphages, F-RNA coliphages and GB124 phages) and those of norovirus genogroups I and II in mussels and oysters from January 2013 to January 2014 were analysed using a two-tailed Spearman's rank correlation at two levels of significance ($P < 0.01$ and $P < 0.05$) representing 99% and 95% confidence interval respectively (Table 6.5). The levels of norovirus genogroups I in *M. edulis* showed positive correlations with levels of somatic coliphages in *M. edulis* (rho = 0.89, $P < 0.01$), levels of somatic coliphages in *C. gigas* from Poole (rho = 0.85, $P < 0.01$), and levels of somatic coliphages in *C. gigas* from Fleet (rho = 0.79, $P < 0.01$). Similarly, the levels of norovirus genogroups II in *M. edulis* showed positive correlations with levels of bacteriophages infecting *B. fragilis* GB124 in *M. edulis* (rho = 0.64, $P < 0.05$), levels of somatic

coliphages in *M. edulis* (rho = 0.74, $P < 0.01$), levels of F-RNA coliphages in *M. edulis* (rho = 0.57, $P < 0.05$), levels of bacteriophages infecting *B. fragilis* GB124 in *C. gigas* from Poole (rho = 0.65, $P < 0.05$), levels of somatic coliphages in *C. gigas* from Poole (rho = 0.77, $P < 0.01$), levels of somatic coliphages in *C. gigas* from Fleet (rho = 0.73, $P < 0.01$), and levels of F-RNA coliphages in *C. gigas* from Fleet (rho = 0.76, $P < 0.01$).

The levels of norovirus genogroups I in *C. gigas* from Poole showed positive correlations with levels of somatic coliphages in *M. edulis* (rho = 0.73, $P < 0.01$), levels of somatic coliphages in *C. gigas* from Poole (rho = 0.62, $P < 0.05$), and levels of somatic coliphages in *C. gigas* from Fleet (rho = 0.65, $P < 0.05$). The levels of norovirus genogroups II in *C. gigas* from Poole showed positive correlations with levels of bacteriophages infecting *B. fragilis* GB124 in *M. edulis* (rho = 0.60, $P < 0.05$), levels of somatic coliphages in *M. edulis* (rho = 0.72, $P < 0.01$), levels of bacteriophages infecting *B. fragilis* GB124 in *C. gigas* from Poole (rho = 0.65, $P < 0.05$), levels of somatic coliphages in *C. gigas* from Poole (rho = 0.82, $P < 0.01$), levels of somatic coliphages in *C. gigas* from Fleet (rho = 0.88, $P < 0.01$), and levels of F-RNA coliphages in *C. gigas* from Fleet (rho = 0.70, $P < 0.01$). The levels of norovirus genogroups II in *C. gigas* from Fleet showed a positive correlation with levels of F-RNA coliphages in *C. gigas* from Poole (rho = 0.63, $P < 0.01$).

The results of this research further demonstrate that the rapid phage lysis technique may offer an effective low-cost surrogate for the detection of pathogenic viruses in shellfish. The results also demonstrate that the target phages are effective surrogates of viral pathogens in two commonly harvested shellfish species (i.e., mussels and oysters).

Table 6.5: Spearman's rank correlation between bacteriophages and norovirus in *M. edulis* and *C. gigas* at selected coastal site in southern England (13 months dataset)

	Mussel NoVI	Mussel NoVII	Oyster-P NoVI	Oyster-P NoVII	Oyster-F NoVI	Oyster-F NoVII	Mussel GB124	Mussel SomC	Mussel F-RNA	Oyster-P GB124	Oyster-P SomC	Oyster-P F-RNA	Oyster-F GB124	Oyster-F SomC	Oyster-F F-RNA
Mussel NoV I	1.00														
Mussel NoV II	0.79**	1.00													
Oyster-P NoV I	0.78**	0.67**	1.00												
Oyster-P NoV II	0.72**	0.87**	0.64*	1.00											
Oyster-F NoV I	0.32	0.24	0.33	0.16	1.00										
Oyster-F NoV II	0.14	0.57**	0.08	0.58*	0.42	1.00									
Mussel GB124	0.51	0.64*	0.43	0.60*	-0.12	0.39	1.00								
Mussel SomC	0.89**	0.74**	0.73**	0.72**	0.16	0.15	0.52	1.00							
Mussel F-RNA	0.47	0.57*	0.21	0.42	-0.16	0.23	0.35	0.62*	1.00						
Oyster-P GB124	0.30	0.65*	0.26	0.65*	-0.16	0.55	0.80**	0.25	0.13	1.00					
Oyster-P SomC	0.85**	0.77**	0.62*	0.82**	0.31	0.37	0.62*	0.80**	0.28	0.49	1.00				
Oyster-P F-RNA	0.24	0.47	0.12	0.48	-0.08	0.63*	0.74**	0.39	0.58*	0.47	0.39	1.00			
Oyster-F GB124	0.12	0.41	0.15	0.41	-0.12	0.27	0.36	-0.03	-0.23	0.81**	0.26	-0.12	1.00		
Oyster-F SomC	0.79**	0.73**	0.65*	0.88**	0.39	0.43	0.41	0.68*	0.34	0.35	0.82**	0.31	0.17	1.00	
Oyster-F F-RNA	0.42	0.76**	0.26	0.70**	-0.22	0.44	0.46	0.45	0.66*	0.68*	0.37	0.35	0.55*	0.45	1.00

Key: SomC – Somatic coliphages; F-RNA – F-RNA coliphages; GB124 – GB124 phages; NoVI and NoVII – Norovirus genogroups I and II; P – Poole; F – Fleet; ** – Correlation is significant at the 0.01 level (2-tailed); * – Correlation is significant at the 0.05 level (2-tailed)

Chapter Seven: Discussion and Conclusions

7.1 Environmental factors influencing the levels of faecal indicator bacteria and phages in shellfish and their harvesting waters

Temporal variability in environmental conditions is associated with variations in the sanitary quality of shellfish and their harvesting waters. Oftentimes, climates are classified based on average annual and monthly temperature and precipitation. These phenomena influence the survival and possible proliferation of microbial pathogens and consequently influence the risk of infectious disease outbreak. Norovirus infection and outbreaks have been documented to occur during the winter season in temperate climates (Lopman *et al*., 2009). Similarly, outbreaks of *Vibrio* infections have been associated with humid and temperate climates (Baker-Austin *et al*., 2010) and environmental conditions and climate are factors that microbiologists, epidemiologists, and ecologists need to take into account in elucidating the processes of pathogen ecology (Lipp *et al*., 2002). Currently, the EU classification criteria for shellfish and harvesting waters are based on levels of *E. coli* (EC No. 854/2004) and in this study the ways in which environmental factors influence the occurrence and survival of faecal indicator bacteria and potential indicators of enteric viruses in mussels harvested from a river estuary in southeast England were investigated. Furthermore, the research investigated for the first time the relationship between levels of several shellfish-related viral pathogens and those of both traditional bacterial indicators and proposed bacteriophage indicators as the basis of a possible addition to sanitary inspection protocol that is more soundly based on human health risk evaluation.

In this study, ambient air temperature and the temperature of the overlying waters showed a negative relationship with the levels of faecal indicator bacteria and somatic coliphages in shellfish and their harvesting waters and there were significant differences in the

recorded concentration of faecal indicator bacteria and bacteriophages in mussels and their harvesting waters between seasons. In addition to seasonal changes in ambient and river water temperature, this is also likely to be related to solar radiation levels, which have been described as a major factor influencing the inactivation of bacteria of enteric origin in river waters (Chandran and Hatha, 2003). Bacteriophages (and enteric viruses) on the other hand are considered to be less susceptible than enteric bacteria to solar radiation (Diston *et al*., 2012). Similarly, salinity exhibited a negative relationship with levels of faecal indicator bacteria and phages. This is in agreement with the findings of Solic and Krstulovic (1992) in which high salinity was demonstrated to have a negative effect on faecal coliforms. During periods of high precipitation, there was a corresponding increase in river flow, agricultural land run-off, thus, influencing the number of counts of faecal indicator bacteria and phages in mussels and their overlying waters. This observation explains the positive correlation between river flow and levels of faecal indicator bacteria and bacteriophages. It is important to note that during periods of high salinity, tides were below 1.0 m (low tide) and rainfall values were low, and during periods of low salinity tides were over 1.0 m and rainfall values were high. Studies have shown that rainfall has a negative impact on the sanitary quality of surface waters by increasing the passage of microbial pathogens into the waters (Hernroth *et al*., 2002; Coulliette *et al*., 2009; Wilkes *et al*., 2013) either by diffuse overland flow from agricultural land or by storm-related intermittent discharges from combined sewer overflows (CSO). The results obtained in this study appear to be similar to those obtained by Lipp *et al*. (2001) in his work on the effects of seasonal variability and weather conditions on microbial faecal pollution and enteric pathogens in the Charlotte Harbour estuary in southwest Florida, USA in which the authors observed that faecal indicator bacteria and enteroviruses exhibited significant association with rainfall, streamflow and temperature and suggested that temperature, streamflow and precipitation

are useful parameters for modelling and predicting poor water quality in coastal environments.

Mallin *et al*. (2000), whilst studying the effects of human development on bacteriological water quality in coastal watersheds, demonstrated that turbidity correlated positively with enteric bacterial abundance throughout the system of coastal creeks, whereas salinity correlated inversely with the abundance and spatial pattern of enteric bacteria in the upper part of the stream. The results obtained in this study clearly showed that the levels of colloidal matter (i.e., degree of turbidity of overlying waters) to which microbes are able to attach influenced positively the levels of faecal indicator bacteria and bacteriophages in mussels and their harvesting waters. Turbidity values were relatively high during the winter months coinciding with periods of low salinity and high input of surface waters due to increased precipitation. Pommepuy *et al*. (1992) reported that enteric bacteria are able to survive for longer periods in turbid waters as a result of the attachment of bacteria to organic substrates and the protection offered by suspended solids. Dissolved oxygen levels showed a negative relationship with rainfall and levels of F-RNA coliphages in mussels. As would be expected, levels of total dissolved solids correlated significantly with the electrical conductivity of the shellfish harvesting waters and air temperature values were closely related to water temperature. Counts of faecal indicator bacteria and phages in shellfish and their harvesting waters increased with increases in rainfall, river flow and turbidity. However, increases in temperature and salinity corresponded with reduced counts of faecal indicator bacteria and phages in shellfish and their harvesting waters. Increases in the levels of precipitation reduced the salinity of the river water and increased overland flow from surrounding agricultural lands resulting in additional faecal loads entering the water and consequently higher levels of faecal indicator bacteria and phages

were detected in the shellfish. The limitation of this study is that environmental survey may have failed to analyse a sufficient number and type of organisms indicating faecal pollution in shellfish and their harvesting waters. In addition, the shellfish species may not be at steady-state with respect to their harvesting waters due to seasonal changes in their physiology during sampling activities over the two-year period. Furthermore, the role of environmental factors (such as physicochemical, meteorological and hydrological characteristics of shellfish harvesting waters from southeast England) in the survival of enteric viruses, bacteriophages and faecal indicator bacteria in shellfish was clearly identified, whereas environmental factors in southwest England were not examined in this thesis, this may have resulted in partial geographical representation of environmental parameters as an alternative tool in environmental water and shellfish monitoring in cases where access to microbial data is limited.

The results from this study suggests that the occurrence, survival and behavioural pattern of *E. coli*, faecal coliforms, intestinal enterococci, somatic coliphages, F-RNA coliphages and bacteriophages infecting *Bacteroides fragilis* GB124 in mussels and their harvesting waters are influenced to a large extent by environmental factors (including physicochemical, meteorological and hydrological factors), and this observation is in agreement with those of several previous studies (Pommepuy *et al.*, 1992; Lipp *et al.*, 2001; Coulliette *et al.*, 2009; Lopman *et al.*, 2009; Diston *et al.*, 2012; Wilkes *et al.*, 2013), thus adding to a greater understanding of the mechanisms underlying the dynamics of environmental transmission of microbial pathogens to shellfish and their harvesting waters. This work supports the hypothesis that environmental parameters (such as temperature, salinity, turbidity, rainfall) provide very useful information to support the use of predictive

modelling to improve shellfish hygiene and water quality monitoring for human health protection.

7.2 Bioaccumulation and uptake studies

7.2.1 Field-based study of bioaccumulation

Previous studies have demonstrated that the accumulation and elimination processes of microorganisms in shellfish are affected by temperature and salinity (Cabelli and Heffernan, 1970; Solic and Krstulovic, 1992) and this may partly be responsible for the seasonal variation of the observed levels of indicator organisms in shellfish (Cabelli and Heffernan, 1971) that were also demonstrated in this study. Solic and Krstulovic (1992) highlighted a 25% reduction in the filtration rate of *Crassostrea gigas* and *Mytilus edulis*; and 45% in *Ostrea edulis* when the temperature decreased from $20^{o}C$ to $10^{o}C$. This corresponds with the results obtained in this study in which mussels demonstrated a higher filtration rate and bioaccumulated *E. coli*, faecal coliforms and intestinal enterococci to considerably higher levels during periods of relatively high temperature between the months of May and October, during both 2013 and 2014. On the other hand, during periods of low temperature, between November 2013 and March 2014 and November 2014 and March 2015, levels of bioaccumulated *E. coli*, faecal coliforms and intestinal enterococci in mussels were lower and this is likely to be due to a reduction in their filtration rate. Clearly, water temperature demonstrated a significant positive correlation with the bioaccumulation of faecal indicator bacteria in mussels. Other environmental parameters that may have a positive influence on the bioaccumulation of faecal indicator bacteria in mussels are pH level and salinity. Other environmental parameters, namely turbidity, rainfall and river flow, showed an inverse relationship with the bioaccumulation of *E. coli*, faecal coliforms and intestinal enterococci in mussels.

Bioaccumulation of F-RNA coliphages in mussels correlated positively with rainfall, so that a seasonal trend was observed, with higher levels of this phage observed during the winter months. This observation corresponds with the findings of previous studies that suggest that the prevalence of enteric viruses in wastewaters fluctuates seasonally, with high levels occurring during periods of low temperature (i.e., winter) (Burkhardt and Calci, 2000) and during periods of high levels of precipitation, as observed in this study. River flow and rainfall demonstrated a positive correlation with the bioaccumulation of F-RNA coliphages in mussels. Increased river flow is the consequence of heavy rainfall, which can result in both diffuse (overland flow) and point source (combined sewer overflows) contamination of the river. Water temperature demonstrated an inverse relationship with the bioaccumulation of F-RNA coliphages in mussels. This may likely be due to the seasonal pattern of occurrence of the phage in the environment, as observed in this study. This may also be as a result of changes in filtration rate in mussels which varies with temperature. This observation is in agreement with the findings of other studies, suggesting that virus retention in shellfish is temperature dependent (Formiga-Cruz *et al*., 2002; Flannery *et al*., 2009).

Somatic coliphages were the most abundant group of phages isolated in this study and were bioaccumulated to significant levels in mussels, in accord with previous findings (Grabow, 2004) that somatic coliphages are likely to be the most abundant group of enteric phages in the environment, being shed by all warm-blooded mammals. Rainfall demonstrated an inverse relationship with the bioaccumulation of somatic coliphages in mussels. The bioaccumulation of GB124 phages in mussels showed a significant positive correlation ($P < 0.05$) with electrical conductivity and total dissolved solids. However, this group of phages were bioaccumulated to the greatest levels during the spring and winter

seasons. Studies have suggested that the GB124 phages are human-specific (Ebdon *et al*., 2007), and that they correlate with levels of human norovirus in mussels (Trajano Gomes Da Silva, 2013). Norovirus is the most common aetiological agent of human gastroenteritis caused by enteric viruses associated with the consumption of bivalve shellfish and infected individuals shed the virus in large quantities during the winter season (Flannery *et al*., 2009). The results from this study further suggest similarities between the behavioural dynamics of the GB124 phages and the norovirus pathogens.

7.2.2 Laboratory-based study of bioaccumulation

The rate of uptake, bioaccumulation and persistence of three faecal indicator bacteria (*E. coli*, faecal coliforms and intestinal enterococci) and three bacteriophages (somatic coliphage, F-RNA coliphages and bacteriophages infecting *Bacteroides fragilis* GB124) was examined in two shellfish species namely oysters (*Crassostrea gigas*) and mussels (*Mytilus edulis*) in artificial seawater at low and high salinities (approximately 5 and 25 ppt) and low and high temperatures (approximately 8 and 24°C) over a 96 hour exposure period. Previous shellfish bioaccumulation studies have predominantly evaluated bacteria (Roslev *et al*., 2009; Ottaviani *et al*., 2013), protozoa (Willis *et al.,* 2014), enteric viral pathogens (Nappier *et al*., 2008; Grodzki *et al*., 2014), and bacteriophages (Burkhardt and Calci, 2000; Nappier *et al*., 2009) in clams (Grodzki *et al*., 2014), mussels (Roslev *et al*., 2009; Ottaviani *et al*., 2013; Grodzki *et al*., 2014) and oysters (Burkhardt and Calci, 2000; Nappier *et al*., 2009; Willis *et al.,* 2014; Grodzki *et al*., 2014). This investigation represents the first known attempt to investigate bioaccumulation of bacteriophages infecting *Bacteroides fragilis* GB124 in controlled laboratory experiments involving oysters. During the experiment, approximately 8% of mussels and 13% of oysters that were exposed to the experimental conditions were shown to die. A common feature of experimental tanks in

which deaths occurred was elevated temperature (approximately 24°C), which may be one of the factors responsible for the observed mortality rate. This may also be partly due to the fact that the shellfish species used in the experiment were harvested from a temperate climate in which ambient temperatures rarely reach 24°C, except occasionally during the summer periods, but a common occurrence (ambient temperature) in non-temperate climates where these risk assessment tools might be applied.

Throughout the exposure period, mussels and oysters were tested for three faecal bacterial indicators and three potential viral indicators (bacteriophages). The flesh and the intravalvular fluids were assessed for faecal indicator bacteria while the digestive glands were assessed for bacteriophages. All the mussels bioaccumulated *E. coli*, faecal coliforms, intestinal enterococci, somatic coliphages and F-RNA coliphages in all experimental tanks including the positive control tank. The recorded levels of GB124 phages were below their detection limit in mussels in two experimental tanks at low and high salinities at low temperatures (approximately 8°C) (Table 5.4). Although phages infecting *Bacteroides fragilis* have been demonstrated to be resistant to unfavourable conditions (Jofre *et al*., 1995), it might be that the low filtration activity of mussels at low temperature (approximately 8°C) prevented the phages from accumulating to detectable levels. This may also be due to changes in the physiology (Polo *et al*., 2014) of mussels caused by the low temperature. This observation is contrary to the findings from the environmental assessment of the bioaccumulation of phages in mussels under natural conditions, in which the GB124 phages were bioaccumulated to high levels during spring and winter period (Figure 5.4). This variation may be because of other physicochemical parameters (such as dissolved oxygen, electrical conductivity, turbidity, total dissolves solids) that were not included in the controlled experiment. All oysters bioaccumulated *E. coli*, faecal coliforms,

intestinal enterococci and somatic coliphages in all experimental tanks, including the positive control tank (in which salinity was approximately 16 ppt and the temperature was approximately 20°C). Levels of F-RNA coliphages were below their detection limit in oysters in all the experimental tanks. This differential selection process observed in oysters may likely be due to the ability of the labial palp and gills to reject certain microbes (Espinosa *et al*., 2008). Again, the GB124 phages were below their detection limit in oysters in the tank containing artificial seawater at low salinity (approximately 5 ppt) and low temperature (approximately 8°C). Changes in temperature and salinity, among other environmental factors can affect the physiological state of oysters, altering their ability to feed, filter and bioaccumulate viruses (Sobsey and Jaykus, 1991; Nappier *et al*., 2008). In this study, statistical analysis revealed that bioaccumulated levels of somatic coliphages, F-RNA coliphages and GB124 phages were significantly greater in mussels than in oysters. Intestinal enterococci also followed this same pattern of bioaccumulation; in that they were observed to be higher in mussels than in oysters. However, *E. coli* and faecal coliforms exhibited a different pattern of uptake and bioaccumulation from the phages and intestinal enterococci. They were recorded at significantly greater levels in oysters than mussels. This may be because of the physiology of the oysters.

Many authors (Šolić *et al*., 1999; Martinez and Oliveira, 2010) have highlighted the effect of temperature and salinity on physiological rates (such as, uptake, clearance and absorption efficiency) in shellfish species. Clearance rate is described as the speed at which microorganisms are removed from shellfish tissues. Resgalla Jr. *et al*. (2007) observed that the clearance rate and absorption efficiency in mussels (*Perna perna*) exhibited inhibition at salinities of 15 and 40 ppt. In this study, the clearance rate of *E. coli*, faecal coliforms, intestinal enterococci, somatic coliphage, F-RNA coliphages and bacteriophages infecting

Bacteroides fragilis GB124 in mussels and oysters after 96 hours exposure period varied. This is partly due to factors such as temperature and salinity of the artificial seawater used for the bioaccumulation experiment.

Statistical analysis, using Pearson's correlation matrix, revealed that, under laboratory-controlled conditions, temperature significantly influenced the bioaccumulation of all microbial indicators in mussels – *E. coli* (R = 0.895), faecal coliforms (R = 0.919), intestinal enterococci (R = 0.884), somatic coliphages (R = 0.924), F-RNA coliphages (R = 0.938), and GB124 phages (R = 0.936). On the other hand, temperature had minimal effect on the bioaccumulation of the microbial indicators in oysters – *E. coli* (R = 0.012), faecal coliforms (R = 0.086), intestinal enterococci (R = 0.389), somatic coliphages (R = 0.018), and GB124 phages (R = 0.117). These bioaccumulation results clearly demonstrate variation between shellfish species under identical laboratory conditions and support the findings of other authors (Burkhardt and Calci, 2000; Nappier *et al*., 2009; Ottaviani *et al*., 2013; Willis *et al.,* 2014; Grodzki *et al*., 2014). The effect of salinity on the bioaccumulation of all microbial indicators in mussels and oysters under controlled conditions in the laboratory was minimal compared with the findings in the environmental assessment of the bioaccumulation of faecal indicator bacteria and phages in shellfish under natural conditions. This suggests that salinity is not driving the bioaccumulation process under laboratory conditions, but 'in the wild' it inversely relates to rainfall and the pollution inputs from land-runoff into estuarine waters. The limitation of the laboratory-based experiment is that shellfish species may have been exposed to a range of microbial contamination that does not overlap with the range of concern in terms of public health risk assessment. Furthermore, shellfish species may not have achieved a steady-state concentration within the exposure period before intermittent removal and microbial

examination. Also, exposure conditions may have been unrealistic compared with what's achievable in the environment. For instance, a water temperature of 24°C is not a common feature in temperate climate, but in non-temperate climate.

7.3 Predicting enteric viral contamination using bacteriophages

Shellfish farming is a major component of the economy in many parts of the world. In the UK, it is a multi-million pound business producing thousands of tonnes of shellfish each year. Commercially valuable species, such as oysters, mussels and clams, account for a large part of the industry, which is an important contributor to the UK economy and is worth over £250 million annually (Defra, 2013). Similarly, the shellfish farming sector contributes significantly to the annual French fisheries economy. This puts France amongst the leading European countries for fisheries production. The sector generated €1.9 billion between 2003 and 2007, with an average of €380 million annually during this period (Kalaydjian *et al*., 2010).

Cases of human bacterial gastroenteritis associated with the consumption of faecally contaminated shellfish are considered to be relatively rare in Europe, which may be due to strict compliance with shellfish sanitary regulations. However, cases of viral gastroenteritis, such as that caused by norovirus, are a public health concern (Flannery *et al*., 2009). Public Health England (2014) reported 1459 norovirus outbreaks between July 2012 and June 2013, of which 1311 (90%) resulted in hospital ward or bay restrictions or closures, and 990 (68%) were reported as laboratory confirmed norovirus outbreaks. In the United States, the CDC estimates that each year on average 19 to 21 million cases of acute gastroenteritis are caused by noroviruses, i.e., about 1 in every 15 individuals will become ill because of norovirus each year. The virus is also estimated to cause between 56,000 and

71,000 hospitalisations and between 570 and 800 deaths each year (CDC, 2014). Figures of incidence and prevalence of foodborne outbreaks published by the UK Health Protection Agency between 2000 and 2009 (Pyke, 2010), showed 679 outbreaks of Infectious Intestinal Disease (IID), of these, 68 (10%) were caused by norovirus and 25 (3%) were attributed to shellfish. The numbers of reported outbreaks are likely to be an underestimate of the total number of outbreaks that occurred during the given periods and this is partly due to the self-limiting nature of the virus. Previous studies (Dore *et al*., 2000; Flannery *et al*., 2009) have highlighted the inadequacy of traditional *E. coli* to predict accurately the risk of enteric viral contamination in shellfish harvesting areas. Viruses have also been detected in shellfish harvested from areas classified as 'category A status' according to EU standards (Muniain-Mujika *et al*., 2003). This suggests that the current hygiene classifications for shellfisheries, based on levels of faecal indicator bacteria, may not necessarily be an accurate indicator of risk of viral contamination.

Enteric viruses, such as norovirus and hepatitis A virus (although HAV were recorded to be below detectable levels in shellfish and water samples during this study), have been observed to pose the greatest risk to public health and they are the most common type of viruses associated with human gastroenteritis from shellfish (Koopmans *et al*., 2002). Others include enteroviruses, adenoviruses, rotaviruses and astroviruses. Molecular methods have been developed and used for detecting these viral pathogens in shellfish (Pina *et al*., 1998) and presently, real-time polymerase chain reaction (qPCR) and reverse transcription polymerase chain reaction (RT-qPCR) are being used to quantify viral nucleic acids in water and shellfish matrices (Campos and Lees, 2014). These methods remain expensive, time-consuming and require expertise, and do not provide information about the infectivity of the quantified viral nucleic acids (Bosch, 2010). There is, therefore, a

demonstrable need to target alternative indicators to maintain the microbial safety of shellfish and for human health protection.

In this study, Spearman's correlation coefficient demonstrated that the use of somatic coliphages, F-RNA coliphages and GB124 phages provide a better indication of the risk of norovirus and adenovirus F and G contamination in mussels and their harvesting waters compared with traditional faecal indicator bacteria. This study represents the first investigation of GB124 phages as an effective low-cost surrogate for the detection of norovirus in oysters. The research findings support the use of somatic coliphage, F-RNA coliphages and bacteriophages infecting *Bacteroides fragilis* GB124 as alternative indicators and effective surrogates of enteric viruses in the two shellfish species studied (mussels and oysters), as well as their harvesting waters. The limitation of this study is that environmental survey may have failed in collection of water and shellfish samples with concentration of contaminant that are within the range of interest for correlation analysis with actual enteric viral pathogens.

Norovirus outbreaks usually occur during the winter season in temperate climates (Lopman *et al*., 2003) when ambient temperatures are lower and rainfall levels are generally higher. During this period, norovirus outbreaks that are shellfish-related have been reported in many studies. These outbreaks may result from the shedding of the virus in high concentration in faeces of infected individuals who may be symptomatic or asymptomatic (Atmar *et al*., 2008; Rajko-Nenow *et al*., 2013). In this study, F-RNA coliphages and GB124 phages exhibited seasonal trends in shellfish harvesting waters that were similar to the pattern of occurrence of norovirus in the overlying waters. However, somatic coliphages, which were the most abundant group of phages detected in shellfish and their

harvesting waters, did not demonstrate this trend. This further suggests that F-RNA coliphages and GB124 phages may be used to predict enteric viral contamination. The results from this study demonstrated that norovirus levels in mussels and oysters correlated positively with levels of GB124 phages in these shellfish. This finding is in agreement with previous studies (Trajano Gomes Da Silva, 2013), further demonstrating the usefulness of simple pollution markers (bacteriophages) as effective surrogates of enteric viral contamination in public health protection efforts. Incorporating the use of this tool into shellfish safety planning, especially for routine monitoring of shellfish and their harvesting waters will provide improved human health protection.

7.4 Conclusions

The principal findings of this research are:

1. Environmental factors (which include physicochemical, meteorological and hydrological factors) influenced the occurrence, survival and behavioural dynamics of *E. coli*, faecal coliforms, intestinal enterococci, somatic coliphages, F-RNA coliphages and bacteriophages infecting *Bacteroides fragilis* GB124 in shellfish and their harvesting waters. Interestingly, positive relationships were observed between levels of the microbial indicators and rainfall, river flow and turbidity, and an inverse relationship was observed between the microbial indicators and temperature and salinity. In circumstances where there are limitations of access to adequate laboratory facilities for microbial quality testing, environmental factors could be used for risk assessment purposes. The findings of this study suggest that monitoring environmental parameters is a useful addition to monitoring plans for ensuring compliance of shellfish and their harvesting waters with public health protection regulations and as a component of predictive modelling in shellfish and water quality monitoring for human health protection.

2. The results from the field-based study demonstrated that the levels of faecal indicator bacteria in mussels show a positive relationship with water temperature, pH level and salinity; and an inverse relationship with turbidity, rainfall and river flow. The levels of F-RNA coliphages in mussels correlated positively with rainfall, turbidity and river flow and negatively with water temperature. The levels of somatic coliphages in mussels correlated negatively with rainfall. The levels of GB124 phages showed a positive correlation with electrical conductivity and total dissolved solids and they were bioaccumulated to the greatest levels during spring and winter, thus demonstrating a similar pattern to those of

enteric viral pathogens such as norovirus. Again, these findings demonstrate the impact of environmental factors on the bioaccumulation of faecal indicator bacteria and bacteriophages in shellfish. Interestingly, coastal waters could be moderately impacted by faecal pollution and in some circumstances contamination might be due to intermittent discharge of wastewaters in low quantities from point or non-point sources. Rapid dilution and high flow velocity of the overlying waters may cause faecal indicator organisms to fall below detectable limits. Examining traditional grab samples of overlying waters for faecal indicators might yield little or no information about the microbiological quality of the overlying waters and this might lead to unsuccessful water quality monitoring. Shellfish, however, have the ability to filter-feed and bioaccumulate indicators of faecal pollution over a period of time. Determining the microbial quality of such shellfish samples by measuring their bioaccumulation factor may provide more useful information on water quality than examining traditional grab samples of the overlying waters. The findings from this study suggest that measuring a 'bioaccumulation index' in shellfish may provide a novel alternative tool for monitoring the microbiological quality of environmental waters and this will be of great benefit to shellfish and water industries.

3. The results from the laboratory-based bioaccumulation studies revealed that the rate of uptake, bioaccumulation and persistence of microbial indicators in shellfish under controlled conditions varies between the shellfish species, and also varies from what is observed in the environment, in which shellfish species may be contaminated naturally during their filter-feeding process in contaminated estuarine or coastal waters. *E. coli* were bioaccumulated to the highest levels after 48 hours in mussels and 6 hours in oysters. Faecal coliforms were bioaccumulated to the highest levels after 48 hours in mussels and 12 hours in oysters. Intestinal enterococci were bioaccumulated to the highest levels after

48 hours in mussels and 24 hours in oysters. Somatic coliphages were bioaccumulated to the highest levels after 48 hours in both mussels and oysters. The GB124 phages were bioaccumulated to the highest levels after 24 hours in both mussels and oysters. The F-RNA coliphages were bioaccumulated to the highest levels after 6 hours in mussels and were below detectable limits in oysters. Similarly, based on the initial high titre of microbial suspension used for spiking, the pattern of persistence of microbial indicators in the shellfish species between 48 and 96 hours varied. During this period, bioaccumulated levels of *E. coli* and faecal coliforms reduced in mussels but increased in oysters. Intestinal enterococci and somatic coliphages reduced in both mussels and oysters. The GB124 phages reduced in mussels and were no longer detected in the oysters, whereas the F-RNA coliphages reduced in mussels and were not detected in oysters. In general, mussels bioaccumulated all phages to higher densities compared with oysters suggesting that mussels may be more sensitive to sporadic viral contamination than oysters. This work has elucidated the rate of uptake, bioaccumulation and persistence of faecal indicator bacteria and bacteriophages in mussels and oysters under controlled conditions and has demonstrated the selective accumulation patterns of pathogens commonly observed in shellfish species. This new knowledge will be useful to the shellfisheries industry to support selective shellfish species harvesting from classified sites and more effective purification processes in terms of duration and conditions necessary to achieve a successful depuration or relaying in clean waters in order to prevent shellfish-related infections.

4. The use of somatic coliphages, F-RNA coliphages and GB124 phages was demonstrated to provide a better indication of the risk of norovirus and adenovirus contamination in mussels, oysters and their harvesting waters than traditional faecal indicator bacteria. These findings are in agreement with those of other recent studies that have demonstrated

the usefulness of bacteriophages as effective surrogates of enteric viral contamination. The results from this study suggest that relatively simple pollution markers (i.e., bacteriophages) may be used to predict enteric viral contamination in shellfish and their harvesting waters.

7.5 Recommendations for further research

1. It is important to explore simple indicators of bioaccumulation of viral pathogens (such as somatic coliphages, F-RNA coliphages and bacteriophages infecting *Bacteroides fragilis* GB124) in shellfish. This will be of great benefit in water quality monitoring, as shellfish have been suggested to have biological characteristics that may reveal contamination events that routine monitoring of water may miss ordinarily.

2. This work has demonstrated that the rapid bacteriophage technique may be an effective surrogate of enteric viruses such as norovirus (genogroups I and II) and adenovirus (F and G) in mussels and oysters. It would now be timely to assess the efficacy of this method in other important commercial shellfish species, such as scallops, clams, etc.

3. This work examined the rate of uptake, bioaccumulation and persistence of microbial indicators in mussels and oysters under controlled conditions using culture-dependent methods. It would be useful to assess actual pathogens (norovirus, adenovirus, etc.) in a similar experimental setup using culture-independent method such as molecular detection technique.

4. In addition, the bioaccumulation experiment was carried out in 'static' tanks. It would be beneficial in the future to examine the rate of uptake, bioaccumulation and persistence of

microbial indicators in the shellfish species under controlled conditions in flow-through tanks.

5. It would be beneficial to examine and compare the rate of depuration of faecal indicator bacteria, bacteriophages and enteric viral pathogens in artificially (laboratory-based) and environmentally (*in-situ* field-based) contaminated shellfish species in standard depuration experiments.

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Appendix 1: Media composition

Bacteriological examination

i. Membrane lauryl sulphate agar (MLSA)

Peptone bacteriological (Oxoid LP37)	39g
Yeast extract	6g
Lactose	30g
Phenol red	0.2g
Sodium lauryl sulphate	1g
Agar	12 – 20g
Distilled water	1000ml

Dehydrated MLSA

Powder	76.2g
Agar	12 – 20g
Distilled water	1000ml

Note: Sterilize in autoclave at 121°C for 15 minutes and store in the dark at 4 ^{o}C for not more than 6 months.

ii. Tryptone bile glucuronide (TBX) agar

Tryptone bile glucuronide agar (Lab M)	36.5g
De-ionised water	1000ml

iii. Difco^{TM} Membrane faecal coliform agar (mFc)

Tryptose	10g
Proteose peptone No. 3	5g
Yeast extract	3g
Lactose	12.5g
Bile salt No. 3	1.5g
Sodium chloride	5g
Aniline blue	0.1g
Agar	15g

Dehydrated mFc agar

Powder	52g
1% Rosolic acid in 0.2N NaOH	10ml
Distilled water	1000ml

Note: Do not autoclave; boil with frequent agitation to dissolve powder for 1 minute.

iv. Difco^{TM} Membrane enterococcus agar (mEnt)

Tryptose	20g
Yeast extract	5g
Dextrose	2g
Dipotassium phosphate	4g
Sodium azide	0.4g
2,3,5-Triphenyl tetrazolium chloride	0.1g
Agar	10g
Dehydrated mEnt agar	
Powder	42g

Distilled water	1000ml

Note: Do not autoclave; boil with frequent agitation to dissolve powder for 1 minute.

v. 0.1% Peptone

Peptone bacteriological (Oxoid LP37)	1g
De-ionised water	1000ml

vi. Mineral modified glutamate broth (MMGB)

Single-strength (ssMMGB)

Ammonium chloride	2.5g
Sodium glutamate (Oxoid L124)	6.4g
Mineral modified medium base (Oxoid CM607)	11.4g
De-ionised water	1000ml

Double-strength (dsMMGB)

Ammonium chloride	5g
Sodium glutamate (Oxoid L124)	12.8g
Mineral modified medium base (Oxoid CM607)	22.8g
De-ionised water	1000ml

Enumeration of F-specific RNA bacteriophages

i. Tryptone yeast extract glucose broth (TYGB)

Basal broth

Tryptone	10g
Yeast extract	1g
Sodium chloride (NaCl)	8g
Distilled water	1000ml

Note: Sterilize in autoclave at 121°C for 15 minutes and store in the dark at 4 ^{o}C for not more than 6 months.

ii. Calcium glucose solution

Calcium chloride ($CaCl_2.2H_2O$)	3g
Glucose	10g
Distilled water	100ml

Note: Filter-sterilize through 0.22µm membrane filter and store in the dark at 4 ^{o}C for not more than 6 months.

Complete broth

Basal broth of TYGB	200ml
Calcium glucose solution	2ml

iii. Tryptone yeast extract glucose agar (TYGA)

Basal agar

Tryptone	10g
Yeast extract	1g
NaCl	8g
Agar	12 – 20g
Distilled water	1000ml

Note: Sterilize in autoclave at 121°C for 15 minutes and store in the dark at 4 °C for not more than 6 months.

Complete agar	
Basal agar of TYGA	200ml
Calcium glucose solution	2ml

iv. Semi-solid tryptone yeast extract glucose agar (ssTYGA)

Basal agar of TYGA but with half mass of agar (6 – 10g) depending on gel strength.

v. Nalidixic acid solution

Nalidixic acid	250mg
Sodium hydroxide (NaOH – 1 mol/litre)	2ml
Distilled water	8ml

Note: Filter-sterilize through 0.22µm membrane filter and store at – 20 °C for not more than 6 months.

vi. Kanamycin monosulfate

Kanamycin monosulfate	1.25g
Distilled water	10ml

Note: Filter-sterilize through 0.22µm membrane filter and store at – 20 °C for not more than 6 months.

Enumeration of somatic coliphages

i. Modified Scholtens' Broth (MSB)

Basal broth	
Peptone	10g
Yeast extract	3g
Meat extract	12g
NaCl	3g
Sodium carbonate (Na_2CO_3) solution (150g/l)	5ml
Magnesium chloride (100g of $MgCl_2.6H_2O$ in 50 ml water)	0.3ml
Distilled water	1000ml

Note: Sterilize in autoclave at 121°C for 15 minutes and store in the dark at 4 °C for not more than 6 months.

ii. Calcium chloride solution

Calcium chloride ($CaCl_2.2H_2O$)	14.6g
Distilled water	100ml

Note: Filter-sterilize through 0.22µm membrane filter and store in the dark at 4 °C for not more than 6 months.

Complete broth	
Basal broth of MSB	200ml
Calcium chloride solution	1.2ml

iii. Modified Scholtens' Agar (MSA)

Basal agar	
Peptone	10g

Yeast extract	3g
Meat extract	12g
NaCl	3g
Sodium carbonate (Na_2CO_3) solution (150g/l)	5ml
Agar	10 – 20g
Magnesium chloride (100g of $MgCl_2.6H_2O$ in 50 ml water)	0.3ml
Distilled water	1000ml

Note: Sterilize in autoclave at 121°C for 15 minutes and store in the dark at 4 °C for not more than 6 months.

Complete agar

Basal agar of MSA	200ml
Calcium chloride solution	1.2ml

iv. Semi-solid Modified Scholtens' Agar (ssMSA)

Basal agar of MSA but with half mass of agar (6 – 10g) depending on gel strength.

Enumeration of bacteriophages infecting human specific *Bacteroides fragilis*

i. *Bacteroides* phage recovery medium broth (BPRMB)

Basal broth

Special peptone	10g
Tryptone	10g
Yeast extract	2g
NaCl	5g
Monohydrate L-cysteine	0.5g
Glucose	1.8g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.12g
$CaCl_2$ solution (0.05 g/ml)	1ml
Distilled water	1000ml

Note: Sterilize in autoclave at 121°C for 15 minutes and store in the dark at 4 °C for not more than 1 week once the lid has been opened or 1 month when the lid is not opened.

ii. Haemin solution

Haemin	0.1g
Sodium hydroxide (NaOH solution – 1mol/l)	0.5ml
Distilled water	99.5ml

Note: Filter-sterilize through 0.22μm membrane filter or in autoclave at 121°C for 15 minutes and store in the dark at 4 °C for not more than 6 months.

iii. Disodium carbonate solution

Na_2CO_3	10.6g
Distilled water	100ml

Note: Filter-sterilize through 0.22μm membrane filter and store in the dark at 4 °C for not more than 6 months.

Complete broth

Basal broth	1000ml
Haemin solution	10ml
Disodium carbonate solution	25ml

35% Hydrochloric acid (HCl)	2.5ml
Kanamycin monosulfate	1ml
Nalidixic acid	4ml

iv. *Bacteroides* phage recovery medium agar (BPRMA)

Basal agar

Basal broth	1000ml
Agar	12 – 20g

Note: Sterilize in autoclave at 121°C for 15 minutes and allow to cool to 45 – 60 ^{o}C

Complete agar

Basal agar	1000ml
Haemin solution	10ml
Disodium carbonate solution	25ml
35% Hydrochloric acid (HCl)	2.5ml
Kanamycin monosulfate	1ml
Nalidixic acid	4ml

Note: Pour into 90mm diameter petri dishes and and store in the dark at 4 ^{o}C for not more than 1 month.

v. Semi-solid *Bacteroides* phage recovery medium agar (ssBPRMA)

Basal agar of BPRMA but with half mass of agar (6 – 10g) depending on gel strength. Before use, melt ssBPRMA and add all the additives.

Appendix 2

A: Detection of faecal indicator bacteria in *M. edulis* and their overlying waters

Year	Month	Overlying river water			Shellfish		
		E. coli	**Faecal coliforms**	**Intestinal enterococci**	***E. coli***	**Faecal coliforms**	**Intestinal enterococci**
2013	May	2.34	2.39	1.48	3.79	3.57	3.61
	June	1.55	1.56	1.18	2.74	2.90	2.47
	July	1.66	1.78	0.99	2.47	2.65	2.55
	August	2.22	2.25	1.43	3.46	3.54	2.81
	September	2.45	2.77	2.18	3.23	3.54	3.54
	October	2.07	2.23	1.88	2.90	3.04	2.23
	November	3.34	3.55	2.89	3.22	3.43	2.91
	December	2.81	3.14	2.18	3.42	3.72	3.43
2014	January	3.96	4.02	3.50	3.72	3.90	3.14
	February	4.00	4.16	2.76	3.62	3.72	2.52
	March	3.47	3.59	2.45	3.64	4.11	3.54
	April	2.73	3.03	2.75	3.75	4.20	3.56
	May	1.94	2.23	1.70	2.66	3.15	2.90
	June	2.00	2.11	1.69	2.80	3.12	2.29
	July	2.46	3.55	2.66	3.22	3.66	3.03
	August	3.41	3.69	3.19	3.79	4.14	3.43
	September	1.61	1.70	3.17	2.43	2.52	3.23
	October	2.67	2.92	2.90	3.75	3.85	3.22
	November	3.99	4.03	3.43	4.27	4.30	3.35
	December	3.59	3.63	3.01	4.20	4.23	3.23
2015	January	3.40	3.69	3.34	3.73	4.23	3.97
	February	3.01	3.21	3.04	3.68	3.87	3.46
	March	2.70	2.97	2.68	3.42	3.48	3.46
	April	2.61	2.82	2.62	2.95	3.55	2.58

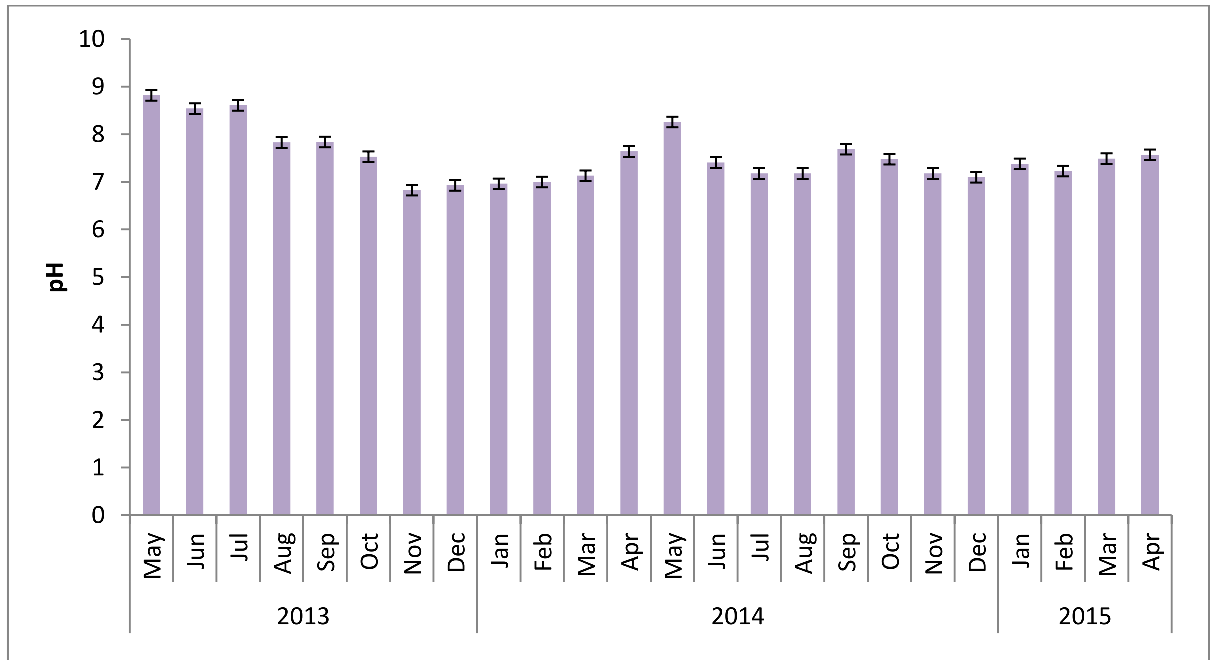
Key: Concentration of faecal indicator bacteria is given as – $\log_{10}$ most probable number per 100 g of shellfish flesh and intravalvular fluid; $\log_{10}$ colony-forming unit per 100 ml of water.

B: Detection of bacteriophages in *M. edulis* and their overlying waters

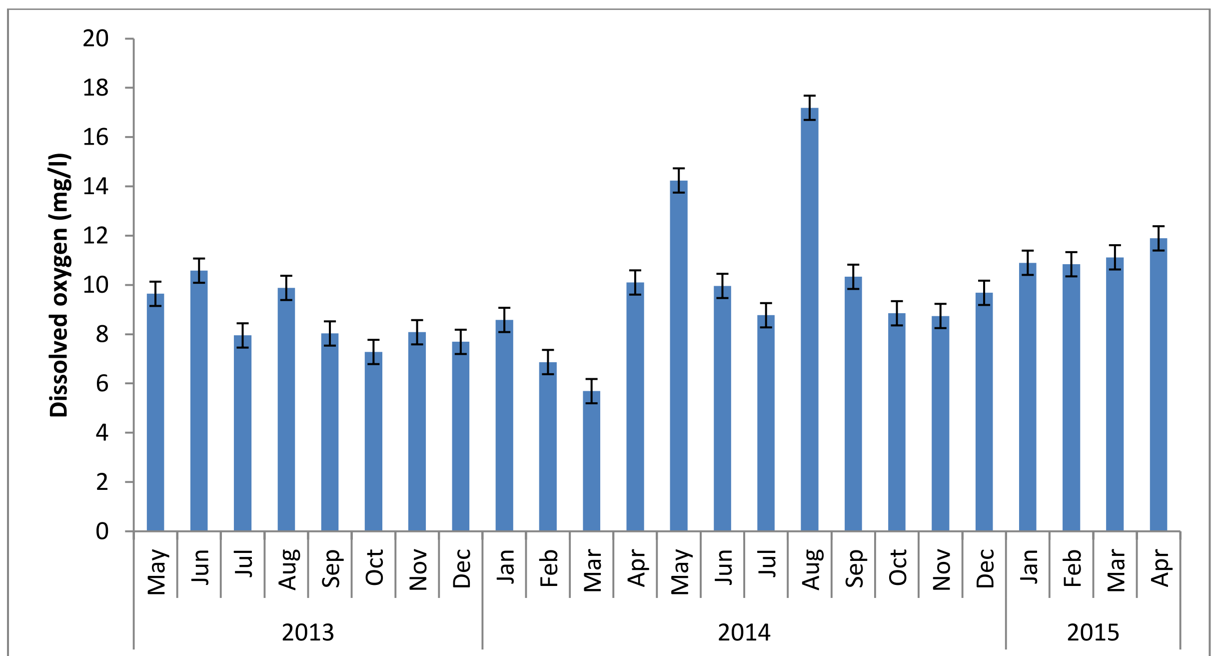
Year	Month	Overlying river water			Shellfish		
		B. fragilis GB124	Somatic coliphages	F-RNA coliphages	*B. fragilis* GB124	Somatic coliphages	F-RNA coliphages
2013	May	ND	2.39	2.30	ND	4.46	2.48
	June	2.00	2.30	ND	ND	4.27	3.18
	July	3.34	2.84	ND	5.29	4.49	3.65
	August	ND	2.30	ND	2.48	4.12	ND
	September	ND	2.90	ND	ND	4.34	ND
	October	ND	2.18	ND	ND	3.43	3.18
	November	2.00	4.02	2.09	2.48	5.30	3.13
	December	ND	3.38	ND	2.85	5.17	2.85
2014	January	3.35	3.62	2.00	2.78	5.20	3.48
	February	ND	3.33	2.18	ND	4.89	3.82
	March	ND	2.48	ND	3.48	4.52	2.48
	April	1.70	2.94	2.00	2.98	4.81	2.78
	May	ND	2.95	ND	ND	4.34	ND
	June	ND	3.10	ND	ND	4.71	3.38
	July	ND	3.08	ND	ND	3.75	ND
	August	ND	3.36	ND	3.26	5.36	2.78
	September	ND	2.00	ND	ND	4.53	ND
	October	ND	2.56	2.00	ND	3.89	3.56
	November	ND	3.54	1.70	2.78	4.84	3.38
	December	ND	3.59	ND	2.78	3.98	ND
2015	January	2.00	3.44	ND	3.38	5.23	ND
	February	ND	2.99	ND	2.48	4.95	ND
	March	ND	3.51	ND	2.78	5.04	ND
	April	1.70	3.44	ND	3.26	4.84	ND

Key: Concentration of bacteriophages is given as – $\log_{10}$ plaque-forming unit per 100 g of shellfish digestive gland or per 100 ml of water; 0.00 = Phage is below detection limit (≤ 1 PFU per ml or g)

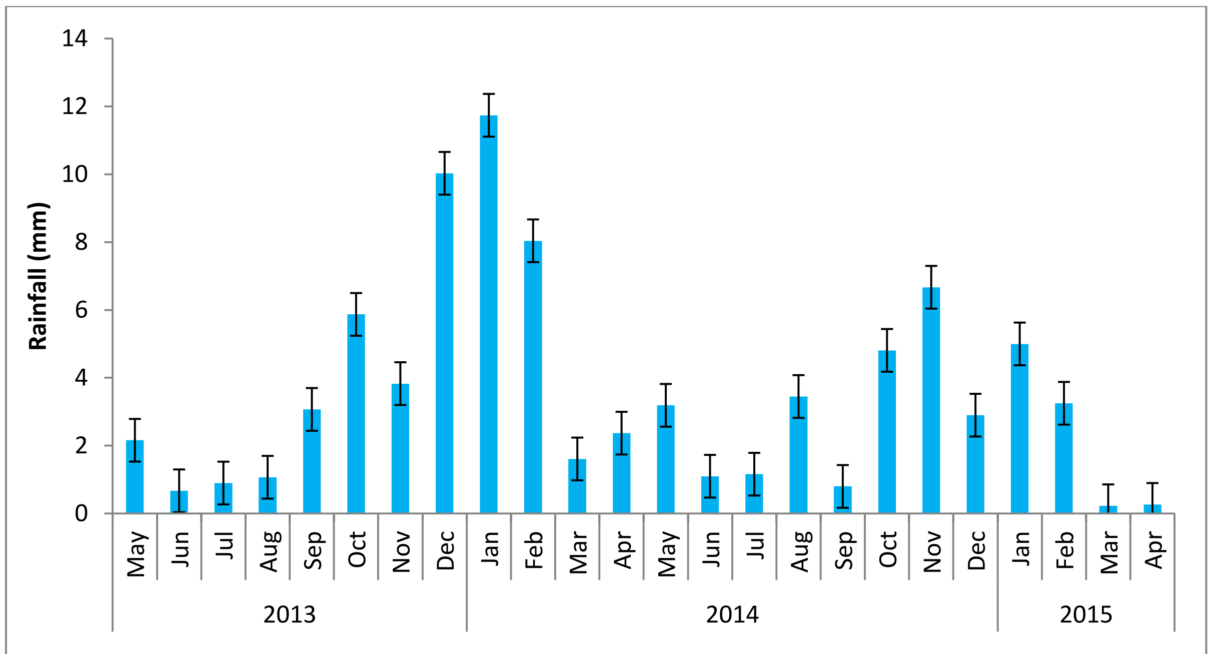
Appendix 3



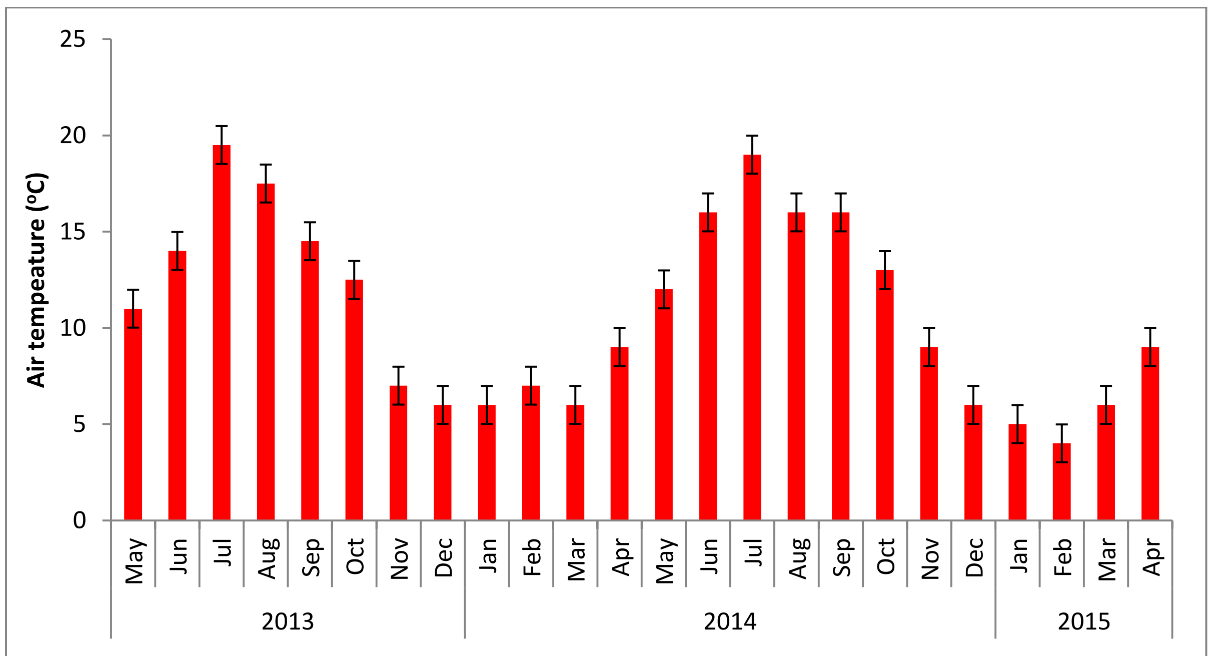
A: Monthly (n = 24, standard error) pH level of river water at the Piddinghoe sampling site during sampling period



B: Monthly (n = 24, standard error) dissolved oxygen (mg/l) in river water at the Piddinghoe sampling site during sampling period



C: Monthly (n = 24, standard error) rainfall (mm) at Piddinghoe during sampling period



D: Monthly (n = 24, standard error) air temperature (^{o}C) at Piddinghoe sampling site during sampling period

Appendix 4
Field-based study of bioaccumulation of bacterial and viral indicators in mussels

4.1 *E. coli*

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.436[a]	.190	.100	.24607

a. Predictors: (Constant), Salinity, Temp

$R^2 = 0.190$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.083	.207		5.233	.000
	Temp	.010	.016	.158	.596	.559
	Salinity	.019	.016	.322	1.213	.241

a. Dependent Variable: EC

E. coli = (0.010) Temp + (0.019) Salinity + 1.083

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.352[a]	.124	.078	.24911

a. Predictors: (Constant), Temp

$R^2 = 0.124$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.027	.204		5.028	.000
	Temp	.021	.013	.352	1.639	.118

a. Dependent Variable: EC

E. coli = (0.021) Temp + 1.027

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.417[a]	.174	.131	.24186

a. Predictors: (Constant), Salinity

$R^2 = 0.174$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.193	.095		12.592	.000
	Salinity	.024	.012	.417	2.002	.060

a. Dependent Variable: EC

E. coli = (0.024) Salinity + 1.193

Salinity ($R^2 = 0.174$) seems to be a better predictor of *E. coli* bioaccumulation in mussels (field study) than temperature ($R^2 = 0.124$)

4.2 Faecal coliforms

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.548[a]	.300	.222	.28851

a. Predictors: (Constant), Salinity, Temp

$R^2 = 0.300$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.915	.243		3.769	.001
	Temp	.016	.019	.204	.825	.420
	Salinity	.029	.018	.400	1.621	.122

a. Dependent Variable: FC

Faecal coliforms = (0.016) Temp + (0.029) Salinity + 0.915

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.445[a]	.198	.155	.30062

a. Predictors: (Constant), Temp

$R^2 = 0.198$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.827	.247		3.354	.003
	Temp	.034	.016	.445	2.163	.043

a. Dependent Variable: FC

Faecal coliforms = (0.034) Temp + 0.827

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.523[a]	.273	.235	.28608

a. Predictors: (Constant), Salinity

$R^2 = 0.273$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.092	.112		9.750	.000
	Salinity	.039	.014	.523	2.673	.015

a. Dependent Variable: FC

Faecal coliforms = (0.039) Salinity + 1.092

Salinity ($R^2 = 0.273$) seems to be a better predictor of faecal coliforms bioaccumulation in mussels (field study) than temperature ($R^2 = 0.198$)

4.3 Intestinal enterococci

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.309[a]	.095	-.005	.71749

a. Predictors: (Constant), Salinity, Temp

$R^2 = 0.095$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.859	.604		1.423	.172
	Temp	.033	.047	.195	.694	.496
	Salinity	.024	.045	.149	.532	.601

a. Dependent Variable: Ent

Intestinal enterococci = (0.033) Temp + (0.024) Salinity + 0.859

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.285[a]	.081	.033	.70383

a. Predictors: (Constant), Temp

$R^2 = 0.081$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.787	.577		1.364	.189
	Temp	.048	.037	.285	1.295	.211

a. Dependent Variable: Ent

Intestinal enterococci = (0.048) Temp + 0.787

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.267[a]	.071	.022	.70765

a. Predictors: (Constant), Salinity

$R^2 = 0.071$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.230	.277		4.439	.000
	Salinity	.043	.036	.267	1.206	.243

a. Dependent Variable: Ent

Intestinal enterococci = (0.043) Salinity + 1.230

Temperature ($R^2 = 0.081$) seems to be a better predictor of intestinal enterococci bioaccumulation in mussels (field study) than salinity ($R^2 = 0.071$)

4.4 GB124 phages

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.259[a]	.067	-.037	.68838

a. Predictors: (Constant), Salinity, Temp

$R^2 = 0.067$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.714	.579		1.232	.234
	Temp	-.010	.045	-.064	-.223	.826
	Salinity	-.033	.043	-.216	-.757	.459

a. Dependent Variable: GB124

GB124 phages = (-0.010) Temp + (-0.033) Salinity + 0.714

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.193[a]	.037	-.013	.68060

a. Predictors: (Constant), Temp

$R^2 = 0.037$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.812	.558		1.454	.162
	Temp	-.031	.036	-.193	-.859	.401

a. Dependent Variable: GB124

GB124 phages = (-0.031) Temp + 0.812

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.254[a]	.065	.015	.67095

a. Predictors: (Constant), Salinity

$R^2 = 0.065$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.599	.263		2.281	.034
	Salinity	-.039	.034	-.254	-1.145	.267

a. Dependent Variable: GB124

GB124 phages = (-0.039) Salinity + 0.599

Salinity ($R^2 = 0.065$) seems to be a better predictor of phages of GB124 bioaccumulation in mussels (field study) than temperature ($R^2 = 0.037$)

4. 5 Somatic coliphages

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.299[a]	.089	-.012	.25705

a. Predictors: (Constant), Salinity, Temp

$R^2 = 0.089$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.424	.216		6.583	.000
	Temp	.004	.017	.060	.214	.833
	Salinity	.015	.016	.258	.917	.371

a. Dependent Variable: SomCol

Somatic coliphages = (0.004) Temp + (0.015) Salinity + 1.424

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.216[a]	.047	-.004	.25598

a. Predictors: (Constant), Temp

$R^2 = 0.047$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.379	.210		6.572	.000
	Temp	.013	.013	.216	.963	.348

a. Dependent Variable: SomCol

Somatic coliphages = (0.013) Temp + 1.379

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.295[a]	.087	.039	.25051

a. Predictors: (Constant), Salinity

$R^2 = 0.087$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.465	.098		14.932	.000
	Salinity	.017	.013	.295	1.344	.195

a. Dependent Variable: SomCol

Somatic coliphages = (0.017) Salinity + 1.465

Salinity ($R^2 = 0.087$) seems to be a better predictor of somatic coliphage bioaccumulation in mussels (field study) than temperature ($R^2 = 0.047$)

4.6 F-RNA coliphages

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.451[a]	.203	.114	.74637

a. Predictors: (Constant), Salinity, Temp

$R^2 = 0.203$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.696	.628		2.700	.015
	Temp	-.067	.049	-.361	-1.369	.188
	Salinity	-.023	.047	-.129	-.491	.629

a. Dependent Variable: F-RNAphages

F-RNA coliphages = (-0.067) Temp + (-0.023) Salinity + 1.696

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.439[a]	.192	.150	.73131

a. Predictors: (Constant), Temp

$R^2 = 0.192$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.765	.600		2.943	.008
	Temp	-.082	.038	-.439	-2.127	.047

a. Dependent Variable: F-RNAphages

F-RNA coliphages = (-0.082) Temp + 1.765

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.346[a]	.120	.074	.76335

a. Predictors: (Constant), Salinity

$R^2 = 0.120$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.935	.299		3.127	.006
	Salinity	-.062	.038	-.346	-1.610	.124

a. Dependent Variable: F-RNAphages

F-RNA coliphages = (-0.062) Salinity + 0.935

Temperature ($R^2 = 0.192$) seems to be a better predictor of F-RNA coliphage bioaccumulation in mussels (field study) than salinity ($R^2 = 0.120$)

Appendix 5
Laboratory-based study of bioaccumulation of bacterial and viral indicators in mussels

5.1 *E. coli*

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.910[a]	.827	.655	.55188

a. Predictors: (Constant), Salinity, Temp

$R^2 = 0.827$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	5.155	.743		6.940	.020
	Temp	-.103	.034	-.896	-3.052	.093
	Salinity	.015	.028	.164	.557	.633

a. Dependent Variable: ECconc

E. coli = (- 0.103) Temp + (0.015) Salinity + 5.155

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.895[a]	.801	.734	.48433

a. Predictors: (Constant), Temp

$R^2 = 0.801$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	5.385	.541		9.945	.002
	Temp	-.103	.030	-.895	-3.472	.040

a. Dependent Variable: ECconc

E. coli = (- 0.103) Temp + 5.385

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.155[a]	.024	-.301	1.07181

a. Predictors: (Constant), Salinity

$R^2 = 0.024$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	3.441	.944		3.643	.036
	Salinity	.015	.054	.155	.272	.804

a. Dependent Variable: ECconc

E. coli = (0.015) Salinity + 3.441

Temperature ($R^2 = 0.801$) seems to be a better predictor of *E. coli* concentration in mussels (lab simulation) than salinity ($R^2 = 0.024$)

5.2 Faecal coliforms

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.925[a]	.855	.710	.50457

a. Predictors: (Constant), Salinity, Temp

$R^2 = 0.855$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	5.378	.679		7.919	.016
	Temp	-.105	.031	-.920	-3.419	.076
	Salinity	.009	.025	.101	.375	.743

a. Dependent Variable: FCconc

Faecal coliforms = (- 0.105) Temp + (0.009) Salinity + 5.378

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.919[a]	.845	.793	.42625

a. Predictors: (Constant), Temp

$R^2 = 0.845$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	5.520	.477		11.583	.001
	Temp	-.105	.026	-.919	-4.043	.027

a. Dependent Variable: FCconc

Faecal coliforms = (- 0.105) Temp + 5.520

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.092[a]	.008	-.322	1.07789

a. Predictors: (Constant), Salinity

$R^2 = 0.008$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	3.623	.950		3.814	.032
	Salinity	.009	.054	.092	.160	.883

a. Dependent Variable: FCconc

Faecal coliforms = (0.009) Salinity + 3.623

Temperature ($R^2 = 0.845$) seems to be a better predictor of faecal coliforms concentration in mussels (lab simulation) than salinity ($R^2 = 0.008$)

5.3 Intestinal enterococci

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.898[a]	.806	.612	.30412

a. Predictors: (Constant), Salinity, Temp

$R^2 = 0.806$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	3.858	.409		9.426	.011
	Temp	-.053	.019	-.882	-2.833	.105
	Salinity	-.008	.015	-.158	-.507	.662

a. Dependent Variable: Ent

Intestinal enterococci = (- 0.053) Temp + (- 0.008) Salinity + 3.858

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.884[a]	.781	.708	.26381

a. Predictors: (Constant), Temp

$R^2 = 0.781$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	3.742	.295		12.688	.001
	Temp	-.053	.016	-.884	-3.272	.047

a. Dependent Variable: Ent

Intestinal enterococci = (- 0.053) Temp + 3.742

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.167[a]	.028	-.296	.55600

a. Predictors: (Constant), Salinity

$R^2 = 0.028$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	2.982	.490		6.086	.009
	Salinity	-.008	.028	-.167	-.293	.789

a. Dependent Variable: Ent

Intestinal enterococci = (- 0.008) Salinity + 2.982

Temperature ($R^2 = 0.781$) seems to be a better predictor of intestinal enterococci concentration in mussels (lab simulation) than salinity ($R^2 = 0.028$)

5.4 GB124 phages

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.936[a]	.875	.751	.59379

a. Predictors: (Constant), Salinity, Temp

$R^2 = 0.875$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-.977	.799		-1.223	.346
	Temp	.136	.036	.936	3.749	.064
	Salinity	-.001	.030	-.010	-.040	.972

a. Dependent Variable: GB124

GB124 phages = (0.136) Temp + (- 0.001) Salinity – 0.977

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.936[a]	.875	.834	.48502

a. Predictors: (Constant), Temp

$R^2 = 0.875$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-.995	.542		-1.835	.164
	Temp	.136	.030	.936	4.590	.019

a. Dependent Variable: GB124

GB124 phages = (0.136) Temp – 0.995

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.001[a]	.000	-.333	1.37367

a. Predictors: (Constant), Salinity

$R^2 = 0.000$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.287	1.210		1.064	.366
	Salinity	-8.982E-005	.069	-.001	-.001	.999

a. Dependent Variable: GB124

GB124 phages = (-8.982E-005) Salinity + 1.287

Temperature ($R^2 = 0.875$) seems to be a better predictor of GB124 phages concentration in mussels (lab simulation) than salinity ($R^2 = 0.000$)

5.5 Somatic coliphages

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.944[a]	.891	.782	.06666

a. Predictors: (Constant), Salinity, Temp

$R^2 = 0.891$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	4.124	.090		45.970	.000
	Temp	-.016	.004	-.942	-4.036	.056
	Salinity	.001	.003	.061	.263	.817

a. Dependent Variable: SC

Somatic coliphage = (-0.016) Temp + (0.001) Salinity + 4.124

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.942[a]	.887	.850	.05536

a. Predictors: (Constant), Temp

$R^2 = 0.887$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	4.138	.062		66.848	.000
	Temp	-.016	.003	-.942	-4.857	.017

a. Dependent Variable: SC

Somatic coliphage = (-0.016) Temp + 4.138

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.052[a]	.003	-.330	.16458

a. Predictors: (Constant), Salinity

$R^2 = 0.003$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	3.851	.145		26.553	.000
	Salinity	.001	.008	.052	.090	.934

a. Dependent Variable: SC

Somatic coliphage = (0.001) Salinity + 3.851

Temperature ($R^2 = 0.887$) seems to be a better predictor of somatic coliphage concentration in mussels (lab simulation) than salinity ($R^2 = 0.003$)

5.6 F-RNA coliphages

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.960[a]	.922	.844	.36132

a. Predictors: (Constant), Salinity, Temp

$R^2 = 0.922$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.222	.486		.456	.693
	Temp	.104	.022	.936	4.737	.042
	Salinity	.019	.018	.205	1.036	.409

a. Dependent Variable: F-RNAphage

F-RNA coliphage = (0.104) Temp + (0.019) Salinity + 0.222

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.938[a]	.880	.840	.36573

a. Predictors: (Constant), Temp

$R^2 = 0.880$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.503	.409		1.229	.307
	Temp	.105	.022	.938	4.690	.018

a. Dependent Variable: F-RNAphage

F-RNA coliphage = (0.105) Temp + 0.503

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.214[a]	.046	-.272	1.03119

a. Predictors: (Constant), Salinity

$R^2 = 0.046$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.963	.909		2.160	.120
	Salinity	.020	.052	.214	.379	.730

a. Dependent Variable: F-RNAphage

F-RNA coliphage = (0.020) Salinity + 1.963

Temperature ($R^2 = 0.880$) seems to be a better predictor of F-RNA coliphage concentration in mussels (lab simulation) than salinity ($R^2 = 0.046$)

Appendix 6
Laboratory-based study of bioaccumulation of bacterial and viral indicators in oysters

6.1 *E. coli*

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.220[a]	.048	-.038	.65718

a. Predictors: (Constant), Sal, Temp

$R^2 = 0.048$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	4.793	.396		12.116	.000
	Temp	.001	.018	.010	.049	.962
	Sal	.015	.015	.219	1.054	.303

a. Dependent Variable: EC

E. coli = (0.001) Temp + (0.015) Salinity + 4.793

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.012[a]	.000	-.043	.65878

a. Predictors: (Constant), Temp

$R^2 = 0.000$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	5.025	.329		15.255	.000
	Temp	.001	.018	.012	.059	.953

a. Dependent Variable: EC

E. coli = (0.001) Temp + 5.025

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.219[a]	.048	.007	.64277

a. Predictors: (Constant), Sal

$R^2 = 0.048$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	4.807	.253		18.979	.000
	Sal	.015	.014	.219	1.079	.292

a. Dependent Variable: EC

E. coli = (0.015) Salinity + 4.807

6.2 Faecal coliforms

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.225[a]	.051	-.036	.53300

a. Predictors: (Constant), Sal, Temp

$R^2 = 0.051$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	4.668	.321		14.550	.000
	Temp	.006	.015	.084	.404	.690
	Sal	.012	.012	.208	1.001	.328

a. Dependent Variable: FC

Faecal coliforms = (0.006) Temp + (0.012) Salinity + 4.668

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.086[a]	.007	-.036	.53302

a. Predictors: (Constant), Temp

$R^2 = 0.007$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	4.847	.267		18.185	.000
	Temp	.006	.015	.086	.414	.683

a. Dependent Variable: FC

Faecal coliforms = (0.006) Temp + 4.847

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.209[a]	.044	.002	.52321

a. Predictors: (Constant), Sal

$R^2 = 0.044$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	4.766	.206		23.114	.000
	Sal	.012	.012	.209	1.024	.317

a. Dependent Variable: FC

Faecal coliforms = (0.012) Salinity + 4.766

6.3 Intestinal enterococci

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.405[a]	.164	.088	.37914

a. Predictors: (Constant), Sal, Temp

$R^2 = 0.164$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	3.267	.228		14.314	.000
	Temp	-.021	.010	-.388	-1.990	.059
	Sal	-.005	.008	-.111	-.571	.574

a. Dependent Variable: IE

Intestinal enterococci = (-0.021) Temp - (0.005) Salinity + 3.267

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.389[a]	.151	.115	.37354

a. Predictors: (Constant), Temp

$R^2 = 0.151$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	3.194	.187		17.101	.000
	Temp	-.021	.010	-.389	-2.026	.055

a. Dependent Variable: IE

Intestinal enterococci = (-0.021) Temp + 3.194

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.115[a]	.013	-.030	.40281

a. Predictors: (Constant), Sal

$R^2 = 0.013$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	2.923	.159		18.416	.000
	Sal	-.005	.009	-.115	-.555	.584

a. Dependent Variable: IE

Intestinal enterococci = (-0.005) Salinity + 2.923

6.4 GB124 phages

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.170[a]	.029	-.059	1.04018

a. Predictors: (Constant), Sal, Temp

$R^2 = 0.029$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-.106	.626		-.169	.867
	Temp	.016	.028	.115	.550	.588
	Sal	.014	.023	.124	.591	.560

a. Dependent Variable: GB

GB124 phages = (0.016) Temp + (0.014) Salinity - 0.106

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.117[a]	.014	-.029	1.02536

a. Predictors: (Constant), Temp

$R^2 = 0.014$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.100	.513		.195	.847
	Temp	.016	.028	.117	.564	.579

a. Dependent Variable: GB

GB124 phages = (0.016) Temp + 0.100

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.125[a]	.016	-.027	1.02428

a. Predictors: (Constant), Sal

$R^2 = 0.016$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.154	.404		.382	.706
	Sal	.014	.023	.125	.606	.551

a. Dependent Variable: GB

GB124 phages = (0.014) Salinity + 0.154

6.5 Somatic coliphages

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.157[a]	.025	-.064	1.52490

a. Predictors: (Constant), Sal, Temp

$R^2 = 0.025$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.497	.918		1.631	.117
	Temp	.004	.042	.020	.095	.925
	Sal	-.025	.034	-.156	-.741	.466

a. Dependent Variable: SC

Somatic coliphages = (0.004) Temp –(0.025) Salinity + 1.497

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.018[a]	.000	-.043	1.50989

a. Predictors: (Constant), Temp

$R^2 = 0.000$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.118	.755		1.481	.152
	Temp	.004	.041	.018	.088	.930

a. Dependent Variable: SC

Somatic coliphages = (0.004) Temp + 1.118

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.156[a]	.024	-.018	1.49169

a. Predictors: (Constant), Sal

$R^2 = 0.024$

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.563	.588		2.658	.014
	Sal	-.025	.033	-.156	-.757	.457

a. Dependent Variable: SC

Somatic coliphages = (-0.025) Temp + 1.563

Appendix 7

A: Detection of enteric viruses in *M. edulis* and their overlying waters

Year	Month	Overlying river water					Shellfish				
		NoV GI	**NoV GII**	**Total NoV**	**HAV**	**AdV F&G**	**NoV GI**	**NoV GII**	**Total NoV**	**HAV**	**AdV F&G**
2013	May	ND	ND	ND	ND	ND	ND	0.23	0.23	ND	ND
	June	ND	0.19	0.19	ND	ND	ND	ND	ND	ND	ND
	July	ND	ND	ND	ND	0.30	ND	1.15	1.15	ND	0.51
	August	ND	ND	ND	ND	ND	0.06	ND	0.06	ND	ND
	September	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	October	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	November	ND	0.98	0.98	ND	0.36	ND	1.11	1.11	ND	0.66
	December	1.09	ND	1.09	ND	0.48	1.15	ND	1.15	ND	0.54
2014	January	ND	0.90	0.90	ND	0.29	ND	2.17	2.17	ND	0.45
	February	ND	0.47	0.47	ND	0.18	ND	ND	ND	ND	ND
	March	ND	ND	ND	ND	ND	ND	1.18	1.18	ND	ND
	April	0.24	ND	0.24	ND	0.09	ND	0.70	0.70	ND	0.27
	May	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	June	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.40
	July	ND	0.78	0.78	ND	ND	ND	ND	ND	ND	ND
	August	ND	0.90	0.90	ND	ND	ND	2.92	2.92	ND	ND
	September	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	October	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	November	ND	2.77	2.77	ND	0.78	ND	2.89	2.89	ND	ND
	December	ND	1.72	1.72	ND	0.95	ND	1.40	1.40	ND	ND
2015	January	ND	1	1	ND	1.34	ND	0.70	0.70	ND	2.94
	February	ND	ND	ND	ND	0.30	ND	2.78	2.78	ND	2.94
	March	ND	ND	ND	ND	ND	ND	1.31	1.31	ND	ND
	April	ND	ND	ND	ND	0.22	ND	ND	ND	ND	0.71

Key: Concentration of viral pathogen is given as Log_{10} detectable virus genome copies /100 ml overlying waters or 100 g digestive gland; ND = Non detect i.e. virus is below detection limit (≤ 10 detectable virus genome copies per 100 ml or 100 g)

B: Detection of bacteriophages in *M. edulis* and *C. gigas* at selected coastal site in southern England

Year	Month	*M. edulis* (Poole Harbour)			*C. gigas* (Poole Harbour)			*C. gigas* (Fleet)		
		GB124 phages	**Somatic coliphage**	**F-RNA phages**	**GB124 phages**	**Somatic coliphage**	**F-RNA phages**	**GB124 phages**	**Somatic coliphage**	**F-RNA phages**
2013	Jan	ND	4.71	3.62	ND	3.82	ND	ND	3.89	3.38
	Feb	2.78	4.56	ND	3.08	4.56	ND	2.78	3.56	3.08
	Mar	3.08	4.69	3.62	2.78	4.38	3.38	ND	3.62	3.26
	Apr	ND	ND	ND	2.78	3.08	ND	2.78	3.26	3.38
	May	ND	3.86	2.78	ND	3.08	ND	ND	ND	2.78
	Jun	-	-	-	-	-	-	-	-	-
	Jul	ND	2.48	ND	ND	2.48	ND	ND	ND	ND
	Aug	-	-	-	ND	2.78	ND	ND	2.95	ND
	Sep	-	-	-	-	-	-	-	-	-
	Oct	ND	4.33	ND	ND	3.82	ND	ND	2.48	ND
	Nov	ND	3.76	ND	ND	3.73	ND	ND	3.30	ND
	Dec	ND	3.64	ND	ND	4.02	ND	ND	3.32	ND
2014	Jan	ND	4.32	ND	ND	4.03	ND	ND	3.84	ND
	Feb	ND	2.78	ND	ND	3.26	ND	ND	2.78	ND
	Mar	ND	2.78	ND	ND	3.68	ND	ND	3.08	ND
	Apr	ND	3.56	ND	ND	3.08	ND	-	-	-
	May	ND	2.48	ND	2.48	2.95	ND	ND	2.48	ND
	Jun	ND	2.95	ND	ND	2.48	ND	ND	ND	ND
	Jul	ND	2.78	ND	ND	2.48	2.48	ND	ND	ND
	Aug	ND	ND	ND	ND	2.78	ND	ND	2.78	ND
	Sep	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Oct	ND	ND	ND	ND	2.95	ND	ND	ND	ND
	Nov	-	-	-	ND	3.91	ND	ND	2.78	ND
	Dec	ND	4.10	ND	ND	3.52	ND	ND	2.78	ND
2015	Jan	ND	3.62	ND	ND	3.82	ND	ND	ND	ND
	Feb	ND	4.36	ND	ND	4.68	ND	ND	3.92	ND

Key: Concentration of bacteriophages is given as Log_{10} PFU/100 g; ND = Non detect i.e. phage is below detection limit (≤ 1 PFU per g).

C: Detection of norovirus in *M. edulis* and *C. gigas* at selected coastal site in southern England

Year	Month	*M. edulis* (Poole Harbour)		*C. gigas* (Poole Harbour)		*C. gigas* (Fleet)	
		NoV GI	**NoV GII**	**NoV GI**	**NoV GII**	**NoV GI**	**NoV GII**
2013	Jan	3.11	3.24	2.36	3.20	1.30	1.30
	Feb	3.30	3.28	2.52	2.76	1.30	1.30
	Mar	2.60	4.16	1.70	3.56	1.30	2.82
	Apr	1.30	2.91	1.30	2.36	1.30	2.71
	May	2.30	2.44	1.30	1.30	1.30	1.30
	Jun	1.30	1.30	1.30	1.30	1.30	1.30
	Jul	1.30	1.30	1.30	1.30	1.30	1.30
	Aug	1.30	1.30	1.30	1.30	1.30	1.30
	Sep	1.30	1.70	1.70	1.30	1.30	1.30
	Oct	1.70	1.70	1.70	1.70	1.30	1.30
	Nov	2.42	1.70	1.70	1.70	1.30	1.30
	Dec	2.20	1.70	1.30	1.70	1.30	1.30
2014	Jan	2.71	2.93	2.04	2.30	1.70	1.70

Key: Concentration of viral pathogen is given as Log_{10} detectable virus genome copies /100 g digestive gland; ND = Non detect i.e. virus is below detection limit (≤ 10 detectable virus genome copies per 100 g)