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Contamination of shellfish waters with human noroviruses: environmental risk factors and management options

by

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A thesis submitted in fulfilment for the

degree of Doctor of Philosophy

in the Department of Geography and Earth Sciences

September 2016

Dedicado a minha ménoma Célia

Aberystwyth University, Department of Geography and Earth Sciences

Abstract

Contamination of shellfish waters with human noroviruses: environmental risk factors and management options

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This thesis reports research undertaken to better understand the factors that influence norovirus (NoV) contamination in shellfish production areas (SPAs). This knowledge is necessary to develop control measures for mitigating risk from NoV contamination in SPAs. Predictive models were developed for concentrations of NoV (as measured by the reverse transcription polymerase chain reaction method) and *Escherichia coli* (as measured by the culture method) in shellfish from 31 harvesting sites and climatic, hydrometric, demographic and pollution source-related characteristics of upstream river catchments. Concentrations of NoV in shellfish increased as water temperature decreased and volume of sewage discharges and river flows increased. Concentrations of *E. coli* increased as rainfall also increased. Field studies were conducted in an estuary and in a coastal embayment to inform risk management measures for these sites. Concentrations of NoV and *E. coli* and their removal efficiencies were quantified in effluents from primary, secondary and tertiary treatments at four sewage treatment works. Shellfish were placed at different distances downstream of sewage discharges and tested for NoV and *E. coli*. Dye tracing and drogue tracking studies were conducted to quantify the dispersion and dilution of sewage effluents. Significant NoV reductions were found as sewage treatment levels increased. Activated sludge was more effective in reducing NoV than trickling filters. Norovirus was frequently detected in sewage effluents and in shellfish. It was found that a NoV limit for shellfish established at 100 copies/g would have a high compliance impact on SPAs. The dispersive characteristics of the dye-tagged effluents were consistent with the variation of NoV contamination in the study sites. A buffer zone established at 1,000:1 dilution of estuarine water to treated effluent would afford a high level of public health protection while a buffer zone established at much lower dilution ratios (e.g. 300:1) would represent a much higher health risk.

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Glossary

Activated sludge   A flocculent microbial mass of bacteria, protozoa and other microorganisms with a significant proportion of inert debris, produced when sewage is continuously aerated.

Amplicon   Portion of DNA or RNA that is the source and/or product of natural or artificial amplification or replication events

Bivalve shellfish   Marine or freshwater molluscs of the class Pelecypoda (formerly Bivalvia or Lamellibranchia) with a laterally compressed body, a shell consisting of two hinged valves, and gills for respiration. The group includes clams, cockles, oysters and mussels.

Buffer zone   An area classified as prohibited to shellfish harvesting for human consumption, normally established adjacent to sewage treatment plant outfalls or other point sources of sewage pollution. The designation of buffer zones is a measure principally aimed at protection against contamination of shellfish with human enteric viruses.

Capsid   Protein coat that surrounds and protects the genome of the virus.

Combined sewer overflow   Combined sewer systems are wastewater collection systems designed to carry sanitary sewage and stormwater in a single piping system to a sewage treatment works. In periods of rainfall, sewage flows can exceed the capacity of the sewer collection systems and STW. When this occurs, the combined sewer system is designed to overflow directly to nearby rivers and estuaries, discharging untreated sewage and stormwater.

Depuration   Process by which shellfish are held in tanks of clean seawater under conditions which maximise the natural filtering activity which results in expulsion of intestinal contents and enhances separation of the expelled contaminants from the shellfish.

Drogue   Instrument used to measure surface water currents. It consists of a surface buoy which keeps the drogue near its required depth and provides a marker which can be located and tracked.

Escherichia coli (*E. coli*)   A species of bacterium that is a member of the faecal coliform group. It is more specifically associated with the intestines of warm-blooded animals and birds than other members of the faecal coliform group. Traditionally, *E. coli* produce indole from tryptophan at 44 °C.

Fluorometer   Instrument used to measure parameters of fluorescence. In this study, WetLabs fluorometers were used to measure Rhodamine dye fluorescence units at a sensitivity of 0.03 ppb.

Phylogenetics   Study of the evolutionary history and relationships among individuals or groups of organisms.

Gastroenteritis   Inflammation of the stomach and intestines caused by pathogenic viruses or bacteria.

Genogroup   Group of related viruses within a genus which may be further subdivided into genetic clusters.

Geometric mean   Mean of n positive numbers obtained by taking the nth root of the product of the numbers

Grit   The heavy mineral matter in sewage, such as silt, sand, gravel, cinders, ashes, metal and glass. It is abrasive in character and may vary in composition seasonally.

Humus tank   A secondary settlement stage succeeding the percolating filter process. Named after the nature of the humus solids separated.

Hypokalaemia   Low concentration of potassium in the blood.

Limit of quantification   Lowest concentration in a test sample that can be quantitatively determined with acceptable level of precision and accuracy under the experimental conditions specified in the method.

Lower super output area   A set of geographical areas of consistent size, whose boundaries would not change, used by the Office for National Statistics for the publication of neighbourhood statistics. Lower super output areas are the lowest level of output area (typically 4–6) with a population of around 1,500.

Norovirus	Formerly called Small Round Structured Viruses and Norwalk-like viruses. Small, structured RNA viruses which have been implicated as the most common cause of non-bacterial gastroenteritis outbreaks.
Population equivalent	The volume and strength of a wastewater expressed in terms of an equivalent population, assuming a production of 0.06 kg biochemical oxygen demand per capita per day.
Primary treatment	The first major stage of treatment following preliminary treatment in a sewage works, usually involving removal of settleable solids.
Primary settlement	Used prior to biological treatment to reduce the velocity of flow of the wastewater such that a proportion of suspended matter settles out.
Production area	Any estuarine or marine area containing either natural beds of bivalve shellfish or sites used for the cultivation of bivalve shellfish, and from which these shellfish are taken for human consumption
Roughing filter	A wastewater treatment method that reduces the quantity of suspended matter in wastewater prior to other treatment processes. It also contributes to the removal of microorganisms.
Sand filter	A wastewater treatment method consisting of two or three chambers or basins. The first is the sedimentation chamber, which removes floatables and heavy sediments. The second is the filtration chamber, which removes additional pollutants by filtering the runoff through a sand bed. The third is the discharge chamber.
Sanitary survey	An evaluation of the sources of faecal contamination in or near a shellfish production area together with an assessment of the potential impact of these sources on the microbiological status of the production area.
Secondary treatment	The second major stage of treatment. It removes the dissolved organic matter that escapes primary treatment. This is achieved by microbes consuming the organic matter as food, and converting it to carbon dioxide, water, and energy for their own growth and reproduction. The biological process is then followed by additional settling tanks to remove more of the suspended solids.
Storm tank	Tank that provides storage and sedimentation for excess flows entering STW as a result of storm events.
Stormwater	Stormwater runoff is generated when precipitation from rain and snowmelt events flows over land or impervious surfaces and does not percolate into the ground. As the runoff flows over land, it accumulates contaminants that could adversely affect water quality if the runoff is discharged untreated.
Shellfish production area	In the context of this thesis, any sea and estuarine area containing either natural beds of bivalve shellfish or sites used for the cultivation of bivalve molluscs, and from which bivalve shellfish are taken for human consumption.
Study catchment	In the context of this thesis, the portion of land draining to a study site.
Study site	In the context of this thesis, the body of water where shellfish sampling stations were established and with one or more shellfish production area(s).
Tertiary treatment	The third major stage of treatment. It is used to further reduce parameter values below the standards set out in national legislation. The term is often used in relation to nutrient removal. It includes UV disinfection, membrane bioreactors and other treatment systems.
Trickling filter (also called percolating filter)	A biological wastewater treatment method using an immobile support medium for the growth of a bacterial slime that consumes organic matter under aerobic conditions.
Ultra-violet disinfection	A type of sewage treatment aimed at destroying disease bearing microorganisms or pathogens using ultra-violet light. The UV light is produced by special mercury discharge lamps. Its effectiveness depends on the dose received by the microorganisms.

Abbreviations

ADCP	Acoustic Doppler current profiler
ANOVA	Analysis of variance
AOD	Above ordnance datum
Av	Average
CAMP	Conditional area management plan
CD	Chart datum
cfu	Colony forming units
CI	Confidence interval
E&W	England and Wales
EFSA	European Food Safety Authority
FIO	Faecal indicator organism
FSA	Food Standards Agency
GI	Genogroup I
GII	Genogroup II
GM	Geometric mean
GPS	Global positioning system
HW	High water
LoD	Limit of detection
log	Logarithm
LoQ	Limit of quantification
LSO	Long sea outfall
LW	Low water
Max	Maximum
MSC	Male specific coliphage
Min	Minimum
MPN	Most probable number
n	Number of samples
NoV	Norovirus
NSSP	National Shellfish Sanitation Program
PE	Population equivalent
SO	Storm overflow
pfu	Plaque forming unit
ppb	Parts per billion
PVC	Polyvinyl chloride
RT-PCR	Reverse transcription polymerase chain reaction
SPA	Shellfish production area
StDev	Standard deviation
STW	Sewage treatment works
SWPA	Shellfish water protected area
UV	Ultra-violet
UWWTD	Urban Wastewater Treatment Directive
WFD	Water Framework Directive

Chapter 1

Research Context

"However, unlike other forms of food poisoning, bacterial cases of gastroenteritis are rarely linked to molluscan shellfish consumption. It is now widely accepted that the clinical symptoms reported in the majority of outbreaks of unknown cause are consistent with the epidemiological criteria for viral gastroenteritis, such as caused by Norwalk-like viruses, and therefore that these agents probably constitute the bulk of the disease problem both in the UK, the USA and elsewhere." Lees (2000, p. 95)

1.1 Rationale

Bivalve shellfish (clams, cockles, oysters, mussels) feed by filtering large volumes of water in which they grow and reproduce. They "pump" hundreds of litres of water per day into their mantle cavity and extract from it the particles that they use as food (Galtsoff, 1964). If pathogenic bacteria and viruses are present in the waters, shellfish accumulate these pathogens in their tissues to levels considerably greater than those in the overlying waters. When consumed raw or lightly cooked, contaminated shellfish can cause illness of variable severity and duration (Butt *et al.*, 2004). Human health problems associated with the consumption of contaminated shellfish have been recognised for many decades (Dodgson, 1928; Fisher *et al.*, 1936; Rippey, 1994; Lees, 2000). In many parts of the world, most cases of shellfish-related illness have an unidentified aetiology (Rippey, 1994; Graczyk *et al.*, 2010). In the past, a large proportion of illness cases were typhoid and gastroenteritis of bacterial origin (Fisher *et al.*, 1936; Rippey, 1994). Currently, epidemiological surveillance systems in the developed world demonstrate that illness cases of known aetiology are predominantly caused by human norovirus (NoV) (Lipp and Rose, 1997; Lees, 2000) (Figure 1A).

Noroviruses, previously known as "small round structured viruses" or "Norwalk-like viruses" (family Caliciviridae, genus Norovirus; International Committee on Taxonomy of Viruses, 2013) were firstly discovered by Albert Kapikian in Norwalk, USA in 1972 (Kapikian *et al.*, 1972). This group of viruses comprises seven valid genogroups (Figure 1B). The strains that infect humans (referred to collectively as "human noroviruses") are found in genogroup I (GI), genogroup II (GII), and genogroup IV. Morphologically, NoV

are non-enveloped, icosahedral viruses with a diameter of approximately 38 nm (Donaldson *et al.*, 2010). The virus genome is a 7.5 kb, positive-sense, single stranded RNA containing three open reading frames which encode both structural and non-structural proteins (Figure 1C).
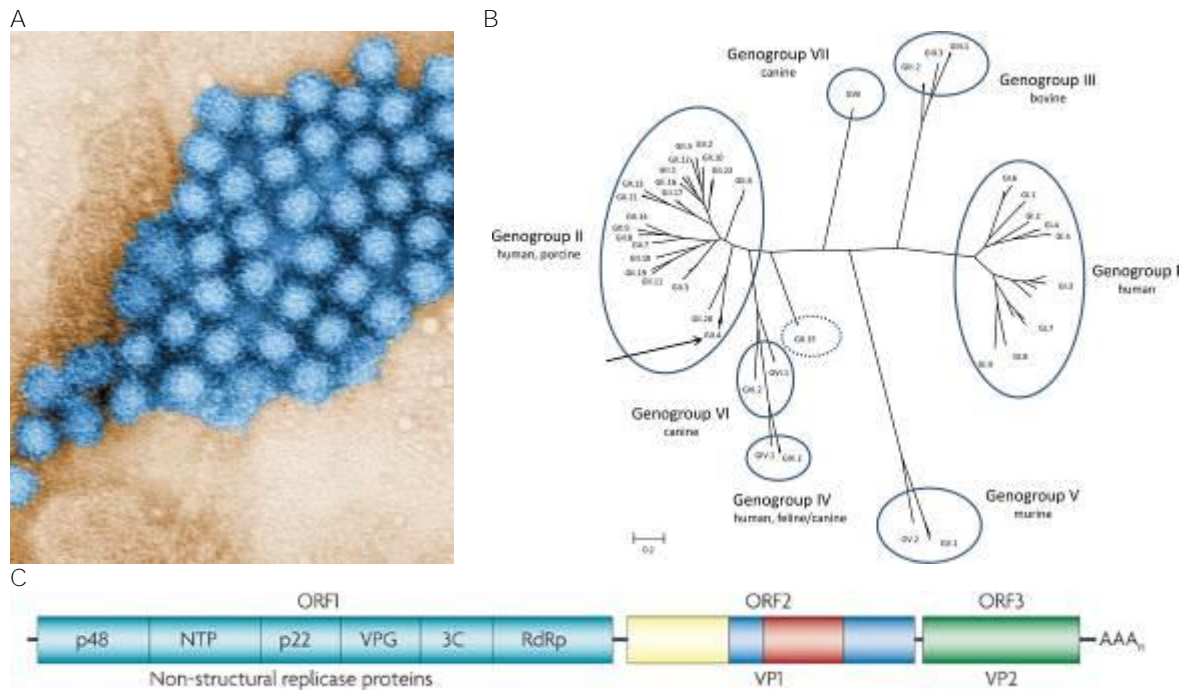
Figure 1 Transmission electron micrograph (A), phylogenetic relationships and nomenclature (B) and genome (C) of norovirus.
A - Source: Centers for Disease Control and Prevention, Public Health Image Library.
B - Phylogenetic tree based on capsid sequences from 105 strains representing the diversity of noroviruses. Viruses belonging to GI, GII, and GIV infect humans, except GII.11, GII.18, and GII.19 viruses, which infect porcine species, and GIV.2 viruses, which infect canine species. GII.15 viruses, which have been detected only in humans, form a tentative new genogroups (dotted circle). GIII viruses infect cows and sheep, GIV.2 infects canines, GV.1 and GV.2 infect mice and rats, respectively, and GVI and GVII infect canine species. GII.4 viruses (arrow) are responsible for most norovirus infections worldwide. Modified from Vinjé (2015).
C - The genome is composed of three open reading frames (ORFs). ORF1 encodes a protein that is auto-processed by a protease (3C) to yield the non-structural proteins that are essential for viral replication. The resultant proteins are: p48, an amino-terminal protein; nucleoside triphosphatase (NTP), a 2C-like protein; p22, a 3A-like protein; viral genome-linked protein (VPG); and RNA-directed RNA polymerase (RdRp). ORF2 encodes the main structural capsid protein VP1. VP1 is divided into two domains, the shell domain (yellow) and the protruding domain, which is further divided into two subdomains known as P1 (blue) and P2 (red). ORF3 encodes VP2. Modified from Donaldson *et al.* (2010).

Norovirus is a category B pathogen under the classification criteria used by the US National Institute of Allergy and Infectious Diseases (Li *et al.*, 2012). These viruses have all the characteristics of an "ideal" infectious agent (highly contagious; shed in large quantities over long periods of time; constantly evolving; resistant in the environment outside the host; multiple transmission routes) which enable them to maintain a large

pool of susceptible populations (Hall, 2012). A summary of the main epidemiological characteristics of human NoV is shown in Table 1.

Table 1 Epidemiological characteristics of human norovirus.

Characteristic	Evidence	References
Human infection	The stool of an infected individual may shed up to 100 billion viral copies/g of faeces. The average period of NoV excretion from infected people living in aged-care facilities is 2 weeks	Lee *et al.* (2007); Atmar *et al.* (2008); Aoki *et al.* (2010)
Human susceptibility	NoV can infect people of all ages. Individuals with type B histo-blood group antigens are protected against infection with NoV GI. The number of NoV-associated deaths in adults (> 65 years) increases as a result of higher number of infected people, but not increased virulence	Rockx *et al.* (2005); Harris *et al.* (2008)
Duration of asymptomatic infection	NoV can be detected in faecal samples for at least 1 month after resolution of symptoms. Children and immuno-compromised individuals usually shed virus for longer periods than healthy adults	Amar *et al.* (2007); Atmar *et al.* (2008); Furuya *et al.* (2011); Parra and Green (2014)
Human immunity	Immunity to NoV ranges from months to years. Infected persons are susceptible to re-infection with the same strain as well as with heterologous strains. Young children (< 5 years old) are more infectious than older children and adults	Johnson *et al.* (1990); Lysén *et al.* (2009); Debbink *et al.* (2013); Simmons *et al.* (2013)
Infectious dose (ID)	Very low concentrations may cause human infection. The ID_{50} for non-aggregated GI NoV can be as low as 18 viral particles.	Lindesmith *et al.* (2003); Amar *et al.* (2007); Teunis *et al.* (2008)
Environmental stability and resistance	NoV are resistant to many forms of chemical disinfection and can survive freezing and heating conditions	Seitz *et al.* (2011); Charles *et al.* (2009); Richards *et al.* (2012) Tung *et al.* (2013)
Transmission routes	Person-to-person: through the faecal-oral route, by ingestion of aerosolised vomitus or by indirect exposure via contaminated surfaces. Foodborne: by contamination of infected food handlers or further upstream of the production system through contamination with human faeces. Waterborne: contact with water contaminated with human faeces	Lopman *et al.* (2012)

The symptoms of NoV gastroenteritis are characterised by nausea, vomiting, abdominal cramps and non-bloody diarrhoea which could persist for 12–60 h following an incubation period of 24–48 h (Hall *et al.*, 2011). Infected persons may experience only vomiting and diarrhoea (Hall *et al.*, 2011). Sequelae of NoV infection include electrolyte

imbalance and hypovolaemia or more severe medical presentations such as hypokalaemia and renal insufficiency (MacCannell *et al.*, 2011). Most cases of NoV gastroenteritis are self-limiting and do not require clinical intervention. Only a small proportion of illness cases are investigated and therefore the official statistics highly underestimate the real public health burden of NoV illness (Tam *et al.*, 2012). There is no long-term immunity to NoV and repeated infections may occur throughout a person's life (Li *et al.*, 2012). There is large variation in the duration of NoV shedding in healthy individuals. Milbrath *et al.* (2013) showed that long-term shedders (105–136 days) increase the probability of an outbreak by 33% and the severity of transmission (as measured by the attack rate) by 20%. NoV can also be shed asymptomatically by infected hosts for over 35 days (Leon *et al.*, 2008). There is large variation in the number of cases/outbreaks between years. Factors driving this variation are associated with the epidemiology of the virus which in turn is associated with cold and dry weather, low population immunity and emergence of new antigenic variants (Lopman *et al.*, 2009).

The literature contains abundant case and outbreak reports of NoV infection associated with the consumption of shellfish (Doyle *et al.*, 2004; Ng *et al.*, 2005; David *et al.*, 2007; Huppatz *et al.*, 2008; Westrell *et al.*, 2010; Wall *et al.*, 2011; Smith *et al.*, 2012; Lodo, Veitch and Green, 2014). Such outbreaks continue to occur on a regular basis worldwide (Bellou, Kokkinos and Vantarakis, 2013). Different profiles of NoV illness are associated with different shellfish species. Most cases of illness are associated with oysters because these are usually consumed whole and raw. Illness cases linked to species that are typically cooked before consumption, such as mussels, have been reported sporadically (Lee and Younger, 2002). Species that are eviscerated (e.g. scallops) prior to sale represent lower risk of infection because most NoV ingested by shellfish is accumulated in the digestive gland (Schwab *et al.*, 1998; Wang *et al.*, 2008).

In the European Union, the first sanitary controls for shellfish intended for human consumption were implemented in 1992. During the period 1992–2014, public health agencies reported 75 outbreaks of diagnosed or suspect viral origin associated with bivalve shellfish in England and Wales (E&W) (Figure 2). Each outbreak had several individual cases. Figure 2 also shows lower numbers of cases of unknown aetiology over the last decade which reflect progress with epidemiological surveillance. However,

comparisons of the number of outbreaks over time is problematic because of the differences in the quality of the data.
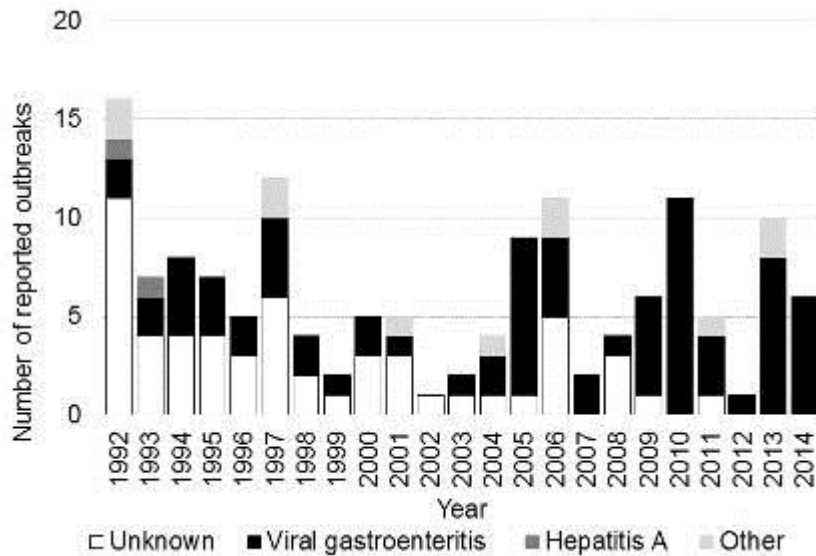
Figure 2 Number of outbreaks of intestinal disease associated with bivalve shellfish in England and Wales, 1992–2014.
Data compiled by Cefas based on information provided by the Communicable Disease Surveillance Centre, Public Health England. These data relate to outbreaks only, not individual cases.

Oysters were the most frequently implicated group of species (87% of outbreaks) as they are often eaten whole and raw. Mussels (10% of outbreaks) were also occasionally reported as vehicles of infection[1]. Univalve molluscs (e.g. winkles, whelks) are not filter feeders and do not present the same risk as that presented by bivalves. Most outbreaks were associated with batches of shellfish harvested from classified production areas, depurated in certified plants and processed in approved establishments in compliance with the legislation (see Section 1.3 below; Cefas, 2011).

Despite being common (millions of cases in the UK each year), NoV gastroenteritis is usually a mild, self-limiting disease and most infected people make a full recovery with no long-lasting effects (Harris *et al.*, 2008; Tam *et al.*, 2012). During periods of peak prevalence, NoV outbreaks cause substantial disruptions to health care systems (Cooper *et al.*, 2011) and represent very substantial financial costs (Cooke, Goddard and Golland, 2003; Lee *et al.*, 2011; Belliott *et al.*, 2014). Besides the clinical burden, NoV outbreaks

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[1] Source: Cefas Norovirus Outbreaks Database.

have a substantial impact on the shellfish industry through loss of sales and loss of consumer confidence (Applied Economics Pty Ltd., 2010).

Cases of NoV gastroenteritis associated with shellfish harvested from contaminated waters following sewage pollution events have been reported in the literature (Huppatz *et al.*, 2008; Wall *et al.*, 2011). In E&W, many shellfish production areas (SPAs) are located in shallow estuaries and other coastal environments close to densely populated areas producing large volumes of sewage pollution and potentially associated human pathogenic viruses. This presents a significant management challenge to the shellfish industry and its regulators because shellfish require specific and targeted measures to control faecal contamination and the resultant health risks. Episodes of poor water quality caused by discharges of untreated or partially treated sewage effluent remain an important barrier to further expansion of the shellfish farming sector (Fitzgerald, 2008; Defra, 2012). This has led the Shellfish Associated of Great Britain to ask the Food Standards Agency (FSA) to place greater emphasis on NoV controls as part of its Foodborne Disease Strategy (T. Pickerell, pers. comm. 29 July 2010). In 2011, the FSA identified NoV as a priority for action to reduce the incidence of foodborne illness in the UK (Food Standards Agency, 2011; Food Standards Agency, 2015). More recently, the UK Advisory Committee on the Microbiological Safety of Food (ACMSF) – *Ad hoc* Group on Foodborne Viral Infections recommended the UK Government to work collaboratively with local authorities, scientists and members of the industry to develop proactive management of shellfisheries to ensure that harvested products are safe for consumers to eat. The committee further recommended that research is undertaken into the effectiveness of sewage treatment processes in reducing NoV and that risk management measures for shellfisheries in relation to sewage discharges are reviewed (ACMSF, 2015). At an international workshop on foodborne viruses jointly organised by the FSA and the European Food Safety Authority (EFSA), the group of experts specifically identified the environmental transmission of NoV contamination in SPAs as a topic requiring further research to effect NoV control.

1.2 Fate and Behaviour in the Environment

Discharges of sewage from municipal and private sewage treatment works (STW) (Nordgren *et al.*, 2009; Rajko-Nenow *et al.*, 2013), overflows from sewerage systems (Rodríguez *et al.*, 2012) and septic tanks (Cook *et al.*, 2009; Borchardt *et al.*, 2011) can introduce high quantities of NoV into the marine environment as measured by molecular methods which do not provide a measure of infectivity. Catchments at higher risk of contamination are those with combined sewerage infrastructure characterised by large variations in flow and contaminant concentrations with the associated requirement to discharge storm overflows (SOs) without treatment. In an urban catchment in Chicago (USA), average levels of NoV in water samples collected near combined sewer overflow (CSO) outfalls increased more than 10 times during wet weather (Rodríguez *et al.*, 2012). In Ireland, oyster samples collected 3 h after a CSO discharge had 2,691 copies/g NoV GII and a further 6 oyster samples collected over the next 48 h had GII concentrations ranging from 1,047 to 3,090 copies/g (Doré *et al.*, 2013). These studies suggest that SOs may be significant sources of NoV contamination of shellfish waters. A review of discharge frequency for 760 SO discharges for the period April 2010–March 2011 undertaken by the Environment Agency showed that 46% of discharges spilled 20 times or less and 10% of the SOs discharged 21–40 times (P. Simmons, pers. comm., May 2012). However, information on NoV concentrations for a range of short-term and long-term sewage spill impacts is lacking in the literature.

Secondary-treated effluents are often contaminated with NoV and this, in general, reflects the viruses circulating in the community. This is regardless of the symptoms shown by the contributing population because subclinical infections are common (Iwai *et al*., 2009). Various studies have suggested that biological sewage treatment processes are less efficient at removing viruses (measured by molecular methods) than faecal indicator organisms (FIOs) (measured by culture methods which prove the ability to metabolise and replicate) (Rose *et al.*, 2004; Palfrey *et al.*, 2011). Most studies report that, on average, activated sludge treatments (including reductions during primary settlement) reduce up to 2 $\log_{10}$ units across the process (Henshilwood, 2002; van den Berg *et al.*, 2005; da Silva *et al.*, 2007; Nordgren *et al.*, 2009; La Rosa *et al.*, 2010; Palfrey *et al.*, 2011; Flannery et al., 2012). Higher average removals have been reported because of the large variability in NoV concentrations in sewage and other factors influencing

suspended growth processes (inflow variations, pH, oxygen, suspended solids, etc.). The available evidence on NoV removal rates for some types of secondary treatment commonly used in the UK (e.g. trickling filters, sand filters) is however extremely limited.

Regarding ultra-violet (UV) disinfection, the magnitude of virus inactivation depends upon the UV dose absorbed by the viruses and their resistance to UV. Reductions of 4 $\log_{10}$ have been reported for murine NoV (a cultivable surrogate for NoV) in bench scale experiments when the virus was exposed to ultra-violet (UV) light doses of 29 mJ/cm^2 (Park, Linden and Sobsey, 2011). Although it is often assumed that most bacteria and viruses require relatively low UV doses for inactivation, the available information on the relationships between NoV removal and the factors influencing the performance of UV disinfection (flow rates/retention time, turbidity, UV intensity) is very scarce. Membrane bioreactor (MBR) technology has been increasingly used in areas requiring high levels of environmental protection. This treatment process combines a suspended growth biological reactor with solids removal via a filtration process. Average NoV removal rates through MBR reported in the literature are quite variable ranging from 1 to 4 $\log_{10}$ units (Ottoson *et al.*, 2006); Sima *et al.*, 2011; Simmons, Kuo and Xagoraraki, 2011). This large variability within and between sites could be associated with maintenance of membranes, membrane imperfections or breakages and microbial regrowth (Hai *et al.*, 2014).

Very few studies have reported data on the geographical extent and duration of NoV contamination from sewage discharges on SPAs. In New Zealand, a gradient of NoV was observed in oysters as distance from the STW outfall. Total levels of NoV (GI + GII) were about 1,000 copies/g adjacent to the outfall and decreased to 130 copies/g at 10 km and to 100 copies/g at 24 km from the outfall (Greening, 2007). In New South Wales (Australia), samples of Sydney rock oysters (*Saccostrea glomerata*) collected from 7 sites along a river impacted by a sewage spill from a SO were positive for NoV GII up to 8.5 km downstream from the sewage source (Brake *et al.*, 2011). These studies demonstrate that the geographical extent of NoV contamination in coastal waters impacted by sewage spills could be large. Concerning temporal variability of NoV in coastal waters, a study conducted in New Zealand, detected NoV in shellfish located 50 m away from the sewage source for up to 3 months following a sewage spill event (Scholes *et al.*, 2009). No

studies have however been conducted on seasonality and persistence of NoV contamination in sites impacted by low levels of sewage pollution or quantified NoV in multiple species exposed to the same environmental conditions. These studies are important to understand if the differences observed in average levels of FIOs between different species of shellfish (Younger and Reese, 2013) also occur for NoV.

The concentrations of viruses bio-accumulated by active, filter-feeding shellfish can exceed 100 times those in the surrounding waters (Seraichekas *et al.*, 1968; Canzonier, 1971). Bio-accumulation of NoV occurs in the gills, digestive glands and other tissues (Wang *et al.*, 2008) within 4–24 h (Schwab *et al.*, 1998). The dynamics of clearance of NoV from shellfish has been studied in microcosms using small scale tanks with disinfected water simulating commercial purification processes (see Section 1.3 below). Studies with Pacific oysters (*C. gigas*) artificially contaminated with NoV GII for 72 h and transferred to clean water for 10 days (water at 10 ± 2 °C containing phytoplankton) demonstrated that oysters can accumulate 5.2×10^3 copies/g within 72 h of exposure. No significant NoV clearance was observed over the following 10 days (7.7×10^3 copies/g) (Ueki *et al.*, 2007). Studies conducted in the natural environment have found that NoV concentrations in oysters can also reduce when contaminated shellfish are transferred to clean waters. In Ireland, Doré *et al.* (2010) conducted one of these experiments and observed that oysters transferred to clean waters over 17 days had lower (0.8 $\log_{10}$) concentrations of NoV. A further reduction of 0.6 $\log_{10}$ was obtained when the re-laid oysters were placed in purification tanks at 17 °C during 4 days.

Outbreaks of NoV occur throughout the year although there is a seasonal pattern of increased activity during the winter months, at least in temperate climates where most epidemiological data are available (Ahmed, Lopman and Levy, 2013). Although NoV is often detected in sewage throughout the year, the peaks of prevalence usually occur during the winter (Katayama *et al.*, 2008; Nordgren *et al.*, 2009). In the UK, average levels of NoV in *C. gigas* during colder months (October–March) were found to be 17 times higher than those during the remainder of the year (Lowther, Henshilwood and Lees, 2008). The oysters were collected at two estuarine locations with substantial urban populations (> 100,000). This seasonal pattern often mirrors variations in temperature (Lowther *et al.*, 2012a), salinity and rainfall (Miossec *et al.*, 2000). This is further discussed in Chapter 2.

1.3 Legislation Intended to Control Microbiological Contamination in Coastal Waters

There are two monitoring programmes of the microbiological quality of shellfish undertaken in UK waters. These programmes are related to the requirements of the food hygiene regulations, primarily Regulations (EC) No 853/2004 and 854/2004 (European Parliament and Council of the European Union, 2004, 2004a), and Directive 2000/60/EC (Water Framework Directive) (WFD).

Regulation (EC) No 854/2004 sets out controls for SPAs comprising microbiological monitoring and classification. The classification of SPAs is based on monitoring of *E. coli* in shellfish flesh. For SPAs with levels of contamination exceeding the end-product standard (230 *E. coli*/100 g), there are additional requirements for post-harvest purification before shellfish can be marketed for human consumption (Table 2).

Table 2 Microbiological standards for classification of shellfish production areas under EU regulations.

Class[1]	Microbiological standard[2]	Post-harvest treatment required
A	Live bivalve molluscs from these areas must not exceed 230 MPN *E. coli*/100 g[3,a]	None
B	Live bivalve molluscs from these areas must not exceed, in 90% of the samples, 4,600 *E. coli*/100 g[a]. In the remaining 10% of samples, live bivalve molluscs must not exceed 46,000 *E. coli*/100 g[4,a]	Purification[b], relaying or cooking by an approved method
C	Live shellfish from these areas must not exceed 46,000 *E. coli* per 100 g of flesh and intra-valvular liquid	Relaying over a long period or cooking by an approved method
Prohibited	> 46,000 *E. coli* per 100 g of flesh and intra-valvular liquid	Harvesting not permitted

[1] The competent authority has the power to prohibit any production and harvesting of bivalve molluscs in areas considered unsuitable for health reasons. [2] The reference method is given as ISO 16649-3 (ISO, 2005). [3] By cross-reference from Regulation (EC) No 854/2004, via Regulation (EC) No 853/2004, to Regulation (EC) No 2073/2005 (European Communities, 2005). [4] From Regulation (EC) No 2073/2005. [a] In flesh and intra-valvular liquid. [b] Usually by means of depuration.

In addition to determining the classification status of the production areas, *E. coli* monitoring also provides an indication of changes in the risk of contamination and thus whether additional short-term controls need to be implemented to protect consumers.

Before classifying a production area, shellfish hygiene authorities are required to undertake a "sanitary survey". This survey comprises:

- An inventory of the sources of faecal contamination of human or animal origin that give rise to contamination of the production area;

- An assessment of any seasonal variations in contamination levels observed as a result of variations of human and animal populations, rainfall and sewage treatment; and

- An examination of the way that contamination levels are influenced by tides, currents and bathymetry in the production area.

The sanitary survey is a catchment-level evaluation of the sources and types of faecal contamination impacting SPAs intended primarily to inform a sampling programme for monitoring and classification of the production areas (European Parliament and Council of the European Union, 2004).

The WFD aims to protect the environmental quality of areas designated for the protection of economically important shellfish species (shellfish water protected areas) (SWPAs). This legislation aims to achieve "Good Ecological Status" and to prevent deterioration of surface waters (European Parliament and Council of the European Union, 2000). The environmental objectives and the monitoring required to protect shellfish water quality are included in the WFD River Basin Management Plans as set out in the Water Environment (Water Framework Directive) (England and Wales) (Amendment) Regulations 2016 (Statutory Instrument, 2016). The WFD does not contain a microbiological standard to confer protection of the microbiological quality of SWPAs. However, for the purposes of meeting the water quality objectives, the administrations for E&W have specified that agencies must endeavour to observe a guideline (G) standard of 300 *E. coli*/100 ml in shellfish flesh (75% of samples taken within any period of 12 months) (Defra and Natural Resources Wales, 2016). From a microbiological point of view, the G standard is laxer than the class A standard of Regulation (EC) No 854/2004. Improvements to STW final effluent quality and SOs have been considered critical to achieving compliance with the G standard (Defra, 2012a). Essentially, the EU food hygiene regulations set quality standards for human

consumption and protect human health from poor quality shellfish while the shellfish flesh G standard is designed to achieve high quality of shellfish products for human consumption (Statutory Instrument, 2016).

Additional requirements intended to ensure that there are adequate levels of sewage treatment in catchments draining to SWPAs are exerted in Directive 91/271/EEC (Urban Waste Water Treatment Directive, UWWTD) (Council of the European Communities, 1991). The UWWTD concerns the collection, treatment and discharge of urban sewage effluents and the treatment of sewage discharges from certain industries. The directive also seeks to protect rivers, estuaries and coasts from the adverse effects of domestic sewage, industrial wastewater and surface water runoff into such waters. Specifically, the UWWTD requires the collection and treatment of wastewater in all agglomerations of > 2,000 population equivalents (PEs); secondary treatment of all discharges from agglomerations of > 2,000 PEs, and more advanced (tertiary) treatment for agglomerations > 10,000 PEs in designated SWPAs and their hydrological catchments (Council of the European Communities, 1991).

In 1999, the UK Government identified a policy objective of achieving class B for all SPAs classified under Food Hygiene legislation in E&W. To achieve this, a baseline water quality standard (95th percentile of 1,500 faecal coliforms/100 ml of seawater) was identified for use as part of STW scheme design and impacting sewer overflows with a limit of 10 significant spills per year on average (over 10 years), or a modelled impact design (97th percentile of 1,500 faecal coliforms/100 ml of seawater) for combined continuous and overflow discharges. Under this policy, the scheme is designed to achieve the G standard for, at least, 97% of the time in the long-term (Environment Agency, 2003).

1.4 Additional Risk Management Options

The number of shellfish-related outbreaks reported in E&W shown in Figure 2 (page 5) suggests that the legislative requirements have been effective in preventing shellfish-borne illness associated with bacterial pathogens but have not reduced the number of viral illnesses. This is a common situation in the developed world. Several countries

have implemented additional measures to address the NoV risks. In the EU, the EFSA Panel on Biological Hazards has recommended the introduction of microbiological criteria for NoV in shellfish in the food hygiene regulations, unless batches are labelled "to be cooked before consumption" (EFSA, 2011). The Panel has further recommended refinement of the regulatory standards and monitoring approaches to improve public health protection (EFSA, 2011). Scope for these recommendations is given in Article 27 of Regulation (EC) No 2073/2005: "In particular, criteria for pathogenic viruses in live bivalve molluscs should be established when the analytical methods are developed sufficiently." (European Communities, 2005, p. 4). Although the implementation of NoV standard(s) would reduce the number of contaminated batches placed on the market, the available evidence base is insufficient to estimate the impact of any potential standards on consumers' exposure to NoV. To obtain this evidence, the EFSA Panel on Biological Hazards recommended undertaking a surveillance study of NoV contamination in oysters to establish levels of NoV in production areas across the European Union (EFSA, 2012). This study has been supported by the EU National Reference Laboratories and members of the industry, and has been commissioned to start in 2016 (Food Standards Agency, 2016).

Affiliated measures have been implemented in other countries. The Food Safety Authority of Ireland has recommended that oysters from a class A production areas implicated in NoV outbreaks can only be sold for human consumption if NoV concentrations in the oysters are < 200 copies/g based on results of two consecutive samples taken at least 24 h apart. For oysters from class B areas, NoV concentrations in the oysters following post-harvest treatment (e.g. depuration at increased temperature) must be < 200 copies/g (Food Safety Authority of Ireland, 2013).

More proactive risk management measures have also been suggested, namely the use of surrogate variables (e.g. antecedent rainfall and information on sewage discharges, river flows) for prediction of NoV contamination in production areas and actions following detection of illness outbreaks. In France, the harvesting of shellfish from areas linked to NoV outbreaks must be prohibited as soon as the first case of illness occurs and investigations demonstrate a link to a contaminated production area. The production area can be re-opened when shellfish samples are negative for NoV or, alternatively, if (a) a rainfall threshold is not exceeded during 28 days following the outbreak; (b) no

further notifications of STW failures are received; and (c) the results of *E. coli* monitoring return to "normal" (D. Lees, Cefas, pers. comm.). A similar closure period of 28 days for harvesting areas implicated in NoV outbreaks has been implemented in New Zealand (Hay, McCoubrey and Zammit, 2013).

The establishment of "buffer or exclusion zones" around STW outfalls and other contamination sources has also been implemented in many countries (ex. New Zealand, USA, Canada, Italy, The Netherlands) in recognition that regulatory monitoring of faecal indicators cannot be relied upon to characterise the NoV health risk (Fitzgerald, 2015). In the USA, the National Shellfish Sanitation Program (NSSP) contains specific requirements for the establishment of buffer zones based on a theoretical calculation of sewage dilution required to meet a bacteriological standard of 14 faecal coliforms/100 ml in the growing water. The calculations are based on worst-case STW loadings (i.e. for untreated effluents in the case of STW) and the buffer zone must incorporate a minimum effluent dilution of 1,000:1 of estuarine water to treated effluent. Waters with dilutions less than 1,000:1 should be classified as "prohibited" for the harvest of shellfish (Goblick, 2015). The dilution and dispersion of the sewage effluent during specific STW discharge conditions are usually estimated using dye tracing studies or computer modelling programmes. For SPAs that are intermittently affected by pollution (classified as "conditionally approved"), there are additional requirements in the NSSP for a written management plan (Conditional Area Management Plan) (CAMP) (USFDA and ISSC, 2013). This CAMP demonstrates how the production area can be closed before contaminated shellfish are marketed and must include information on:

- STW flow conditions (peak effluent flow, average flow, and infiltration flow);

- Bacteriological or viral quality of the effluent;

- Physical and chemical quality of the effluent;

- Conditions which cause plant failure;

- STW or collection system bypasses;

- Design, construction, and maintenance of the STW to minimize mechanical failure, or overloading;

- Provisions for monitoring and inspecting the STW; and

- Establishment of an area in the prohibited classification adjacent to a STW outfall in accordance with the "prohibited" classification criteria.

In Europe, the Food Hygiene Regulations do not contain requirements for the establishment of buffer zones although some EU Member States have implemented some form of zone-based controls based on geographical proximity to pollution sources as required by national legislation (Cefas, 2013). The Guide to Good Practice on Microbiological Monitoring of Bivalve Mollusc Harvesting Areas includes recommendations on closure areas around sewage outfalls, harbours and marinas (EU Working Group on the Microbiological Monitoring of Bivalve Mollusc Harvesting Areas, 2014). In 2013, representatives of European National Reference Laboratories for monitoring bacteriological and viral contamination of bivalve molluscs considered that the establishment of buffer zones would improve health protection against enteric viruses and that further work is required to develop criteria (e.g. based on geographic or dilution approaches) for the establishment of buffer zones in Europe (Cefas, 2013a).

In summary, contamination of SPAs with NoV is one of the most significant barriers to expansion of the shellfish industry. The epidemiological characteristics of NoV enable these viruses to be the leading cause of shellfish-related illness worldwide. Current regulatory standards based on *E. coli* monitoring do not fully protect consumers from NoV infection and there is opportunity to develop more targeted risk management approaches, namely focused on the establishment of NoV standards for shellfish and/or the identification of buffer zones in production areas. Currently, there is an almost complete absence of data on NoV behaviour in the environment on which to base these risk management decisions. This presents a significant problem for the shellfish and water industries and regulators considering the likely substantial economic and human health consequences of NoV outbreaks.

1.5 Aims of the Thesis

The overarching aim of this thesis is to evaluate the main factors influencing the abundance and distribution of NoV in SPAs. Water companies and shellfish hygiene regulators require this information to develop pollution reduction programmes and risk management measures for NoV. Members of the shellfish industry also require this information to better understand when and where it is safe to harvest shellfish for human consumption. The specific objectives of the study are:

1. To investigate the effect of climatic, hydrometric, demographic and pollution source factors on NoV and *E. coli* contamination in shellfish;

2. To assess the effectiveness of sewage treatment processes in reducing NoV and *E. coli* from sewage;

3. To study the relationships between NoV in shellfish and the dispersion and dilution of sewage effluent in the production areas; and

4. To evaluate the impact of establishing NoV limits and buffer zones as measures to control the risk of NoV contamination in SPAs.

1.6 Outline of the Thesis and Research Questions

This thesis is structured around seven chapters. **Chapter 2** presents the results of a generic investigation into the relationships between concentrations of microbiological contaminants (NoV and *E. coli*) in shellfish and a selection of climatic, hydrometric, demographic and pollution source characteristics of the adjacent surface water catchments (Objective 1). This investigation was undertaken using existing data on NoV and *E. coli* levels in oysters collected from 31 harvesting sites in E&W over two years and additional environmental and pollution source data held by Cefas and provided by other organisations. Site-specific investigations were also undertaken in two SPAs and their catchments to address the data gaps identified in the generic modelling and obtain empirical data to develop NoV risk management measures. **Chapter 3** provides quantitative information on NoV and *E. coli* levels in untreated sewage and treated

effluents and rivers at the experimental sites (Objective 2). It also provides information on reduction efficiencies of these microbiological contaminants at four full scale STW operating treatment processes characteristic of primary-, secondary- and tertiary-treated effluents. The results of NoV and *E. coli* monitoring in shellfish placed in the receiving waters of these discharge points are presented in **Chapter 4** (Objective 3). **Chapter 5** presents the results of a characterisation of surface water movements and sewage dispersion and dilution in the experimental sites to contextualise the microbiological impacts described in Chapter 4 (Objective 3). In **Chapter 6**, the results of the field studies were used to develop risk management measures for NoV (Objective 4). The measures focus on the establishment of buffer zones around sewage discharges and the establishment of NoV limits for SPAs. The thesis concludes with **Chapter 7**, which summarises the results obtained in the desk and field studies and provides a set of recommendations to the shellfish and water industries, regulators and the research community.

Research questions were identified for each objective to help inform the approach taken in the desk and field studies. These research questions are listed in Table 3.

Table 3 Structure, objectives and research questions of the thesis.

		Objective		Research Questions
CHAPTER 1 Research Context				
CHAPTER 2 Generic Analysis of Factors Influencing Concentrations of Norovirus and *E. coli* in Shellfish Production Areas	—	To study the relationships between NoV and *E. coli* in 31 oyster production sites and the characteristics of the adjacent catchments	—	Which environmental factors correlate with the levels of NoV and *E. coli* contamination in shellfish production areas?
CHAPTER 3 Field Studies on Norovirus and *E. coli* in Sewage, Treated Effluents and Rivers	—	To quantify concentrations of *E. coli* and NoV in untreated sewage and treated effluents and their removal efficiencies at four STW operating primary, secondary and tertiary treatments	—	Which level(s) and type(s) of sewage treatment(s) are more effective in reducing NoV? Are removal rates for *E. coli* different from those of NoV? Which factor(s) influence the removal of NoV from sewage?
CHAPTER 4 Field Studies on Norovirus and *E. coli* in Shellfish Production Areas	—	To obtain data on concentrations of NoV and *E. coli* in three species of shellfish in two sites with different characteristics during low and high prevalence of NoV	—	How does the abundance of NoV in shellfish vary between years, between species and between production areas? Is the NoV abundance different from that of *E. coli*?
CHAPTER 5 Drogue Tracking, Current Profiling and Dye Tracing in Shellfish Production Areas	—	To study the surface water movements and the time of travel, dispersion and dilution of sewage effluent in the sites studied in Chapter 4	—	How does the degree of dispersion and dilution of sewage effluent vary between shellfish production areas? Is there a relationship between sewage dilution and NoV in shellfish?
CHAPTER 6 Measures to Control Norovirus Contamination in Shellfish Production Areas	—	Using the empirical data presented in Chapters 3–5, to evaluate the feasibility of implementing NoV limits for shellfish and buffer zones in shellfish production areas	—	What is the impact of NoV limits on shellfish production areas? What is the impact of dilution ratios of estuarine water to treated effluent on shellfish production areas?
CHAPTER 7 Conclusions and Recommendations				

1.7 Publication Details

This thesis is not submitted under the Alternative Thesis Procedure[2] set out in the Aberystwyth University regulations. However, the research described in this thesis is the focus of six manuscripts which are either published or have been submitted to international peer reviewed journals. These manuscripts are first-authored by the author of this thesis and co-authored by the research team at Cefas and, where relevant, by scientists of Southern Water and US Food and Drug Administration. Additional publications and dissemination activities are listed for reference in Appendix I.

Citations:

Campos, C.J.A., Goblick, G., Till, D. and Lees, D.N. Determining the zone of impact of norovirus contamination in shellfish production areas through microbiological monitoring and hydrographic analysis. In draft for *Applied and Environmental Microbiology*.

Campos, C.J.A., Kershaw, S., Morgan, O.C. and Lees, D.N. (2017) Risk factors for norovirus contamination of shellfish water catchments in England and Wales. *International Journal of Food Microbiology* 241: 318–324.

Campos, C.J.A., Avant, J., Lowther, J. Till, D. and Lees, D.N. (2016) Human norovirus in untreated sewage and effluents from primary, secondary and tertiary treatment processes. *Water Research* 103: 224–232.

Campos, C.J.A., Avant, J., Gustar, N., Lowther, J., Powell, A., Stockley, L. and Lees, D. (2015) Fate of human noroviruses in a shellfish water impacted by frequent sewage pollution events. *Environmental Science & Technology* 49(14): 8377–8385.

Campos, C.J.A. and Lees, D. (2014) Environmental transmission of human noroviruses in shellfish waters. *Applied and Environmental Microbiology* 80(12): 3552–3561.

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[2] See paragraphs 18 to 28 at https://www.aber.ac.uk/en/regulations/contents/phd/.

Campos, C.J.A., Avant, J., Lowther, J., Lees, D. and Till, D. (2013) Levels of norovirus and *E. coli* in untreated, biologically treated and UV-disinfected sewage effluent discharged to a shellfish water. *Journal of Water Resources and Protection* 5(10): 978–982.

1.8 Author Contributions and Data Confidentiality

The author of this thesis designed the study, conducted all the field sampling, compiled the data, developed the database, analysed the data and drafted the thesis and the papers that derive from this work. Colleagues at Cefas assisted with GIS mapping and performed the microbiological analyses. Further details on authorship are given in the individual chapters of the thesis.

One of the conditions for conducting the research reported in this thesis agreed with project stakeholders was that all study sites should remain anonymous because of the sensitivities in the shellfish industry about the identification of NoV in SPAs. To preserve anonymity, the study sites were assigned site codes and specific geographic locations are not disclosed in the thesis.

Chapter 2

Generic Analysis of Factors Influencing Concentrations of Norovirus and *E. coli* in Shellfish Production Areas

"To ensure that the regulatory and control bodies have good science upon which to base legislation poses significant challenges to the scientific community. To accomplish that, science needs to provide means to resolve fundamental issues. Thus we must be able to track contaminants back through system to source, which implies accurate techniques to detect and measure contaminants in water and/or flesh." Rees *et al.* (2010, p. 48)

This chapter reports the results of a generic investigation into the relationships between concentrations of microbiological contaminants (NoV and the regulatory parameter *E. coli*) in shellfish from commercial harvesting sites and a selection of climatic, hydrometric, demographic and pollution source characteristics of the adjacent hydrological catchments. The aim of this investigation was to develop predictive models for NoV and *E. coli* based on variables that include measures of potential sources of NoV within catchments and factors that may influence the abundance and distribution of NoV in the receiving waters. The assumptions of this work were that, if these models can be developed, then they could be used as risk management tools for characterisation of NoV and *E. coli* contamination in SPAs. From a policy perspective, these models provide information on the key sources of NoV within catchments, thereby informing the development of pollution reduction plans to help achieve compliance with the *E. coli* standard of the SWPAs. Importantly, too, these models could be used to identify catchments associated with low risk of NoV contamination, where future shellfish farming operations could be developed more sustainably.

This study used an existing database of concentrations of NoV and *E. coli* in oysters from 31 commercial harvesting sites on the coast of E&W published by Lowther *et al.* (2012a), which is the largest database of this kind available in the UK. Access to the database was kindly provided by the FSA. Oyster harvesting sites were selected for this study because epidemiological data show that the risk of human infection is greater for these species.

2.1 Study Sites

The oyster harvesting sites were selected according to a risk scoring process based on the historical classification status of the production areas, number of high *E. coli* results in the previous three years, human population in the catchment, and number NoV outbreaks as detailed in Lowther (2011). The selected sites had proportionate representation in relation to risk scores, geographical locations and shellfish beds within the same classified production area. The classifications of the sampled sites were class A (1 site), class B (28 sites) and class C (2 sites). These broadly reflect the current number of production areas classified as A, B and C in E&W (Food Standards Agency, 2016a). Shellfish sampling was undertaken on a random basis with respect to the likely influencing environmental factors (e.g. tidal state, rainfall, wind, etc.) to avoid introducing any bias in the microbiological results.

2.2 Data Sources

The microbiological database contains concentrations of NoV (genogroups I and II; reported as genome copies/g) and *E. coli* (reported as MPN/100 g) quantified in native oysters (*Ostrea edulis*) from 11 sampling points and in Pacific oysters (*Crassostrea gigas*) from 20 sampling points around the coast of E&W during the period 2009–2011. The database also contains water temperature measurements taken at the time of shellfish sampling by local food authorities. The shellfish samples were collected on a monthly basis by the local authority sampling officers in parallel to the collection of samples for the statutory classification monitoring programme during the period May 2009–April 2011. During this period, the NoV GII.4 New Orleans 2009 was the most prevalent strain worldwide (Vinjé, 2015). All production areas were classified under Regulation (EC) No 854/2004. All samples were obtained directly from production areas and prior to any further commercial processing (e.g. depuration) which may have been performed prior to placing the oysters on the market. The microbiological dataset contained a total of 669 valid NoV results for the 31 sampling sites.

Data on environmental factors that may influence the levels of NoV and *E. coli* contamination in the 31 sites were added to the microbiological database. The factors

selected include climatic, hydrometric, demographic, catchment morphology and pollution source-related characteristics and represent the environmental drivers operative as the virus passes through the catchment and seawater to the measurement sites. The data parameters used in the study and corresponding data sources are listed in Table 4. The variables were selected based on the outcomes of the literature review summarised in Chapter 2.

Table 4 Independent variables and data sources used in the regression models to predict variations in $\log_{10}$-transformed concentrations of *E. coli* and norovirus in oysters.

Variable (unit of measurement)	Data source
Hydrometric	
Rainfall (day of sampling) (mm)*	Environment Agency
Rainfall (cumulative 7 days before sampling) (mm)*	Environment Agency
River flows (day of sampling) (m^3/s)*	Environment Agency and NERC-CEH National River Flow Archive
River flows (cumulative 7 days before sampling) (m^3/s)*	Environment Agency and NERC-CEH National River Flow Archive
Human population	
Total population in the catchment	Census 2011 data. Office for National Statistics
Population density in the catchment (people/km^2)	Census 2011 data by lower super output area. Office for National Statistics
Catchment morphology	
Catchment area (hectares)	Environment Agency
Urban area (hectares)	Department for Communities and Local Government database
Pollution source-related variables	Environment Agency national discharge permits database and Cefas Shellfish Hygiene System database of consented water company and private sewage discharges to controlled waters. The database contains geographically-referenced information on the location of classified shellfish production areas, location of sampling points and location where effluent discharges enter the environment, effluent type and amount that can be discharged
Number of continuous sewage discharges	
Distance from the nearest continuous sewage discharge to the sampling point (km)	
Number of intermittent sewage discharges	
Number of trade discharges	
Volume of continuous sewage discharges (m^3/day)	
Number of continuous sewage discharges in the catchment	
Number of intermittent sewage discharges in the catchment	
Number of trade discharges in the catchment	
Total volume of continuous sewage discharges in the catchment (m^3/day)	
Other variables	
Base Flow Index	NERC-CEH National River Flow Archive
Tidal range	Nautical charts issued by Imray Laurie Norie & Wilson Ltd.

The rainfall and river flow gauging stations were selected using the catchment summaries available in the UK Hydrometric Register (Marsh and Hannaford, 2008) and are therefore representative of the hydrological catchments analysed. Two different time windows were chosen for analysis of the effect of rainfall and river flows: day of sampling and cumulative for 7 days before sampling. This time window was chosen because previous investigations indicated elevated concentrations of *E. coli* in shellfish up to 7 days after rainfall events (Campos *et al.*, 2011; Derolez *et al.*, 2012). To study the influence of tides on the levels of microbiological contaminants, the oyster production areas were classified as microtidal (< 2 m), mesotidal (2–4 m), macrotidal (4–6 m) and hypertidal (> 6 m).

In the database of sewage discharges to controlled waters, it was found that only a small proportion of intermittent discharges (mainly SOs) impacting the study sites have telemetry installed and have information on the frequency and duration of sewage spills to SWPAs. For SOs that have telemetry, not all assets had spill frequency and duration data for the preceding 10 years. Therefore, it was not possible to assess compliance against the 10 spills requirement of the EA policy for consenting sewage discharges impacting SWPAs (see Section 1.3). A limited study was however undertaken for 10 sites for which spill frequency data were available for the period of time when oyster sampling was undertaken.

2.3 Microbiological Methods

Concentrations of NoV and *E. coli* were quantified using quantitative real-time reverse transcription (RT)-PCR (ISO/TS 15216-1: 2013) (ISO, 2013) and MPN (ISO/TS 16649-3: 2005) (ISO, 2005) methods, respectively. Both methods are UKAS accredited. In the ISO/TS 15216-1 method, levels of NoV genogroup I (GI) and II (GII) RNA are liberated from tested samples and viral RNA is extracted by lysis with guanidine thiocyanate and adsorption on silica. Target sequences within the viral RNA were amplified and detected by real-time RT-PCR. Standard curves for quantification of NoV levels were subject to quality control parameters for slope (-3.10 to -3.60) and R^2 values (> 0.99). Analysis of

RT-PCR efficiency/inhibition was conducted using RNA external controls as described in Lowther *et al*. (2012a). Method ISO 16649-3 uses a two-stage, five-tube three-dilution MPN technique in which there is an initial resuscitation step requiring inoculation of minerals modified glutamate broth with a series of diluted shellfish homogenates and incubation at 37 ± 1 °C for 24 ± 2 h. The presence of *E. coli* was subsequently confirmed by sub-culturing acid producing tubes onto agar containing 5-bromo-4-chloro-3-indolyl-β-D glucuronide and detecting β-glucuronidase activity. The MPN method is as used in the official control monitoring under EC Regulations in the UK and testing was accredited to ISO 17025 standard.

2.4 Statistical Analyses

Positive NoV results for each genogroup were expressed as genome copies/g. Concentrations of *E. coli* in shellfish at the lower limit of quantification (LoQ) of the MPN method (< 20 MPN/100 g) were adjusted to 19 MPN/100 g as recommended by the US National Shellfish Sanitation Program (USFDA and ISSC, 2013). Stepwise multiple regression analyses were conducted to examine the relationships between concentrations of microbiological contaminants in shellfish (*E. coli* and NoV) (dependent variables, *y*) and the various potential risk factors (independent variables, *x*). In the regression models, the following relationships were generated: $y = a + b_1x_1 + b_2x_2 + \ldots + b_ix_i + e$ where: a is the intercept (*y* at x = 0), *b* is the slope (change in *y* per unit change in *x*) and *e* is a random error term. Independent variables with a variance inflation factor > 5 were excluded to minimise multicollinearity (Rogerson, 2001). The probability of F to enter was set at 0.05. The level of explained variance was assessed using the coefficient of determination (R^2), adjusted for degrees of freedom and expressed as a percentage. The data distributions for each variable were assessed by probability plots. Log_{10}-transformations were applied to variables for which skewness exceeded 1.00. All statistical tests were assessed at 95% confidence level.

Regression models were developed for the following sets of data:

- Relationships between concentrations of NoV/*E. coli* and water temperature and hydrometric variables (rainfall, river flows) using all data from the 31 study sites;

- Relationships between concentrations of NoV/*E. coli* and water temperature and hydrometric variables (rainfall, river flows) using data for the period October–March (period of high prevalence of NoV in the UK) from the 31 study sites; and

- Relationships between geometric mean levels of NoV/*E. coli* and demographic, catchment morphological, pollution source-related variables using all data from the 31 sites.

For significantly correlated variables, modified boxplots showing minimum (Min), maximum (Max), geometric mean (GM), 95% confidence intervals (CI) were used to characterise the distributions of microbiological results in sewage. The GM was calculated as the antilog of the mean of $\log_{10}$-transformed concentrations (Krzywinski and Altman, 2014). Linear (first order) regression models were also computed to investigate the associations between the levels of microbiological contaminants and the independent variables to investigate if these variables could be used to predict levels of microbiological contaminants. The seasonal variation of NoV results in oysters was investigated using one-way ANOVA followed by Tukey's method for comparisons of confidence intervals for all pairwise differences. The Levene's test for homogeneity of variances was used to determine whether to use ANOVA.

In this study, no distinction was made between results for *O. edulis* and *C. gigas* since these species have been shown to accumulate *E. coli* (Younger and Reese, 2013) and NoV (Lowther, 2011) to the same extent in previous studies in E&W.

2.5 Results

Table 5 shows summary statistics for the independent variables tested in the models. Water temperature data were available for 28 of the 31 oyster harvesting sites and ranged from -1 °C to 21.5 °C. This range includes the minimum temperatures for oyster growth (8–9 °C) and for oyster survival (3–4 °C for native oysters; 5–6 °C for Pacific oysters) in the UK (Laing and Spencer, 2006) and therefore represents the full range of temperatures in growing waters during the life cycle of the oysters. Rainfall data were available for all sites while river flow data were available for only 17 sites.

Table 5 Summary statistics of independent variables used in the regression models to predict variations in $\log_{10}$-transformed concentrations of *E. coli* and norovirus in oysters.

Variable (unit of measurement)	Mean	Minimum	Maximum	Standard deviation
Water temperature (°C)	11.6	-1	21.5	4.915
Hydrometric				
Rainfall (day of sampling) (mm)*	1.7	0	22	3.24
Rainfall (cumulative 7 days before sampling) (mm)*	13.4	0	82	15.93
River flows (day of sampling) (m³/s)*	3.968	0	64.1	6.614
River flows (cumulative 7 days before sampling) (m³/s)*	28.7	0	541.9	46.480
Human population				
Total population in the catchment*	223,008	11,919	1,469,089	322,514
Population density in the catchment (people/km²)	1,408	72.5	5,988	1,210
Catchment morphology				
Catchment area (hectares)*	72,953	5,866	280,319	72,756
Urban area (hectares)*	5,624	44	26,782	7,546
Pollution source-related variables				
Number of continuous sewage discharges*	4	0	13	4
Distance from the nearest continuous sewage discharge to the sampling point (km)*	4	0.7	12.9	2.8
Number of intermittent sewage discharges*	27	0	127	34
Number of trade discharges*	11	0	64	17
Volume of continuous sewage discharges (m³/day)*	19,902	50	108,853	31,078
Number of continuous sewage discharges in the catchment*	21	1	71	19
Number of intermittent sewage discharges in the catchment*	77	6	230	65
Number of trade discharges in the catchment*	47	1	237	59
Total volume of continuous sewage discharges in the catchment (m³/day)*	73,410	300	1,344,000	250,386
Other variables				
Base Flow Index*	0.59	0.33	0.96	0.16
Tidal range (m)*	1.7	0	3	0.9

* Indicates $\log_{10}$ transformation applied to variables where this reduces the coefficient of skewness.

The population density in the study catchments is higher than the average density for England (403 people/km²) and Wales (149 people/km²) (Office for National Statistics, 2013a). Twelve oyster harvesting sites are located in catchments with a small to medium number of built-up areas and 8 sites are located in catchments with a large

number of built-up areas according to the classification system used by the Office for National Statistics (Office for National Statistics, 2013). These data indicate that the majority of the study sites are in catchments with high levels of development and human activity.

The modelled catchments display substantial variability in mean base flow index (BFI) with values ranging from 0.33 to 0.96 (Table 5). Catchments with higher BFI are well-drained soils and substrates (e.g. areas of Chalk downland, Tertiary sandstones, etc.) where there is a substantial groundwater component, while lower BFI values are associated with less-permeable catchments in which there is more surface runoff and a correspondingly greater high-flow component (Crowther *et al.*, 2011a). In general, the mobilisation and transport of microbiological contaminants are greater in less permeable catchments (Wilkinson *et al.*, 1995).

2.5.1 Water Temperature, Rainfall and River Flow Models

Statistically significant ($p < 0.05$) regression models were obtained for *E. coli* and NoV (Table 6). For all samples, concentrations of *E. coli* in oysters were positively associated with cumulative rainfall 7 days before sampling; concentrations of NoV GI were negatively associated with water temperature and with river flows on the day of sampling and positively associated with cumulative river flows. Concentrations of NoV GII were negatively associated with water temperature and rainfall on the day of sampling. Concentrations of total NoV (GI + GII) were negatively associated with water temperature only. Water temperature entered at step 1 was the most significant factor associated with NoV contamination in oysters. However, the highest level of explained variance for NoV was associated with rainfall on the day of sampling ($R^2 = 26.5$).

For the seasonal dataset (October–March), concentrations of *E. coli* were positively associated with cumulative rainfall. Concentrations of NoV GI were negatively associated with water temperature, and positively associated with river flows and cumulative river flows. Concentrations of NoV GII were negatively associated with water temperature and rainfall. Concentrations of total NoV (GI + GII) were negatively associated with water temperature and river flows and positively associated with cumulative river

flows. Overall, the *E. coli* models are dominated by rainfall variables while the NoV models are associated with temperature and river flow variables (Table 6).

Table 6 Summary of results of stepwise multiple regression models of relationships between $\log_{10}$-transformed *E. coli* and norovirus in oysters and water temperature and hydrometric variables.

Step	Variable	Sign of b	Adjusted R^2 (%)	Significance level (*p*)
	All data			
	Escherichia coli (n = 137)			
1	Rainfall (cumulative 7 days before sampling)	+	9.15	< 0.001
	Norovirus (GI) (n = 102)			
1	Water temperature	-	8.97	
2	River flows (day of sampling)	-?	13.19	
3	River flows (cumulative 7 days before sampling)	+	16.63	0.001
	Norovirus (GII) (n = 86)			
1	Water temperature	-	23.19	
2	Rainfall (day of sampling)	-?	26.53	< 0.001
	Norovirus (GI + GII) (n = 111)			
1	Temperature	-	23.94	< 0.001
	October–March			
	Escherichia coli (n = 76)			
1	Rainfall (cumulative 7 days before sampling)	+	7.92	0.008
	Norovirus (GI) (n = 69)			
1	Water temperature	-	8.44	
2	River flows (cumulative 7 days before sampling)	+	10.11	
3	River flows (day of sampling)	+	14.96	0.009
	Norovirus (GII) (n = 62)			
1	Water temperature	-	17.57	
2	Rainfall (day of sampling)	-?	21.58	< 0.001
	Norovirus (GI + GII) (n = 73)			
1	Water temperature	-	21.60	
2	River flows (cumulative 7 days before sampling)	+	23.50	
3	River flows (day of sampling)	-?	26.50	< 0.001

? indicates that the sign does not conform with the expectation.

Concentrations of NoV GI and GII in oysters were grouped into five seawater temperature ranges. Figure 3 shows that mean concentrations of NoV GI decreased from 187 copies/g to 9 copies/g as water temperatures increased from the range < 4.9 °C to > 20 °C. For the same temperature ranges, mean NoV GII concentrations decreased from 662 copies/g to 32 copies/g. Table 7 summarises the results of one-way ANOVA followed by Tukey tests to examine statistical differences between mean $\log_{10}$-transformed levels of NoV for the five ranges in water temperatures. For GM levels

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above the LoQ, the results provide evidence of statistically significant differences between GM concentrations of NoV GII in samples collected at < 5 °C and those in samples collected at > 10 °C.
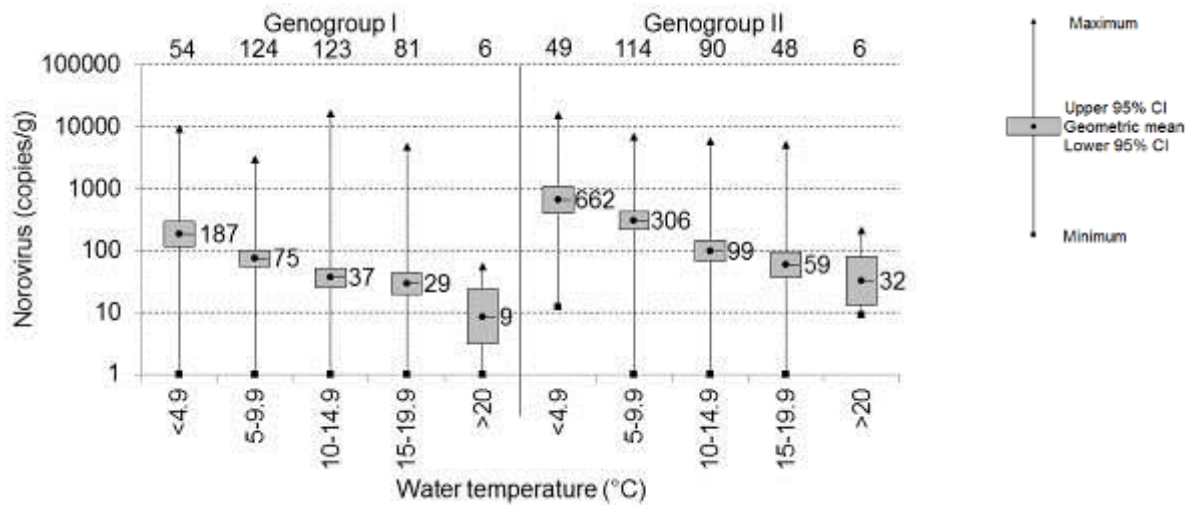
Figure 3 Comparison of geometric means, ranges and 95% confidence intervals of concentrations of norovirus in oysters in relation to five ranges of seawater temperatures measured at the time of sampling.
The numbers of samples with quantitative results are shown above the box plots. Limit of quantification = 100 copies/g.

Table 7 Summary of one-way analyses of variance and *post-hoc* **tests (Tukey's)** comparing mean $\log_{10}$-transformed concentrations of norovirus in oysters collected under different water temperatures.

	Genogroup I					Genogroup II				
	Water temperature range (°C)									
Water temperature range (°C)	<4.9	5–9.9	10–14.9	15–19.9	>20	<4.9	5–9.9	10–14.9	15–19.9	>20
0–4.9										
5–9.9	s*					ns				
10–14.9	s*	s*				s*	s*			
15–19.9	s*	s*	ns			s*	s*	ns		
>20	s*	s*	ns	ns		s*	s*	ns	ns	

s - significant; ns - not significant. * Statistically significant ($p < 0.01$).

2.5.2 Demographic and Pollution Source-Related Models

Statistically significant regression models were also generated for mean concentrations of NoV and the demographic and pollution source-related variables tested (Table 8). Concentrations of NoV GI were positively associated with both the total volume of continuous sewage discharges in the catchment and the total population in the catchment and negatively associated with urban area and the number of intermittent discharges in the catchment. These latter two variables have -ve *b* values and do not

conform with prior expectation. Concentrations of NoV GII were positively associated with both the number of continuous discharges and the total volume of continuous sewage discharges in the catchment. Concentrations of total NoV (GI + GII) were positively associated with the total volume of continuous sewage discharges in the catchment and catchment area. Overall, the volume of sewage discharged in the catchment entered at step 1 for GI and GI + GII and at step 2 for GII was the key variable in the regression models. Interestingly, catchment area was entered in the model describing the variation of total NoV (GI + GII) but not in the models for the individual genogroups. None of the demographic, pollution source-related and other explanatory variables tested were entered in the *E. coli* models.

Table 8 Summary of results of stepwise multiple regression models of relationships between $\log_{10}$-transformed *E. coli* and norovirus in oysters and $\log_{10}$-transformed levels of human population, catchment morphology and pollution source-related variables.

Step	Variable	Sign of b	Adjusted R^2 (%)	Significance level (p)
	All data			
	Norovirus (GI) (n = 19)			
1	Total volume of continuous sewage discharges in the catchment	+	27.54	
2	Total population in the catchment	+	33.72	
3	Urban area	-?	60.51	
4	Number of intermittent discharges in the catchment	-?	59.72	0.002
	Norovirus (GII) (n = 19)			
1	Number of continuous discharges	+	37.83	
2	Total volume of continuous sewage discharges in the catchment	+	48.98	0.004
	Norovirus (GI + GII) (n = 19)			
1	Total volume of continuous sewage discharges in the catchment	+	32.35	
2	Catchment area	+	50.80	0.001

? indicates that the sign does not conform with the expectation.

Linear regression functions were fitted to geometric mean concentrations of NoV in oysters and four explanatory variables (human population in the catchment; catchment area; volume of continuous sewage discharges in the catchment and number of continuous discharges) usually considered in sanitary survey assessments for SPAs (see Section 1.3) (Figure 4A–D). Overall, the tendency of mean NoV concentrations to increase as the explanatory variables also increase is evident. However, the levels of

explanatory variance (R^2) are low (Table 9). This indicates that, although the explanatory variables can be used to provide an indication of catchments at higher risk of NoV contamination, these models are limited for risk management purposes. In particular, most data points in the catchment population model are below the limit of quantification (LoQ) of the method (100 copies/g) and therefore the application of the model to other sites should be treated with caution.
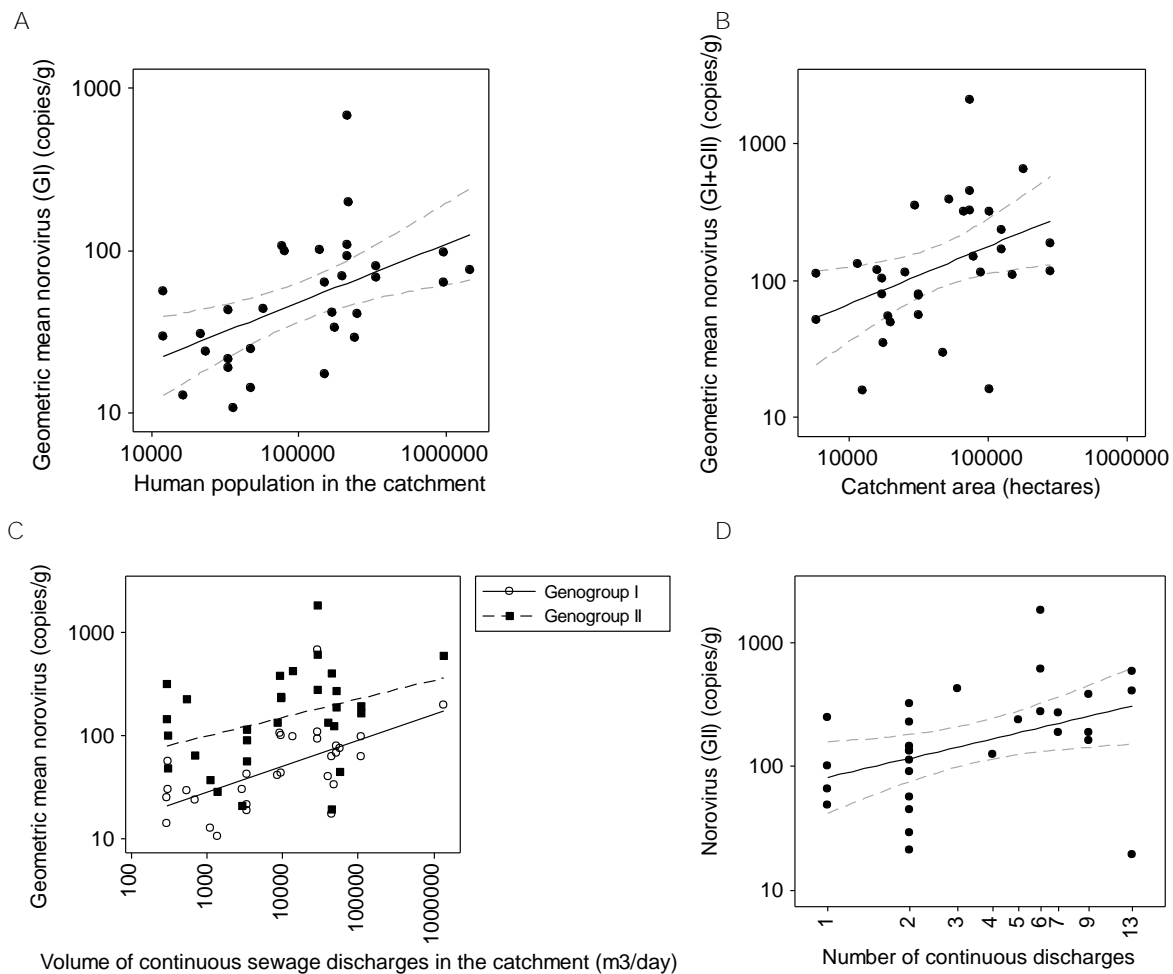
Figure 4 Geometric mean concentrations of norovirus in oysters as a function of human population in the catchment (A), catchment area (B), total volume of continuous sewage discharges in the catchment (C) and number of continuous discharges (D) in 31 sites.
Limit of quantification = 100 copies/g.

Linear regression was also used to model the variation of NoV GI + GII as a function of the number of CSO spills at 10 sites for which CSO spill data were available. The R^2 is high (74.1%) and can be considered operationally useful for risk management purposes (Figure 5). This model predicts that a NoV concentration of 100 copies/g would correspond to 14 sewage spills. This indicates that the average of 10 spills set out in the

policy for consenting sewage discharges impacting SWPAs (Environment Agency, 2003) would correspond to a NoV concentration below 100 copies/g. The model also predicts that NoV concentrations in shellfish at 200 copies/g and 500 copies/g would correspond to average numbers of 45 and 130 sewage spills, respectively. It should be noted however that this model is based only on data from 10 sites and further data are needed to confirm this relationship.
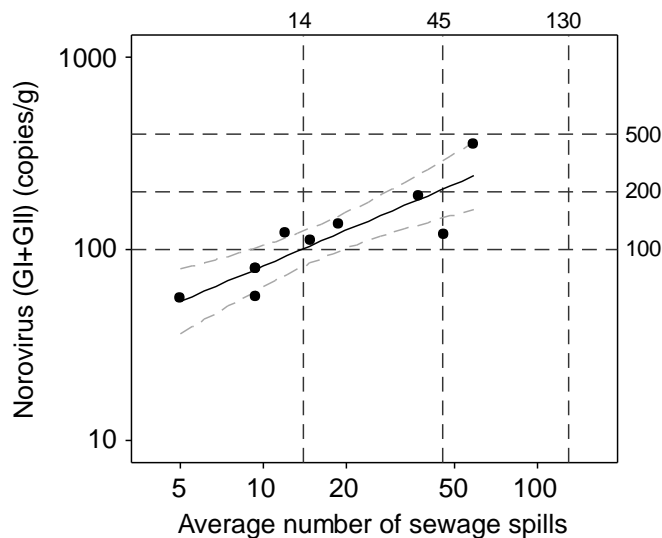
Figure 5 Mean concentrations of total norovirus (GI + GII) in oysters as a function of the average number of combined sewer overflow discharges into 10 harvesting sites.
Data period: April 2010–March 2011. Information on the frequency of sewage spills provided by the EA. The average number of spills was calculated as the total number of recorded spills divided by the number of discharges for which spill data are available. Limit of quantification = 100 copies/g.

Table 9 Model coefficients for simple linear regression models describing relationships between $\log_{10}$-transformed concentrations of norovirus in shellfish and $\log_{10}$-transformed levels of human population, catchment area and pollution source-related variables.

X ($\log_{10}$)	b_0[a]	b_1[b]	R^2 (adjusted) (%)[c]	p[d]	S[e]
Data from 31 sites					
Norovirus (genogroup I)					
Volume of continuous sewage discharges in the catchment (m³/day)	0.690	0.251	34.5	0.000	0.313
Human population	-0.115	0.358	24.8	0.003	0.331
Norovirus (genogroup II)					
Volume of continuous sewage discharges in the catchment (m³/day)	1.455	0.180	10.1	0.048	0.437
Number of continuous discharges	1.912	0.519	14.0	0.026	0.423
Norovirus (GI + GII)					
Catchment area (hectares)	0.139	0.422	14.5	0.020	0.427
Volume of continuous sewage discharges in the catchment (m³/day)	1.116	0.249	21.8	0.005	0.415
Data from 10 sites					
Norovirus (GI + GII)					
Average number of sewage spills	1.296	0.613	74.1	0.001	0.124

Model form: y = b0 + b1*x. y = $\log_{10}$ norovirus (copies/g). [a] Intercept. [b] Coefficient. [c] Variance in y explained by x. [d] Significance. [e] Standard error of the regression.

2.6 Discussion

In this modelling study, different factors were found to influence concentrations of NoV and *E. coli* at the 31 oyster sampling sites. Overall, the predictive environmental factor for *E. coli* contamination in the oysters was rainfall (cumulative 7 days before sampling) while the predictive factors for NoV were water temperature, river flows and the total volume of continuous sewage discharges in the catchment. For NoV GI, human population in the catchment and, for NoV GII, the number of continuous sewage discharges, were also significant variables.

Rainfall enhances the wet weather connectivity between the sources of bacterial contamination in the catchments, which include both human and agricultural (diffuse) sources, and the receiving waters (Campos, Kershaw and Lee, 2013a). Rainfall-induced *E. coli* contamination has been found to persist in shellfish flesh as much as 1 week after the rainfall event (Campos *et al.*, 2011). In this study, rainfall was not found to be positively associated with NoV contamination in the oysters. However, cumulative river flows (7 days before sampling) were positively associated with NoV (GI, GI + GII) concentrations in oysters during the period of high prevalence of the virus in the UK (October–March). These differences are possibly attributed to the effect of lag times between maximum rainfall levels and peak levels of NoV in the shellfish. A lag time of approximately 3 months between the rainfall events and the increase in NoV outbreak incidence has been observed in Victoria, Australia (Bruggink and Marshall, 2010). When all data were used, river flows and rainfall on the day of sampling were negatively associated with concentrations of NoV GI and GII, respectively. These results did not conform with prior expectation. However, at some sites in the UK, *E. coli* levels in shellfish have been found to reduce shortly after rainfall events possibly due to either a suspension of shellfish filtration activity at low salinities or to the dilution of impacting sewage plumes (Lee and Morgan, 2003). It is possible that a similar process also influenced NoV accumulation in the oysters.

Of the various explanatory variables used in the regression modelling, water temperature emerged consistently as the key variable for NoV. In the seasonal model (October–March), water temperature explained 21.6% of the variance of total NoV (GI +

GII) concentrations in oysters. This compares with an R^2 of 7.9% for cumulative rainfall in the *E. coli* model. Geometric mean concentrations of the virus were higher in oysters collected from waters with temperatures < 5 °C than in oysters collected from waters > 10 °C. In the UK, the survival of *C. gigas* becomes compromised at temperatures lower than 5 °C (Laing and Spencer, 2006) and *O. edulis* demonstrates low growth rates at temperatures below 5 °C (Kamphausen, 2012). Therefore, the processes of bioaccumulation and clearance of NoV in shellfish could be affected by periods of low metabolic activity associated with low temperatures. However, NoV concentrations were still significantly higher in samples collected at 5–9.9 °C which are typical temperatures in SPAs in the UK during the period of high prevalence of NoV (October– March). Norovirus RNA titres have been detected in the environment for several months at low temperatures (Kukkula *et al.*, 1999; Richards *et al.*, 2012) and even under freezing conditions (Richards *et al.*, 2012). It has been suggested that, at lower water temperatures, FIOs can substantially underestimate the presence of viruses in the marine environment and this could lead to large errors in predicting the impact of sewage discharges on shellfish water quality (Burkhardt *et al.*, 2000). Furthermore, Burkhardt and Calci (2000) suggested that the incidence of shellfish-related illness is the result of a dynamic relationship between the levels of faecal pollution in the growing waters and the ability of the shellfish to accumulate and retain pathogens. In the Gulf Coast (USA), these authors found that the period of hyper-accumulation of F^+ coliphage begins as water temperature decreases in the autumn and ends when temperature begins to rise in early spring (Burkhardt and Calci, 2000). Although seawater temperature is a good predictor of the temporal variation of NoV contamination, it clearly cannot, on its own, predict the overall degree of NoV contamination occurring within a site. For this, the role of other variables was investigated. In the regression models describing the relationships between geometric mean NoV concentrations and the demographic, pollution source and other variables, the volume of continuous sewage discharges in the catchment was the only variable that entered in the models for GI, GII and GI + GII. This indicates that the NoV risk can be estimated based on the volume of sewage discharged into the SPAs and therefore that NoV contamination will be dependent on the site-specific sewerage discharge arrangements. However, effective risk management tools that incorporate both profiling of NoV levels in sewage discharges and abundance and distribution in SPAs do not currently exist in the peer

reviewed literature. Other variables significantly associated with NoV in shellfish detected in this study were total population in the catchment (positive association), and urban area and the number of intermittent discharges in the catchment (negative association) (NoV GI); number of continuous discharges (positive association with GII); and catchment area (positive association with GI + GII). These results are useful for identifying catchments at risk of NoV contamination as part of sanitary surveys for SPAs and/or for identifying suitable areas for new shellfish farming operations. Although current policy for designation of SWPAs takes into consideration the hygiene status of the classified production area(s), the health risk is not always apparent from *E. coli* monitoring and this is an area where these results could be used to inform policy on SWPA designations. It should be noted, however, that the levels of explained variance of the linear regression models for human population, catchment area and pollution source-related variables were consistently low and therefore not useful for NoV risk management purposes. There are two possible justifications for this. Firstly, the shellfish sampling points were not chosen to represent the worst-case scenario of sewage contamination at the study sites. Secondly, models describing the relationships between microbiological contamination in shellfish and environmental factors usually have lower levels of explained variance than those based on microcosm data (Kay *et al.*, 2008a). The most operationally useful model described the relationship between geometric mean levels of NoV GI + GII in oysters and the number of sewage spills (R^2 = 74%) at 10 study sites. The model predicted that a NoV concentration of 100 copies/g would correspond to 14 sewage spills. Lowther *et al.* (2012) compared NoV concentrations in oyster samples strongly linked to NoV-type illness with the concentrations typically found in commercial production areas. The geometric mean levels in non-outbreak samples was 121 copies/g while the mean levels in outbreak samples was 1,048 copies/g. These results suggest that the current policy objective for discharges impacting SWPAs based on an average of 10 spills over 10 years could be protective of public health. This assessment does not consider the known large variations in volumes discharged and concentrations which are typical of these intermittent discharges. To develop a policy to reduce human exposure to NoV associated with SOs, more data are needed on NoV concentrations in these intermittent discharges. This data gap has been addressed in this research and the results are presented in Chapter 3 below.

In this study, no association was found between any of the human faecal pollution-related variables and the *E. coli* levels in the oysters. Concentrations of FIOs in estuarine and coastal waters are associated with fluxes from the catchments and from point sources along the coast. In E&W, grasslands and their associated grazing livestock are key sources of *E. coli* contamination, particularly during high flow conditions. Loadings from agricultural sources can be as high as those from urbanised areas (Kay *et al.*, 2010). Furthermore, water companies have been required to install UV disinfection systems at STW discharging to SWPAs and reductions in average levels of *E. coli* in shellfish have been detected at many sites as a result of these sewage improvement schemes (Campos *et al.*, 2013). The lack of association between *E. coli* levels and the sewage pollution-related variables can therefore be attributed to the potentially large contribution of diffuse sources from agricultural land to total loadings and/or to the effect of UV disinfection in reducing fluxes of bacteria from sewage discharges impacting the study sites. More comprehensive predictive modelling studies focused on FIOs that incorporate contributions from both agricultural and urban sources have been given in the literature (Kay *et al.*, 2005; Crowther *et al.*, 2011).

In conclusion, statistically significant predictive regression models were developed for concentrations of NoV and *E. coli* in oysters. The NoV models were dominated by water temperature, river flow and volume of sewage discharge variables while the *E. coli* models were only associated with cumulative rainfall. The regression models had universally low levels of explained variance and were not found to be of practical use for risk management. To develop risk management measures from NoV based on sewage pollution variables, data are needed on typical concentrations of NoV in untreated sewage and treated effluents and NoV levels in shellfish to represent transport pathways in the receiving waters. The present thesis has addressed these data needs and the results of these investigations are subsequently presented in the experimental Chapters 4–6 below.

Chapter 3

Field Studies on Norovirus and *E. coli* in Sewage, Treated Effluents and Rivers

"The conservation of the shell-fish industry in polluted waters may be aided by the sterilization of sewage before discharge therein, but shell-fish should not be harvested or marketed from water exposed to sewage pollution except in such localities where properly instituted technical authorities shall determine the origin of pollution to be sufficiently remote from the shell-fish beds to avoid contamination." Webster *et al.* (1915, p. 1354)

As suggested by the results of the generic modelling studies reported previously, current risk management measures based on *E. coli* monitoring may not adequately characterise the NoV risk because fluxes of the virus within catchments are mostly associated with sewage-related sources of pollution. Future risk management measures for NoV could consider discharge concentration limits for sewage discharges and rivers. To develop these measures, the policy community requires data on NoV concentrations in untreated sewage and treated effluents to support catchment profiling initiatives. These data do not currently exist for many sewage treatment types used in the UK and, where these data exist, studies have been limited in temporal coverage (La Rosa *et al.*, 2010; Palfrey *et al.*, 2011). In addition, few studies have quantified typical NoV concentrations in primary settlement and certain types of biological treatment such as trickling filters (Nordgren *et al.*, 2009). In communities served by combined sewerage systems, sampling has not been undertaken during wet weather periods (Katayama *et al.*, 2008) and data are lacking on how NoV concentrations in SOs compare with those in untreated sewage and treated effluents. From a health risk perspective, this is essential because these discharges are more likely to contain infective viruses.

A study protocol was developed to address these data gaps. Concentrations of *E. coli* and NoV were quantified in sewage samples collected from four full scale STW operating treatment processes characteristic of primary-, secondary- and tertiary-treated effluents. In addition to characterising the quality of effluent discharges, this study evaluated the *E. coli* and NoV removal efficiencies at these works. Comparative data on

concentrations of *E. coli* and NoV in rivers were also obtained to provide an indication of the relative risks of NoV contamination in catchments with complex riverine networks.

Two catchments on the south coast of England with representative STW and different characteristics relating to pollution sources and hydrodynamics were selected for this sampling programme. Both sites contained areas classified under European regulations for commercial production of shellfish. One of the catchments drains into a shallow estuary principally impacted by a large discharge at the head of the estuary (site number 13 studied in the generic modelling reported in Chapter 2). This site is representative of most commercial shellfisheries in E&W. The second study site (designated site 32) drains into a deep coastal embayment with offshore areas leased for production of mussels. A more detailed description of the STW and associated catchments is given below. Concentrations of *E. coli* and NoV were also monitored in shellfish at these sites and these data are presented in Chapter 4. Information on commercial fisheries in these sites is however given in this chapter to avoid duplication.

Author contributions: the author of this thesis designed the study, liaised with the water company asset managers, carried out all the fieldwork and data analyses and drafted the manuscript. Colleagues at Cefas carried out the microbiological testing and gave final approval for publication of the manuscript.

3.1 Study Catchments

Study catchment 13

The total catchment area is also relatively large (242 km^2). The total resident population in the catchment is approximately 106,000. Land use is dominated by improved grassland used for livestock production; areas of arable land, mixed woodland, coniferous forest and woodland shrub occur in the upper catchment. Urbanised areas are concentrated in the lower catchment and these are also interspersed by arable land and pastures. Most of the urban areas are served by combined sewerage systems. Mean annual rainfall is 958 mm. Topography gently slopes from 254 m AOD in the upper

catchment to 21 m AOD in the lower catchment. The catchment is drained by two rivers and several smaller streams discharging at various locations around the estuary. The largest river (mean flow = 0.328 m³/s) flows on the eastern side of the catchment through rural and urban areas in the lower catchment before it discharges into a shallow estuary. This river has an ephemeral flow regime with extended periods of no flow.

The estuary (intertidal area = 2,342 hectares) is a shallow semi-enclosed tidal inlet with four confluent channels (Figure 6). The portion of the estuary subject to detailed analysis is about 13 km in length and meanders from the mouth of the estuary. The mean spring tidal range in the estuary is 4.2 m (macro-tidal). Surface water currents in the estuary are driven by a combination of tides, wind effects and freshwater inputs. Tidal currents follow the morphology of the channels. The flow ratio (volume of freshwater:tidal exchange) is low indicating relative good mixing of the water column in the channels. Wind-driven currents are more pronounced near the estuary entrance.

Native oysters (*Ostrea edulis*), common cockles (*C. edule*) and clams (*Tapes* spp.) are commercially harvested in the estuary and classified under Regulation (EC) No 854/2004. Oyster beds occur throughout the main subtidal channels. In 2014, oyster beds were class C in the main estuary channel and class B in the adjacent channels closer to the estuary mouth. Oyster harvesting is prohibited from March to October, inclusive, for stock conservation reasons. Cockles and clams are harvested in the upper reaches of one of the confluent channels and is class C under Regulation (EC) No 854/2004. The estuary has three SWPAs designated under the WFD.

In the lower reaches of the catchment, there are two STW operated by the water company which provide secondary treatment followed by UV-disinfection and discharge directly to the estuary (STW A and STW B; Figure 6). These UV disinfection plants were added to the existing treatment works in 2008 to satisfy the requirements of the UWWTD. A third STW provides secondary treatment and discharges to a watercourse. In the upper catchment, there is a further STW which provides secondary treatment but, based on information published in the sanitary survey report for this area, this discharge is unlikely to impact on the study site (https://www.cefas.co.uk/cefas-data-hub/food-safety/sanitary-surveys/). In addition to these continuous discharges, there

are 18 intermittent sewage discharges, including CSO, storm tank overflows (STOs) and overflows from pumping stations. Of these, five discharge directly to the estuary.

The main source of microbiological contamination impacting the estuary is STW A which discharges into the main meandering channel of the estuary (Figure 6). This is supported by the results of catchment investigations, microbiological source apportionment and microbial source tracking studies previously undertaken in this catchment by Cefas (Dancer *et al.*, 2013). The estuary has three SWPAs designated under WFD.
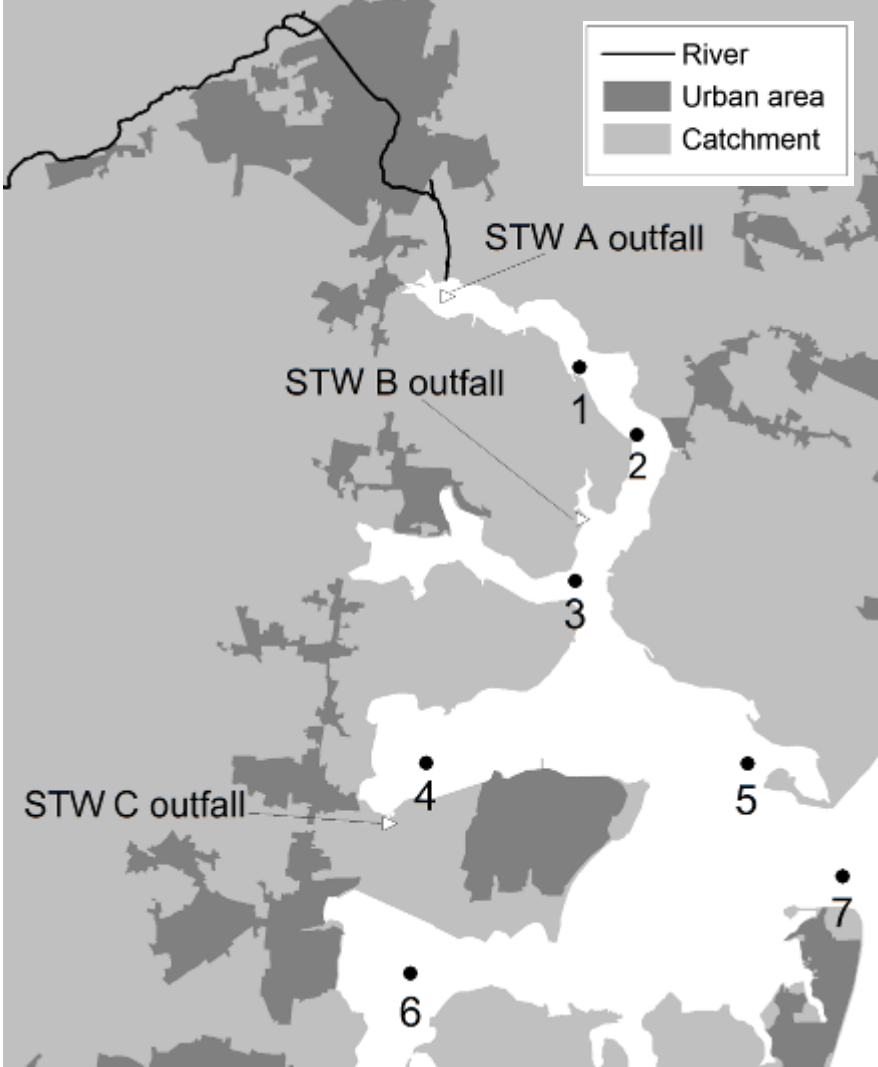
Figure 6 Schematic representation of the study site 13.
The sampling stations used in the field studies described in Chapters 5 and 6 below are also shown.

Study catchment 32

The total catchment area is 317 km². Land use in this catchment is predominantly improved grassland used for livestock production with some areas of arable land and deciduous woodland along the river valleys and areas of mineral extraction in the upper reaches. The lower catchment is relatively more urbanised (Figure 7). The urban areas are served by a central combined sewerage system while more recently developed areas are served by separate systems. Mean annual rainfall is 1,506 mm. Topography is moderately sloping in the lower catchment, but rapidly becomes steeply sloping in the upper catchment (maximum altitude = 400m above ordnance datum (AOD)). The largest river flows on the east of the catchment from 130 m at the headwaters and discharges into a coastal embayment near a sandy beach. Several smaller watercourses and surface water drains discharge at various locations on the shoreline.

The bay is approximately 6 km wide and recessed by 3.5 km. It is bounded by maritime cliffs and slopes and beaches. The bathymetry gently slopes from about 5 m (Chart Datum; CD) near the shore down to about 15 m (CD) in the centre of the bay. The western part of the bay is generally deeper than the eastern part. The tidal range is moderately large (mean spring range = 4.5 m; macro-tidal). In the approaches to the bay, tidal currents often exceed 0.25 m/s.

In the bay, there are areas leased for production of common mussels (*Mytilus* spp.). These shellfish farms use longlines with droppers suspended from headlines submerged at about 2 m depth to minimise disturbance from wave action. Both mussel production areas are class B under Regulation (EC) No 854/2004. Mussel harvesting takes place on a year round basis. The bay is a SWPA under the WFD.

The sanitary survey report for this area indicates that the largest source of sewage pollution impacting the bay is a STW lying just south of the urban area (https://www.cefas.co.uk/cefas-data-hub/food-safety/sanitary-surveys/; Figure 7). This STW discharges secondary-treated effluent via a 1.25 km long sea outfall (LSO) with a 45 m long diffuser head at a depth of 12 m (CD). The outfall is approximately 1.5 km to the northeast of the nearest mussel production area.
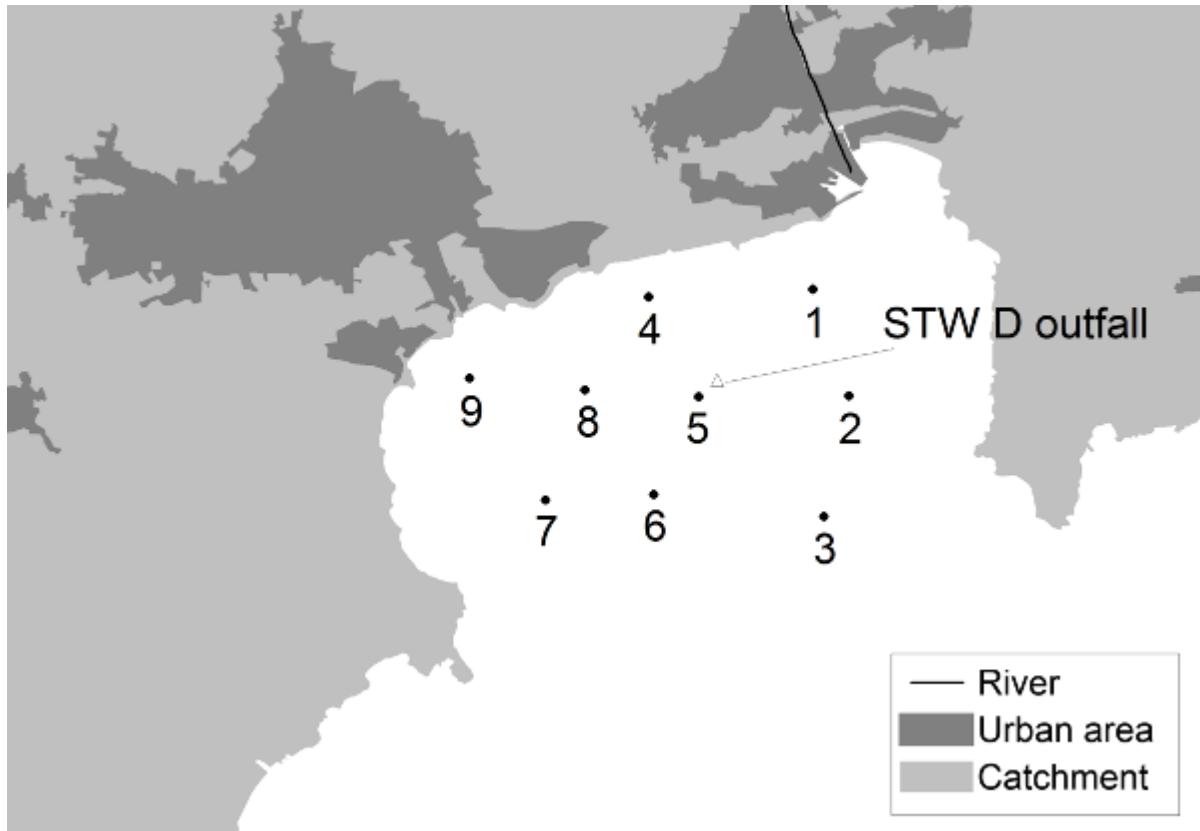
Figure 7 Schematic representation of the study site 32.
The sampling stations used in the field studies described in Chapters 5 and 6 below are also shown.

3.2 Sewage Treatment Works

Sewage samples were collected at screened influent, primary, secondary (biological) and tertiary (where applicable) stages of the treatment processes and at SO discharges associated with the STW (Figure 8).

Figure 8 Aerial photographs of the sewage treatment works monitored in the study.
Inf - influent (screened); PST - primary settlement tank; ST - storm tank; AS - activated sludge; TF - trickling filter; HT - humus tank; BAF - biological aerated filter; RF - roughing filter; FST - final settlement tank; UV - ultra-violet disinfection.

The characteristics of the STW in relation to individual types of treatment, PEs and design flows are summarised in Table 10. In STW A, STW B and STW C, monitoring was undertaken during the period October 2012–January 2015. In STW D, monitoring was undertaken during February 2014–July 2016. These different monitoring periods are associated with logistical constraints however the monitoring data are representative of periods of both high and low prevalence of NoV. During the study period, a new UV plant was installed in the storm tank at STW A to disinfect SO discharges. The same sampling protocol was used in all STW. Sampling was scheduled to represent periods of low and high prevalence of NoV in the catchment populations based on information on NoV outbreaks in hospitals published weekly by Public Health England. Opportunistic sampling was undertaken in the storm tank at STW A to characterise NoV concentrations when this sewerage network was surcharged with stormwater (see section 3.3 below). Sewage samples were collected in the morning to coincide with

higher flow rates through the STW. Sewage sampling was usually undertaken no longer than 2 h before the shellfish sampling.

Freshwater samples were also taken from the main rivers discharging into the SPAs shortly after the sewage sampling. In both catchments, the sewage sources are situated downstream from the river discharge points as illustrated in Figures 6 and 7.

Table 10 Characteristics of sewage treatment works monitored in the study.

	STW A	STW B	STW C	STW D
Study catchment ID	13	13	13	32
Dry weather flow of discharge (m³/day)	11,458	1,221	6,565	8,414
Average daily flow (1.25 x DWF) (m³/day)	14,322	1,526	8,206	10,517
PE	36,300	3,847[a]	20,381	22.140[b]
Primary treatment	Grit removal; primary settlement	Grit removal; primary settlement	Grit removal; primary settlement	Grit removal; primary settlement;
Secondary (biological) treatment	Optimised activated sludge (MLE) followed by Nitrogen removal	Trickling filters; sand filters; humus tanks	Sand filters; humus tanks	Roughing filter; biological-aerated filters; humus tanks
Tertiary treatment	UV disinfection	UV disinfection		
Event duration monitor (EDM) recording spills from storm tank (Yes/No)	Yes[c]	Yes	Yes	No

[a] Population equivalent reported in the discharge consent. [b] Estimated population equivalent for 2011. [c] During the study, a new UV disinfection plant was installed to treat settled stormwater and reduce the impact of frequent discharges from the storm tank on the receiving waters.

3.3 Sewage Treatment Works Operational Performance Data

Notifications of spills of settled stormwater from STW A were received from the water company in real-time by e-mail during the study period. These notifications were used to identify sampling dates. Data on flows to full treatment, average retention times and measured applied UV dose for STW A were also supplied by the water company on

request. Records of flows to full treatment (15-minute interval measurements) for the most significant discharges impacting the study sites (STW A and STW D) were also obtained from the water companies on request.

3.4 Sewage and Freshwater Sampling

A total of 13 samples of freshwater and 170 samples of sewage (single grab) were collected manually directly into 250 ml sterile polysterene containers (Sterilin™) using a telescopic sampling pole. Samples were collected manually because auto-samplers do not guarantee aseptic conditions. The sample containers were wiped with alcohol impregnated towel, placed in labelled plastic bags and stored in the dark inside Icey-Tek Ocean Blue cool boxes containing freezer packs and transported to Cefas Weymouth Laboratory for testing. The time lapse between sample collection and beginning of microbiological testing did not exceed 24 h. The same sampling protocol was utilised in all STW.

3.5 Microbiological Methods

Concentrations of *E. coli* were quantified in sewage samples following standard UK methods based on membrane filtration (Standing Committee of Analysts, 2009). Coliform bacteria were isolated by incubation on membrane lauryl sulphate broth for 4 h at 30 °C followed by 14 h at 44 °C (± 0.5 °C). *E. coli* were isolated by sub-culture of up to 10 colonies from each membrane on nutrient agar at 37 °C for 24 h. The pure cultures were tested for oxidase then inoculated onto MacConkey agar and incubated at 44 °C for 24 h to confirm lactose fermentation. Cultures were also inoculated onto tryptone nutrient agar and incubated at 44 °C for 24 h. Indole formation was demonstrated by adding two or three drops of Kovacs' reagent to each plate and the development of a pink-red colour in the agar. Colonies that were oxidase negative and positive for lactose and indole were recorded as confirmed *E. coli*. The proportion of *E. coli* from each membrane was then used to calculate the *E. coli* count on the corresponding coliform

plate. *Escherichia coli* concentrations were reported by the laboratory as colony forming units (cfu)/100 ml.

Concentrations of NoV were quantified in sewage and freshwater using an ultra-centrifugation method described in Appendix II. The preparation of wastewater concentrates followed the procedure developed by Cross (2004) as modified by Puig *et al.* (2004). These methods employ PCR to target NoV GI and GII. NoV concentrations were reported by the laboratory as detectable genome copies/ml.

3.6 Statistical Analyses

The distribution of microbiological concentrations found in sewage samples showed a closer approximation to normality when $\log_{10}$-transformed (Appendix III). These data were, therefore, $\log_{10}$-transformed prior to statistical analyses. Descriptive statistics (Min, Max, GM, standard deviation of $\log_{10}$-transformed concentrations [$\log_{10}$SD], 95% CI) were used to characterise the distributions of microbiological results in sewage. Modified boxplots showing Max, Min, GM and 95% CI were used to graphically display the distributions of microbiological concentrations (Krzywinski and Altman, 2014). Student's t-test was used to examine differences between arithmetic means of the $\log_{10}$-transformed microbiological concentrations between different monitoring periods and between different types of sewage treatment following Levene's homogeneity of variances test. The reduction of microorganisms through the sewage treatment processes was calculated using the formula:

$\log_{10}$ reduction = ($\log_{10}$initial concentration) - ($\log_{10}$final concentration)

The removal efficiency was also calculated using the formula:

%removal = 100%(initial concentration - final concentration/initial concentration)

The formula used to convert $\log_{10}$ reduction to percent reduction was:

$P = (1 - 10^{-L}) \times 100$ where P is the percent reduction and L is the $\log_{10}$ reduction

3.7 Results

3.7.1 Prevalence, Seasonality, and Variation of *E. coli* and Norovirus in Influent and Effluent

During the study period (October 2012–June 2015) and considering all the STW studied, NoV was frequently detected in samples of screened influent with 86% (n = 31) of samples positive for GI and 94% (n = 34) of samples positive for GII. Of these, 30 samples had quantifiable NoV GI concentrations and 33 had quantifiable NoV GII concentrations. NoV concentrations ranged over 4 $\log_{10}$ orders of magnitude for GI and 5 $\log_{10}$ orders of magnitude for GII while corresponding *E. coli* concentrations ranged only 2 $\log_{10}$ orders of magnitude. The geometric means of NoV GI and GII in influent samples collected during the period of high prevalence (October–March) were 335 copies/ml and 2,806 copies/ml, respectively while means of GI and GII during the period of low prevalence (April–September) were 1,734 copies/ml and 6,571 copies/ml, respectively. It should be noted however that only 10 samples were collected during this period. During the period of high prevalence, 20 samples had quantifiable results for GI and 23 samples had quantifiable results for GII. However, there was no evidence of significant differences in NoV concentrations (student's t-test) between the two periods.

In final effluent samples, mean and ranges of concentrations of NoV GI and GII during the period October–March were 7 copies/ml (1–1,060) copies/ml and 42 (1–2,756) copies/ml. During the period April–September, mean concentrations of GI and GII were 107 (1–44,904) copies/ml and 188 (1–40,899) copies/ml, respectively.

Figure 9 shows geometric means, 95% CI and ranges of $\log_{10}$-transformed concentrations of NoV in influent and effluent samples collected in the four STW studied for three periods of typical high prevalence of NoV (October–March). Mean concentrations of GI and GII in the influent were higher in 2012/13 and 2014/15 than those in 2013/14. For GI, the seasonal differences in NoV concentrations between periods were less than 1 $\log_{10}$ while for GII the differences were more than 1 $\log_{10}$. Many data groups contained fewer than 10 samples with quantitative NoV results which precluded statistical analyses of differences between means.

Information on NoV outbreaks in hospitals published by Public Health England, which is a good indicator of NoV levels in the general population, showed lower numbers of

outbreaks in the winter of 2013/14 (Public Health England, 2015). This is consistent with the lower mean concentrations of NoV in influent samples during this period however more site-specific data would be required to confirm this relationship. The same variation of NoV concentrations between years was not evident in final effluent samples.
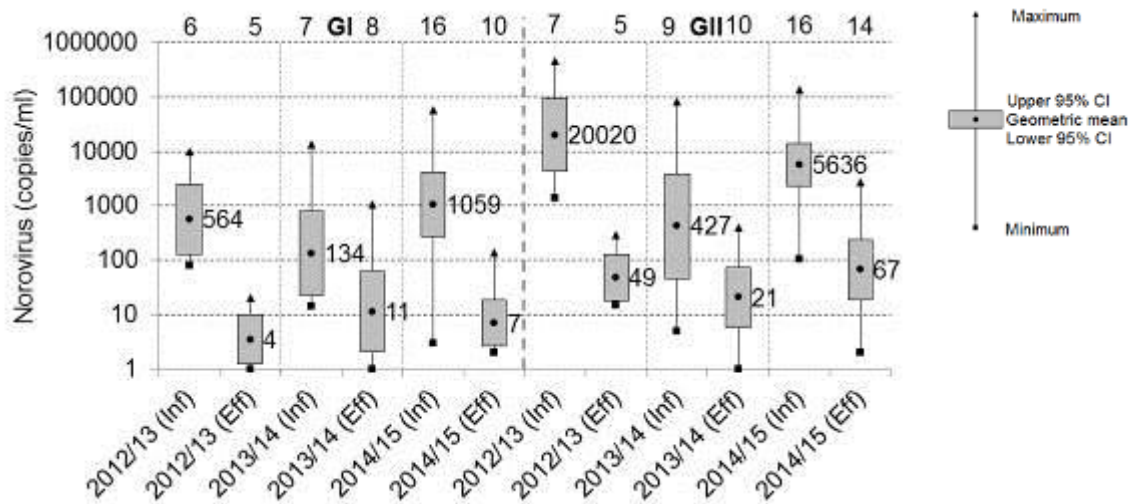
Figure 9 Box and whisker plots of concentrations of norovirus in untreated influent and final effluent for three periods of high virus prevalence at four sewage treatment works.
Data periods: October 2012–March 2013, October 2013–March 2014 and October 2014–March 2015. The numbers of samples with quantitative results are shown above the boxplots.

3.7.2 Comparison of Levels of *E. coli* and Norovirus Between Untreated Sewage and Storm Tank Overflow Discharges

Geometric means, 95% CI and ranges of $\log_{10}$-transformed concentrations of NoV and *E. coli* in samples of untreated sewage and STOs collected in STW A are shown in Figure 10. These samples were collected under high-flow conditions (i.e. during or shortly after rainfall events) through the treatment works. Concentrations of *E. coli* ranged from 1,800,000 to 15,800,000 cfu/100ml in untreated sewage and from 975,000 to 5,480,000 cfu/100ml in STOs. For NoV GI, concentrations in untreated sewage ranged from 80 to 13,475 copies/ml while concentrations in STOs ranged from 11 to 75,174 copies/ml. For NoV GII, concentrations in untreated sewage ranged from 514 to 449,783 copies/ml while concentrations in STOs ranged from 48 to 73,953 copies/ml. T-tests revealed significant ($p < 0.001$) differences in GM concentrations of *E. coli* between untreated and STOs. However, no significant differences were found in GM concentrations of NoV between these two types of sewage samples.
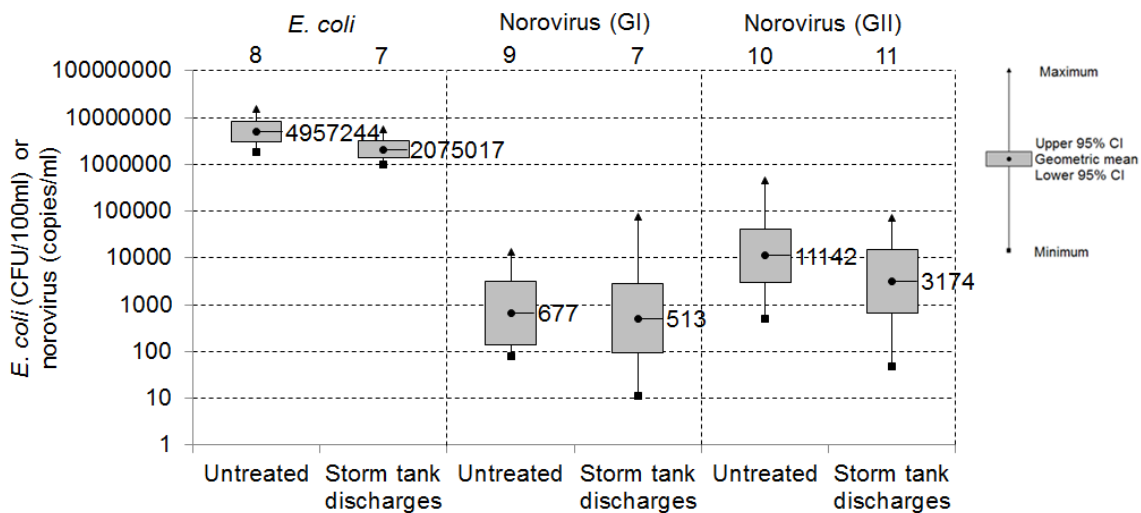
Figure 10 Comparison of concentrations of *E. coli* and norovirus in untreated sewage and storm tank discharges from sewage treatment works A.

The numbers of samples with quantitative results are shown above the boxplots.

3.7.3 Comparison of Norovirus in Sewage Influent and Freshwater

To compare concentrations of NoV in sewage influent at STW A and STW D with those in the rivers discharging to the respective estuary and bay, 13 sets of samples were collected on the same occasions. The rivers discharge upstream of the sewage outfalls and therefore provide a measure of NoV contamination from all pollution sources at the catchment outlet locations. Of the freshwater samples tested, 77% were positive for at least one genogroup. The results indicated consistently more positive samples and higher virus titres in untreated sewage than in freshwater samples (Table 11). Mean levels of GII in freshwater samples were 71 copies/ml while mean levels in influent sewage were 4,681 copies/ml (positive samples with zero values were removed from the dataset). These results confirmed the assumption that sources of sewage pollution in the upper reaches of the catchment were of minor significance concerning NoV contamination impacting the study sites. In the estuary (site 13), the main sources were continuous and intermittent sewage discharges on the shoreline, whilst in the bay (site 32) the main source would be the identified marine LSO.

Table 11 Comparison of concentrations of norovirus in sewage influent and rivers discharging to the study sites.

Date	Weather condition	Freshwater		Influent (screened)		Study catchment
		GI	GII	GI	GII	
12/11/2012	Wet weather	(-)	(+) 63	(+) 9,765	(+) 449,783	13
05/12/2012	Wet weather	(+) 0	(+) 1,116	(+) 0	(+) 16,257	13
13/02/2013	Wet weather	(+) 0	(+) 132	(+) 167	(+) 8,076	13
29/01/2014	Wet weather	(-)	(-)	(+) 13,475	(+) 80,363	13
17/02/2014	Wet weather	(-)	(-)	(+) 104	(+) 102	32
26/03/2014	Wet weather	(-)	(-)	(+) 26	(+) 12,611	32
02/06/2014	Dry weather	(+) 0	(+) 0	(+) 10	(+) 809	32
18/08/2014	Wet weather	(+) 4,891	(+) 0	(+) 56,557	(+) 7,136	32
17/11/2014	Wet weather	(+) 0	(+) 0	(+) 281	(+) 1,128	13
06/01/2015	Wet weather	(+) 0	(+) 0	(+) 961	(+) 1,384	13
04/03/2015	Wet weather	(-)	(+) 2	(-)	(+) 104	13
25/03/2015	Wet weather	(-)	(+) 25	(+) 184	(+) 18,884	13
10/06/2015	Dry weather	(+) 0	(+) 272	(+) 1,240	(+) 4,789	13

Samples were classified as dry weather if there had been no rainfall in the contributing catchment in the week preceding sampling. GI - genogroup I; GII - genogroup II; (-) - negative sample; (+) - positive sample; 0 - sample with no quantitative result.

3.7.4 Levels of *E. coli* and Norovirus in Different Levels and Types of Sewage Treatment

Ranges, GM and 95% CI of *E. coli* and NoV levels in all samples of untreated sewage and treated effluents regardless of STW flow conditions and risk periods are summarised in Table 12. Overall, mean *E. coli* concentrations decreased as levels of treatment increased from 10^6 cfu/100ml in untreated (screened) sewage to 10^1 cfu/100 ml in UV disinfected effluent. Evidence was found of significant differences (t-test; $p < 0.001$) in GM levels of *E. coli* between untreated and primary-treated effluents. *E. coli* concentrations in secondary-treated effluents were 1.5 $\log_{10}$ lower than those in primary-treated effluents while *E. coli* concentrations in secondary-treated effluents were 3.2 $\log_{10}$ lower than

those in tertiary treated effluents (UV disinfected). Mean concentrations of NoV for individual treatment levels also decreased as treatment levels increased. There was no evidence of changes in the relationship between NoV GII and NoV GI (Levene's homogeneity of variances test > 0.05) over the different treatment stages (Figure 11).
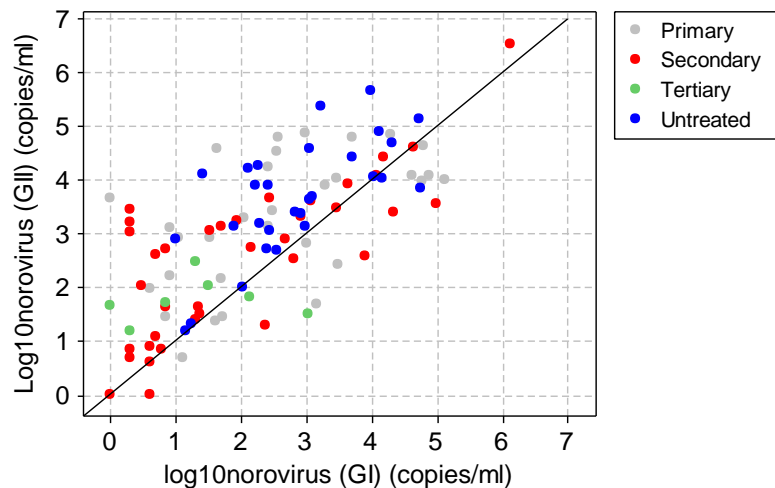
Figure 11 Relationship between norovirus genogroups I and II in untreated sewage and effluents from primary, secondary and tertiary treatments.
The line of equality is shown in black.

In contrast to *E. coli*, considerable variability was found in virus concentrations as indicated by the standard deviation of $\log_{10}$-transformed results. The largest ranges were detected in secondary-treated effluents (a range spanning 6.1 $\log_{10}$ for GI and 6.5 $\log_{10}$ for NoV GII) (Figure 12). The ranges of NoV concentrations in UV disinfected effluents (3 $\log_{10}$ for GI and 2.9 $\log_{10}$ for GII) were lower than those in other levels of sewage treatments.

Paired t-tests evidenced differences in GM concentrations of NoV GI and GII between primary and secondary ($p < 0.001$) and between secondary and tertiary ($p < 0.001$; GII only) levels of treatment at the 0.05 level of significance. Paired t-tests were also conducted to evaluate if there were significant differences in mean $\log_{10}$ NoV levels between individual types of treatment. Only datasets with ≥ 10 results were tested. These analyses evidenced differences in the concentrations of NoV GII in influent (screened) and those in primary settled effluents ($p = 0.025$) and stored settled sewage ($p = 0.025$).

Table 12 Summary statistics of concentrations of *E. coli* and norovirus in untreated sewage and treated effluents.

	E. coli (cfu/100ml)					Norovirus (genogroup I) (copies/ml)								Norovirus (genogroup II) (copies/ml)							
	n	GM	Log₁₀ StDev	Lower 95% CI	Upper 95% CI	n	% (pos.)	GM	Log₁₀ StDev	Lower 95% CI	Upper 95% CI	Min.	Max.	n	% (pos.)	GM	Log₁₀ StDev	Lower 95% CI	Upper 95% CI	Min.	Max.
Influent	34	5,745,971	0.474	3,982,293	8,290,746	30	86	579	1.123	230	1,462	3	56,557	33	94	3,631	1.172	1,446	9,121	5	449,783
Primary																					
SS	9	1,541,086	0.340	924,539	2,568,792	10	83	398	1.189	73	2,168	11	75,174	11	92	1,251	1.371	194	8,078	15	73,953
PST	25	3,909,678	0.540	2,402,084	6,363,468	22	88	388	1.528	89	1,688	1	134,345	24	96	1,543	1.173	524	4,545	5	69,217
Secondary																					
AS	14	104,210	1.105	27,477	395,230	10	88	1,839	1.845	132	25,580	3	1,378,444	12	88	1,489	1.431	231	9,606	18	3,396,249
TF	15	279,402	0.746	117,086	666,739	10	73	77	1.650	7	809	1	21,992	10	62	466	0.840	141	1544	12	2,909
HT	18	103,132	0.641	52,137	204,005	14	94	25	1.446	4	146	1	44,904	15	95	150	1.373	30	742	1	40,899
BAF	11	31,469	0.783	10,839	91,365	9	75	28	1.221	4	173	2	8,152	11	92	79	1.055	19	332	1	1,770
Tertiary																					
UV	24	60	0.963	25	146	14	82	9	0.900	3	27	1	1,060	18	90	45	0.628	23	88	1	715

Inf. - influent (screened); primary - primary treatment; SS - stored settled sewage; PST - primary settlement tank; secondary-secondary treatment; AS - activated sludge; HT - humus tank; TF - trickling filter; BAF - biological aerated filter; tertiary - tertiary treatment; UV - UV disinfected effluent; n - total number of samples; StDev - standard deviation; CI - confidence interval; n (pos.) - number of positive samples; Min. - minimum; Max. - maximum.
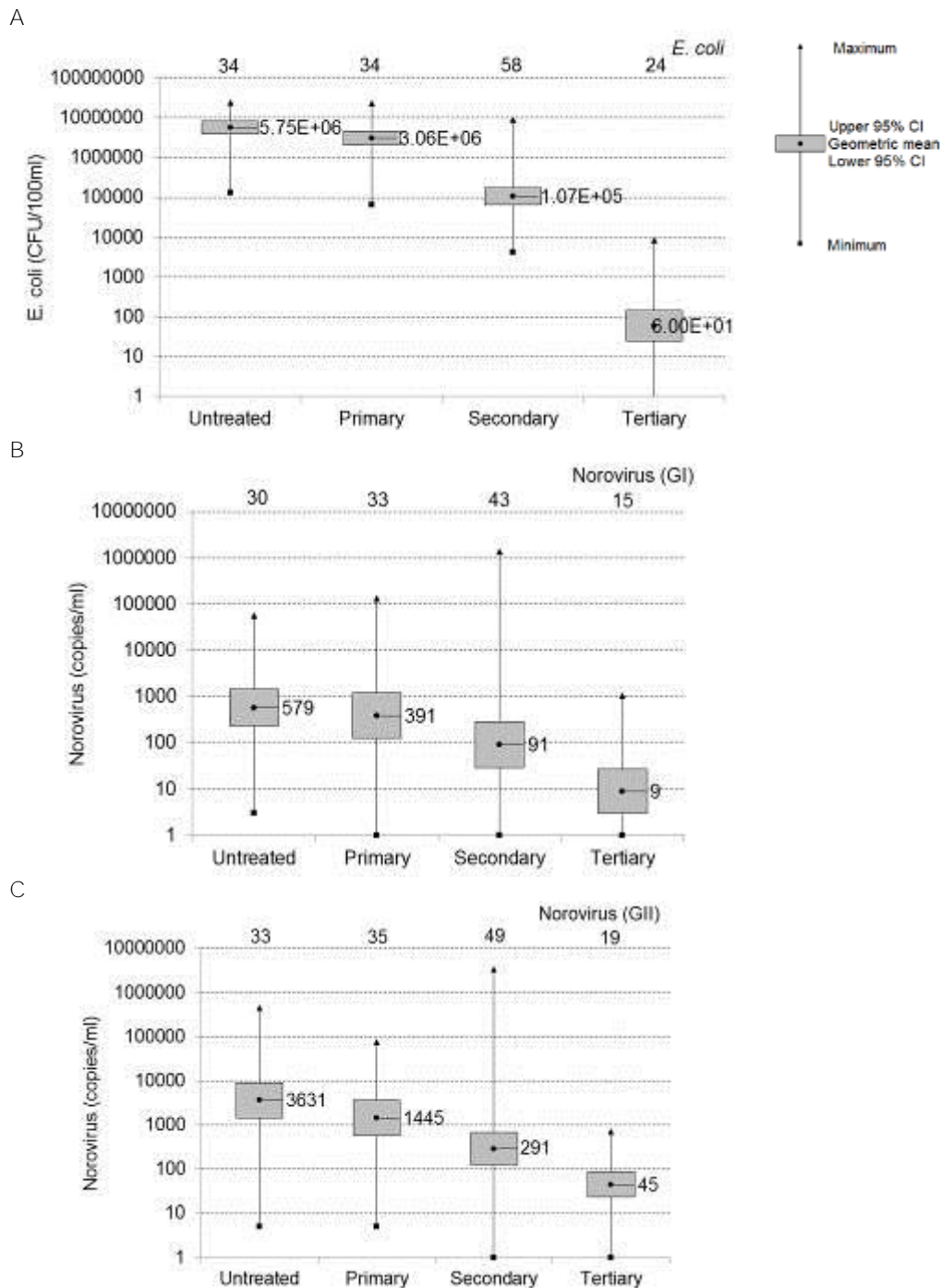
Figure 12 Box and whisker plots of concentrations of *E. coli* (A) and norovirus (GI) (B) and GII (C) in sewage subject to different levels of treatment.
Data from four sewage treatment works. The numbers of samples with quantitative results are shown above the boxplots.

Different STW delivered markedly different removal rates of microbiological contaminants (Table 13). Biological treatment followed by UV disinfection was the most effective in removing *E. coli* contamination from sewage with STW A and B achieving 5.3 $\log_{10}$ (99.9995%) and 4.7 $\log_{10}$ (99.9980%) removals, respectively. The least effective in

removing *E. coli* was STW C (trickling filters and humus tanks) (2.1 $\log_{10}$ or 99.2%) and STW D (biotower and humus tanks) (2.4 $\log_{10}$ or 99.6%), respectively.

Concerning NoV removal, STW A operating activated sludge followed by UV disinfection was more effective than the other STW studied with average removal rates of 2.3 $\log_{10}$ (99.5%) for GI and 2.6 $\log_{10}$ (99.7%) for GII (Table 13). Sewage treatment works A, C and D achieved maximum NoV GII removals in excess of 3 $\log_{10}$ (99.9%).

Table 13 Total removal ($\log_{10}$) of *E. coli* and norovirus achieved in the sewage treatment works studied.

					Norovirus (copies/ml)							
	E. coli (cfu/100 ml)				Genogroup I				Genogroup II			
	n	Min.	Max.	Av.	n	Min.	Max.	Av.	n	Min.	Max.	Av.
STW A	13	3.25	6.41	5.31	9	1.27	3.22	2.31	10	1.97	3.69	2.60
STW B	6	3.86	5.77	4.71	1	-	-	2.54	2	2.02	2.13	2.08
STW C	3	0.37	3.41	2.11	3	0.63	2.84	1.80	4	1.32	3.41	2.40
STW D	9	0.94	3.45	2.42	7	0.72	2.94	1.71	8	0.43	3.67	1.93

STW A - activated sludge + UV disinfection; STW B - trickling filters+sand filters; STW C - sand filters; STW D - roughing filter+biological aerated filters. n - number of samples with quantitative results; Min. - minimum; Max. - maximum; Av. - average.

For individual types of treatment and on average, primary settlement achieved less than 0.5 $\log_{10}$ reduction for *E. coli* and approximately 1.0 $\log_{10}$ reduction for both NoV genogroups (Table 14). Of the secondary treatments, the suspended growth process (optimised Ludzack-Ettinger activated sludge plant) was more effective than the attached growth processes (trickling filters, biological aerated filter) and humus tanks in removing NoV. Occasionally, the activated sludge process achieved NoV removal rates in excess of 3.0 $\log_{10}$ for both genogroups. UV disinfection was the most effective process in removing *E. coli* (2.5–2.8 $\log_{10}$) while, for NoV, this treatment process achieved less than 1.0 $\log_{10}$ removal (datasets with more than 1 pair of samples considered).

Table 14 Removal ($\log_{10}$) of *E. coli* and norovirus achieved by the different types of sewage treatment studied.

| | *E. coli* (cfu/100 ml) | | | | Norovirus (copies/ml) | | | | | | | |
| | | | | | Genogroup I | | | | Genogroup II | | | |
	n	Min.	Max.	Av.	n	Min.	Max.	Av.	n	Min.	Max.	Av.
STW A												
Primary settlement	7	0.00	2.09	0.52	3	0.57	0.73	0.64	4	0.01	2.50	1.17
Activated sludge	7	0.35	2.45	1.45	2	2.89	3.33	3.11	3	0.67	3.81	2.34
UV disinfection	10	1.18	3.81	2.77	4	0.19	1.56	0.84	5	0.05	0.89	0.41
STW B												
Primary settlement	4	0.02	0.38	0.14	3	0.30	2.18	1.01	1	-	-	0.01
Trickling filters	6	0.31	2.41	1.66	3	0.54	2.77	1.72	3	1.45	2.95	2.13
Humus tanks	5	0.19	2.01	0.73	1	-	-	0.00	1	-	-	1.34
UV disinfection	6	1.46	3.22	2.49	1	-	-	2.00	3	0.78	1.56	1.09
STW D												
Primary settlement	6	0.15	0.70	0.39	8	0.13	2.26	1.00	5	0.29	2.13	1.01
Humus tanks	10	0.32	1.54	1.11	4	0.60	1.14	0.77	6	0.21	2.41	1.10
Roughing filters	10	0.02	1.19	0.38	5	0.00	1.85	0.60	4	0.23	0.79	0.47
Biological aerated filters	7	0.58	1.39	1.04	4	0.00	0.83	0.39	3	0.46	0.94	0.72

n - number of samples with quantitative data; Min. - minimum; Max. - maximum; Av. - average.

5.2.2 Comparison of Levels of *E. coli* and Norovirus Between Different Levels and Types of Sewage Treatment

The process control data for STW A were combined with the microbiological data to evaluate the effect of flows to full treatment, average retention times and measured applied UV dose on NoV and *E. coli* removal. A low number of samples with quantitative NoV concentrations were available to perform these analyses. However, the relationships between NoV removals and the concentrations of NoV in the final effluent were studied for STW A (activated sludge followed by UV disinfection) and STW D (biological treatment only). In both STW, NoV GII removal increased as the concentration of the virus in the influent also increased. Figure 13 shows the results of linear regression analysis for STW A. The regression shows good fit to the data ($p = 0.000$) and high explained variance ($R^2 = 80\%$). The regression shows that an increase in influent concentrations by a factor of 10 would correspond to an increase of 0.5 $\log_{10}$ virus removal.
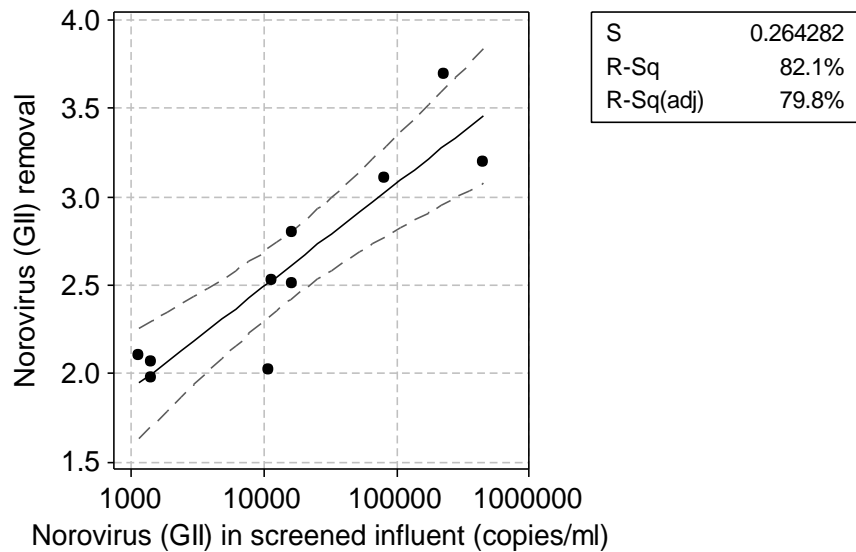
Figure 13 Removal (log$_{10}$) of norovirus (GII) as a function of the concentrations in screened influent to sewage treatment works A.
Linear model: log$_{10}$removal norovirus (GII) = 0.1655 + 0.5817*norovirus (GII) in influent.

3.8 Discussion

Concentrations of *E. coli* and NoV, and their reduction efficiencies, were quantified in four full scale STW operating different treatment processes characteristic of primary-, secondary- and tertiary-treated effluents commonly used in the UK. Since NoV cannot yet be routinely cultured, this study, like similar studies (Myrmel *et al.*, 2006; Katayama *et al.*, 2008; Nordgren *et al.*, 2009; Flannery *et al.*, 2012; Hata *et al.*, 2012), used RT-PCR for detection and quantification whereas *E. coli* was quantified using cultivation techniques. PCR does not distinguish between infectious and non-infectious NoV and this can be considered a limitation of the study. A positive PCR assay for NoV in sewage indicates the presence of nucleic acid in sewage effluents but cannot conclusively demonstrate the presence of live virus. Since sewage treatment processes subject microorganisms to a range of chemical and physical stresses that could impact on virus viability (Richards, 1999; Pecson, Martin and Kohn, 2009) it is possible that PCR methods underestimate reduction of NoV infectivity (Flannery *et al.*, 2013). This is particularly relevant in relation to UV disinfection treatments which may produce significant reductions in the numbers of infectious viral particles without showing equivalent reductions in the level of viral genomes quantified by PCR (Girones *et al.*, 2010). Studies have however demonstrated links between NoV quantified by PCR in

sewage effluents, shellfish and clinical samples from patients with NoV infection (Ueki *et al.*, 2005; Nenonen *et al.*, 2008; Wall *et al.*, 2011). Detection of NoV by PCR has also been useful in identifying the cause of outbreaks associated with drinking water contaminated with sewage (Häfliger, Hübner and Lüthy, 2000; Hewitt *et al.*, 2007). The results of this study therefore provide useful information to inform exposure assessments, particularly in relation to untreated and SO discharges in which NoV have not been subject to the same physical and chemical stresses that secondary and UV treated effluents produce.

This study found a high percentage of untreated sewage samples (86% for NoV GI and 94% for NoV GII) and final effluent (UV-disinfected) (82% for NoV GI and 90% for NoV GII) positive for NoV. This indicates that the virus was continuously shed by the populations connected to the STW and that effluents contaminated with NoV were released into the receiving waters during the study period. This high prevalence of NoV in sewage effluents is similar to that obtained in other geographical areas such as Toyama (Japan) (Iwai *et al.*, 2009) and Shandong Province (China) (Tao *et al.*, 2015).

In addition to sewage discharges, rivers and streams are important routes for NoV contamination impacting SPAs. Phylogenetic analysis of NoV capsid genes demonstrated similarity of gene sequences between infected humans, sewage effluents, contaminated freshwater and cultivated oysters in a study conducted in China demonstrating the linkage of these transmission pathways (Ueki *et al.*, 2005). In a further study conducted in Sweden, trace back linkage of NoV strains in mussels, and in human cases, highlighted the role of rivers in the environmental transmission of NoV causing large scale outbreaks (Nenonen *et al.*, 2008). In this study, freshwater samples were collected in the main rivers discharging into the study sites. Samples were collected at the catchment outlets to assess the relative contribution of the river network to NoV contamination in the receiving waters. The results indicated that 77% of freshwater samples were positive for at least one NoV genogroup. This is a larger percentage of positive samples than those reported in other studies. In a Europe-wide surveillance study of human enteric viruses in recreational waters, Wyn-Jones *et al.* (2000) found that NoV (either GI, GII or both) were detected more frequently in marine waters (16.4%) than in freshwater samples (6.3%). Maunula *et al.* (2012) found 31% of freshwater samples positive for NoV in the River Vantaa (Finland) while Jurzik *et al.* (2010) found 26% of

samples positive for NoV GII in the rivers Ruhr and Rhine (Germany). However, in this study, the frequency of NoV positive samples and virus concentrations in untreated sewage were higher than those in freshwater samples. Maximum levels of NoV in freshwater were 4,891 copies/ml (GI) and 1,116 copies/ml (GII), respectively while maximum levels in untreated influent were 56,557 copies/ml (GI) and 449,783 copies/ml (GII). For both GI and GII, the minimum NoV differences found were that freshwater contained approximately 1.0 $\log_{10}$ lower concentrations compared to sewage influent. Higher concentrations of NoV in rivers than in sewage effluents were found in the Maas and Waals catchment (The Netherlands) (Lodder and de Roda Husman, 2005). This information can help inform pollution reduction programmes and risk management strategies for SPA.

As mentioned in Chapter 1, NoV outbreaks occur throughout the year although there is a seasonal pattern of increased activity during the winter (Ahmed, Lopman and Levy, 2013). Consistent with this seasonality, higher prevalence of NoV in the colder months has also been found in sewage (Myrmel *et al.*, 2006; Katayama *et al.*, 2008; Pérez-Sautu *et al.*, 2012). Similarly, in this study, concentrations of NoV GII in untreated sewage were higher in the winter (December–February) than in the summer (June–August). This seasonality was consistent with laboratory reports of NoV outbreaks in hospitals reported to national surveillance in the local area (Public Health England, 2015). These results suggest that monitoring and typing NoV in raw sewage could provide early warning of the potential occurrence of NoV outbreaks in the community, as demonstrated by studies conducted in Sweden (Hellmér *et al.*, 2014) and France (Prevost *et al.*, 2015).

In this study, NoV GII was more prevalent, and found in higher concentrations, than NoV GI. This reflects the higher prevalence of GII strains, and particularly strain GII.4, in NoV outbreaks in recent years in the UK (Public Health England, 2015). Other studies have similarly found higher prevalence of GII strains in sewage and river water (Lodder and de Roda Husman, 2005; Myrmel *et al.*, 2006). In a study of NoV removal at four STW in France, da Silva *et al.* (2007) found that NoV GI concentrations arriving at the works were more variable than those of NoV GII. It has also been suggested that GI strains may be more resistant to sewage treatments than GII strains (da Silva *et al.*, 2007; Nenonen *et al.*, 2008; Nenonen *et al.*, 2009). However, molecular characterisation and genotyping

of NoV in wastewater and stool samples carried out in Ireland detected multiple genotypes in environmental samples with predominance of the GII.4 variant in sewage which was consistent with the prevalence of GII strains in the community at the time of the study (Rajko-Nenow *et al.*, 2013). In this study, although NoV GII was more prevalent than NoV GI, no evidence was found of changes in the ratio of GI:GII in either treated or untreated effluents. Therefore, it seems appropriate to consider the total NoV load (GI + GII) when evaluating the effectiveness of STW in reducing NoV and estimating the overall NoV risk from sewage effluents, as proposed by the EFSA Panel on Biological Hazards (EFSA, 2012).

The concentrations of *E. coli* found in different treatment types were of the same order of magnitude of those found by Kay *et al.* (2008) in 162 sewage discharge sites in the UK and Jersey indicating that the works studied here are typical for the UK. Under these typical conditions, NoV concentrations in sewage sampled from different levels of treatment varied substantially. The main factors driving virus removal in sewage treatments are sedimentation, adsorption, coagulation and precipitation (Gerba, 1981). Primary settlement (settlement and retention), is commonly less effective than secondary treatment in removing NoV from sewage (Flannery *et al.*, 2012). In the STW studied, primary settlement was conducted in continuous flow sedimentation tanks where 50–70% of suspended solids are removed. The results indicate large variations, for example from 0.01 to 2.5 $\log_{10}$, in NoV removal through primary settlement both between and within different STW. However, on average, primary settlement achieved approximately 1.0 $\log_{10}$ NoV removal for both NoV genogroups in most of the STW studied. Lower average removal rates were found by Nordgren *et al.* (2009) in the Ryaverket STW, Sweden (0.2 ± 0.1 $\log_{10}$ for GI and 0.7 ± 0.3 $\log_{10}$ for GII) and by Flannery *et al.* (2012) in a STW in Ireland (0.1 ± 0.6 $\log_{10}$ for GI and 0.1 ± 0.6 $\log_{10}$ for GII). These differences could be attributed to site-specific factors such as the design of tanks or temperature of the sewage and/or differences in the precision of the methods for detection and quantification.

Secondary treatments delivered 1.5 $\log_{10}$ average reductions for in the STW studied which are consistent with those found by Kay *et al.* (2008) for 162 sewage discharge sites in the UK and Jersey operating under high-flow conditions. NoV reductions during secondary treatment averaged 0.6 and 0.7 $\log_{10}$ for NoV GI and GII respectively. Among

the different STW average removal rates observed during secondary treatment varied significantly (0–3.1 $\log_{10}$ for GI and 0.5–2.3 $\log_{10}$) and were (highest to lowest) activated sludge > trickling filters > humus tanks > biological aerated filters. Within a process, NoV removal was also very variable. The largest variations were 2.2 $\log_{10}$ in trickling filters for GI and 3.1 $\log_{10}$ in activated sludge for GII. Of the types of secondary treatment investigated, the optimised form of activated sludge (modified Ludzack-Ettinger; biological nutrient removal system) operating at STW A was the most efficient process for NoV removal (average removal 3.1 $\log_{10}$ for GI and 2.3 $\log_{10}$ for GII). This is equivalent to or greater than the NoV removal rates reported in comparable studies. In the UK, Palfrey *et al*. (2011) found 2.6 $\log_{10}$ and 1.4 $\log_{10}$ NoV GII removals in a conventional non-nitrifying activated sludge plant and an advanced activated sludge plant with nutrient removal, respectively. In Japan, Hata *et al*. (2012) found 1.6 $\log_{10}$ and 1.8 $\log_{10}$ removals of NoV GI and GII, respectively in a conventional activated sludge plant. In contrast, in Sweden, Nordgren *et al*. (2009) found only 0.3 ± 0.4 $\log_{10}$ for GI and 0.3 ± 0.3 $\log_{10}$ for GII removal in an anaerobic phase for de-nitrification and an aerobic phase for decomposition of organic material. However, among secondary treatments studied, a common finding is the higher efficiency of activated sludge in removing viruses than trickling/sand filters (Gerba, 2007; Gerba, 2008; Nordgren *et al*., 2009; Palfrey *et al*., 2011; Hata *et al*., 2012). This could be associated with the shorter contact times in filter media (short hydraulic residence time) than in activated sludge (Berg, 1973) or with the elution of viruses in trickling filters. In secondary treatments, microorganism reduction is associated with adsorption to solids and biological predation within the microbial communities (Wen *et al*., 2009). The results of this study and the information available in the peer reviewed literature indicate that there is the potential to optimise NoV reduction in secondary treatments. This is an important area for future studies. However, the large variations in NoV reduction observed pose a challenge to regulators since control measures focusing on effluent treatment criteria may produce very variable levels of environmental protection.

UV disinfection systems have been in use for tertiary treatment of effluents in the UK and USA for many years and are known to be effective for bacteriological reduction (Institution of Water and Environmental Management, 1994). In the UK, monitoring of UV disinfection efficacy is required to ensure appropriate protection of sensitive areas.

Monitoring includes microbiological parameters (faecal coliforms and F+ coliphage, as a surrogate indicator for viruses) and parameters characterising maintenance of the UV disinfection process (Environment Agency, 2011). In this study, UV disinfection of final effluent delivered 1.0 $\log_{10}$ and 0.8 $\log_{10}$ NoV (GI and GII, respectively) reductions while the corresponding *E. coli* reduction through UV disinfection was 3.2 $\log_{10}$. The higher removal rates for *E. coli* may reflect either the optimisation of this treatment process for bacterial rather than viral reduction or the underestimation of virus viability reduction by RT-PCR (Pecson, Martin and Kohn, 2009). Further studies comparing NoV removal using RT-PCR and FRNA bacteriophage using both RT-PCR and plaque assay, as conducted by Flannery *et al.* (2013), would help inform this question.

The discharge consenting policy for UV disinfected discharges impacting shellfish protected areas in E&W requires that water companies demonstrate a 5.25 $\log_{10}$ reduction in the levels of faecal coliforms between influent and the shellfish water. The policy assumes that the initial concentration in the sewage is $2 \times 10^7/100$ ml and that the $\log_{10}$ reduction required by UV disinfection would be at least 2.25 (Environment Agency, 2003). In this study, STW A achieved a total 5.3 $\log_{10}$ reduction in *E. coli* concentrations and therefore meets the policy criteria. In contrast, STW B achieved a total 4.7 $\log_{10}$ reduction and therefore an additional 0.5 $\log_{10}$ reduction would be needed in the receiving water to meet the criteria.

In STW A, a positive association was found between $\log_{10}$ removal of NoV across the works and the absolute concentrations of the virus in untreated sewage arriving at the treatment works. This indicates that the removal of the virus increased as the abundance of the virus also increased and highlights the variability in NoV removal during the activated sludge + UV disinfection treatment process. These results contrast with those of Katayama *et al.* (2008) who found no association between NoV reduction and the concentrations of the viruses in the influent to six STW in Japan. These differences could be associated with different epidemiological profiles of NoV occurrence and/or differences in the design and operation of the STW.

The result of highest public health interest is the lack of significant differences between GM concentrations of NoV in SOs and in untreated sewage detected at STW A. This result was not expected since there is a known dilution effect in stormwater which results

from inputs to sewerage systems of relatively 'clean' water (e.g. road and roof drainage) during periods of high flows (Kay *et al.*, 2008). Associations have been found between rainfall causing sewage overflows and increased frequency of hospital admissions for treatment of gastrointestinal illness (Redman *et al.*, 2007). In the UK, many commercial shellfisheries are impacted by SOs (see Chapter 2). Therefore, the policy implication of these results is that stormwater discharges represent high risk to sensitive downstream receiving waters and should therefore be the highest priority for remediation.

In summary, the sewage sampling protocol used successfully provided robust empirical data on the likely concentrations of NoV in sewage-related discharges. The dataset is one of the largest of this kind in Europe and can be used in catchment-level assessments of pollution sources impacting SPAs conducted as part of sanitary surveys or WFD Article 11 programmes of measures.

The results demonstrated large variability of NoV concentrations in untreated sewage and treated effluents and provided insight into some of the factors influencing this variability. Among these, the seasonal occurrence of NoV in the local population plays a key role in driving NoV loads arriving at the treatment works. However, the health risk associated with untreated discharges should not be ignored during the period of low prevalence (April–September) because the infectious dose of the virus is very low (Teunis *et al.*, 2008) and its resistance in the environment is high (Seitz *et al.*, 2011). Statistically significant reductions were observed in GM concentrations of NoV as a result of secondary treatments. Of these, the suspended growth process (activate sludge) was more effective than the attached-growth processes (trickling filters, biological filters) in reducing NoV from sewage. Future research should focus on optimisation of suspended growth processes for NoV reduction, particularly in sensitive areas such as SWPAs. In E&W, elimination and/or treatment of intermittent discharges is a priority action for reduction of the NoV risk in SPAs. This risk is however not only determined by the number of people in the catchment shedding the virus and the STW effectiveness in reducing this load, but also on the dispersion and dilution of the sewage effluents in the receiving waters. To address this, detailed studies were undertaken in two SPAs to better understand the variability of NoV contamination in shellfish (Chapter 4) and the dispersion and dilution of sewage effluents in these sites (Chapter 5).

Chapter 4

Field Studies on Norovirus and *E. coli* in Shellfish Production Areas

"The quantitative levels of NoV within production areas and batches should be investigated further, in order to optimise sampling strategies." EFSA Panel on Biological Hazards (2012, p. 32).

In addition to requiring quantitative information on NoV concentrations produced by different types of sewage effluents, risk managers require data on NoV levels in shellfish samples collected from representative sites downstream of the sewage discharge points. Previous studies in this area have suggested large geographical areas of NoV contamination in estuarine (Gentry *et al.*, 2009; Brake *et al.*, 2011) and offshore (Greening, 2007; Winterbourn, 2014) shellfish farming sites impacted to some degree by sewage discharges. However, most of these studies have been limited in temporal coverage. Furthermore, few studies have investigated NoV contamination in multiple species exposed to similar environmental conditions (Vilariño *et al.*, 2009). A sampling programme was undertaken to obtain field data on concentrations of NoV and *E. coli* in shellfish in the experimental sites 13 (estuary) and 32 (bay) during periods of low and high prevalence of NoV. In the estuary, concentrations of NoV and *E. coli* were quantified in native oysters (*O. edulis*) during the period October 2012–January 2015. In the bay, the concentrations of these microbiological contaminants were quantified in co-located mussels (*Mytilus* spp.) and Pacific oysters (*C. gigas*) during the period March 2014–June 2015 to investigate if there were differences in NoV concentrations between species. The monitoring undertaken in the estuary includes the winter of 2012–13 when the NoV strain Sydney 2012 emerged in the UK (Allen *et al.*, 2014). The underlying assumption for this work was that if NoV contamination in the experimental sites is primarily from sewage-related sources, then levels of the virus in shellfish should decrease as distance between the sampling points and sewage discharges increases. This spatial variability of NoV contamination could be used by the industry and regulators to identify areas that would require prohibition of harvesting and areas that would benefit from other form of management to protect public health. Monitoring of NoV levels in co-located species should also inform consideration of the impact of NoV limits for shellfish at the point of

harvest. It also provides information on suitable areas for future expansion of shellfish farming operations.

Author contributions: the author of this thesis designed the field studies, liaised with external organisations on sampling arrangements, carried out all the fieldwork and data analyses and drafted the manuscript. Colleagues at Cefas carried out the microbiological testing and gave final approval for publication of the manuscripts.

4.1 Establishment of Sampling Stations and Shellfish Sampling

In the estuary, a similar quantity (10 kg) of native oysters was placed in each of seven mesh bags used for sampling (Figure 14). The oysters were harvested in Scotland and depurated at an approved plant in England before the experiments. These bags were deployed on the riverbed at different distances downstream from the STW A outfall representing the anticipated path of sewage effluent in the main estuary channel. Sampling stations were also established in confluent channels to represent contamination from other sewage discharges on the shoreline (Figure 7). The locations of these stations were identified based on the outcomes of a sanitary survey assessment reported by Cefas (Cefas, 2016). Each location was identified by latitude/longitude to an accuracy of 10 metres. The oysters were allowed to acclimatise for 2 weeks before the first sampling campaign.

In the bay, 15 kg of Pacific oysters and 15 kg of mussels were used for sampling at each sampling station. These shellfish quantities were placed in each of nine cages which were positioned at different locations from the STW D LSO representing a grid of stations across the bay (Figure 8). Each cage was at 3 m depth from the surface and was connected to a 100 l PVC float and to an iron chain of 23 m length placed on the seabed to prevent dislocation of the sampling station (Figure 15).

Figure 14 Sampling stations established in the estuary (site 13) showing bag with oysters for sampling (A), mooring chain (B) and mooring buoy (C).

Figure 15 Sampling stations established in the bay (site 32) showing float and mooring (A), shellfish cage (B), float after deployment (C), Pacific oysters (D) and mussels inside the cage (E).

The cages were placed at 3 m depth because it was hypothesised that the upper level of the water column would be more contaminated by the buoyant sewage and freshwater plumes and therefore would yield the highest microbiological results. The mussels were sourced from the local production area and the oysters were sourced from a different production area in England. These shellfish were not subject to purification treatment (depuration) prior to the experiments because of logistical constraints. However, the animals were allowed to acclimatise for 12 days before the beginning of the sampling campaigns. Doré et al. (2010) found approximately 1 log10 reduction in NoV concentrations in shellfish transferred from a contaminated site to a class A site over a period of 10 days. Therefore, the acclimatisation period of 12 days used in this study was considered appropriate.

The bathymetry at the sampling locations and the distances between the sewage discharges and the sampling locations in the study sites are shown in Table 15.

Table 15 Depth of the water column and distance between the main sewage discharges and the sampling stations in the estuary (site 13) and bay (site 32).

Station number	Study site 13		Study site 32	
	Depth (m) at chart datum	Distance (km) from the STW outfall	Depth (m) at chart datum	Distance (km) from the STW outfall
1	0.3	2.3	5	1.3
2	1.1	3.5	10	1.3
3	2.3	5.9	15	1.6
4	3.4	11.5	5	1.1
5	6.8	9.7	10	0
6	6.1	15.3	16	1.5
7	9.8	12.3	15	2.0
8	-	-	8	1.4
9	-	-	5	2.5

Shellfish samples were collected on a random basis with respect to the likely influencing environmental factors discussed in Chapter 2 to avoid introducing any bias to the results as recommended by the EU Guide to Good Practice on Microbiological Monitoring of Bivalve Mollusc Harvesting Areas (Cefas, 2014). Upon collection, samples were immediately transported under temperature-controlled conditions inside Icey-Tek Ocean Blue cool boxes to Cefas Weymouth Laboratory for microbiological testing

following the protocol used in the regulatory classification monitoring programme (Cefas, 2015). Where required, samples were rinsed with seawater from the immediate area and allowed to drain as recommended by the protocol. Testing of all shellfish samples commenced within 24 h of collection.

4.2 Microbiological Methods

Concentrations of NoV and *E. coli* were quantified using quantitative real-time reverse transcription (RT)-PCR (ISO/TS 15216-1: 2013) (ISO, 2013) and MPN (ISO/TS 16649-3: 2005) (ISO, 2005) methods, respectively as described in Section 2.3.

4.3 Hydrometric Data

Rainfall (total daily) and river flow (daily average) data for gauging stations located in the larger rivers discharging into the estuary and bay were supplied by the Environment Agency. At site 13, rainfall data were recorded by an automated station located approximately 1 km upstream of the tidal limit of the bay while at site 32, rainfall data were recorded using a tipping bucket gauging station located approximately 4 km upstream of the tidal limit. Additional water velocity measurements were taken using a Valeport™ 801 EM flow meter. The flow meter was calibrated at Valeport prior to use.

4.4 Temperature and Salinity Profiling

Water temperature and salinity measurements were taken in the water column at each shellfish cage location immediately after the collection of shellfish samples using a handheld SonTek CastAway® conductivity, temperature and depth profiler. The instrument was provided with a calibration certificate. Calibration with artificial seawater was conducted prior to the field studies.

4.5 Statistical Analyses

The statistical analyses followed the procedures described in Section 3.6. Similar to that observed for sewage, the distribution of microbiological concentrations in shellfish showed a closer approximation to normality when $\log_{10}$-transformed (Appendix III). The EU Guide to Good Practice on Microbiological Monitoring of Bivalve Mollusc Harvesting Areas does not provide guidance on how *E. coli* results below the LoQ of the MPN method should be handled for the purposes of statistical analysis (Cefas, 2014). In this study, these concentrations were adjusted to 19 MPN/100 g as recommended by the US National Shellfish Sanitation Program (USFDA and ISSC, 2013). For NoV concentrations, statistical analyses were conducted on detectable genome copies/g of digestive tissues as reported by the testing laboratory. No transformations were applied to NoV results in shellfish that had concentrations below the LoQ of the method (100 copies/g). This approach is supported by studies indicating that the commonly used substitution methods for results below the LoQ often result in poor estimates of the true mean (Helsel and Hirsch, 2002). However, it should be noted that NoV concentrations below LoQ reflect less accurate estimates of concentration than those above LoQ. The implications of this in relation to the results obtained are discussed below.

4.6 Results

4.6.1 Prevalence and Seasonality of Norovirus and *E. coli* in Shellfish

During the study period (October 2012–June 2015), NoV was frequently detected in shellfish sampled from both study sites. The relationship between NoV GII and NoV GI did not change over the monitoring period (Levene's homogeneity of variances test > 0.05) (Figure 16).
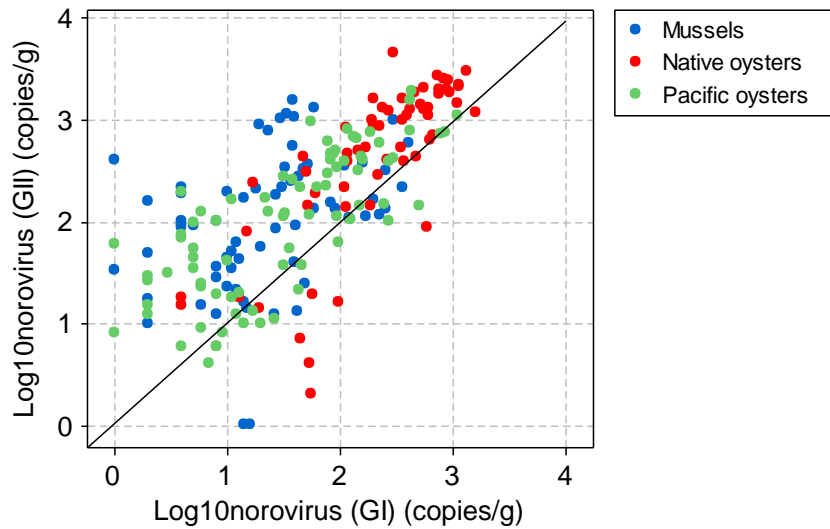
Figure 16 Relationship between norovirus genogroups I and II in mussels and oysters.

In the bay, the percentage of NoV-positive oyster samples was 98% for GI and 92% for GII while the percentage of mussel samples positive for the virus was 88% for GI and 86% for GII. Norovirus concentrations were generally very low with mean levels frequently below the established LoQ (100 copies/g). At this site, maximum concentrations in oysters were 1,105 for GI and 1,921 for GII and in mussels were 405 GI and 1,528 GII (all in NoV copies/g) (Table 16).

Table 16 Summary statistics of concentrations of *E. coli* and norovirus in shellfish sampled from the bay (site 32).

Station (distance from outfall, km)	n	n (pos.)	% (pos.)	GM	Log$_{10}$ St. Dev.	95% confidence interval		Min.	Max.
						Lower	Upper		
E. coli (MPN/100 g) in Pacific oysters									
1 (1.3)	9	5	56	26	0.363	15	46	<18	210
2 (1.3)	8	6	75	43	0.381	23	79	<18	130
3 (1.6)	6	2	33	23	0.266	14	37	<18	78
4 (1.1)	9	6	67	37	0.506	18	80	<18	330
5 (0)	8	5	63	31	0.457	15	65	<18	220
6 (1.5)	9	4	44	21	0.214	15	29	<18	78
7 (2.0)	9	5	56	25	0.300	16	39	<18	130
8 (1.4)	9	6	67	44	0.402	24	80	<18	130
9 (2.5)	11	9	82	94	0.743	34	259	<18	1,300

Norovirus (genogroup I) (copies/g) in Pacific oysters									
1 (1.3)	10	10	100	42	1.626	14	128	4	863
2 (1.3)	9	9	100	52	1.713	18	152	3	415
3 (1.6)	7	7	100	41	1.610	17	97	11	160
4 (1.1)	10	10	100	21	1.320	8	56	4	498
5 (0)	10	10	100	101	2.004	30	339	1	1,105
6 (1.5)	10	10	100	11	1.030	4	32	2	243
7 (2.0)	10	10	100	18	1.249	9	37	4	124
8 (1.4)	10	10	100	31	1.494	11	88	4	274
9 (2.5)	11	9	82	15	1.163	5	47	1	273
Norovirus (genogroup II) (copies/g) in Pacific oysters									
1 (1.3)	10	10	100	95	1.978	35	259	8	768
2 (1.3)	9	9	100	105	2.023	28	391	6	1,546
3 (1.6)	7	7	100	99	1.997	36	271	13	424
4 (1.1)	10	10	100	43	1.633	15	120	6	495
5 (0)	10	10	100	344	2.536	130	910	8	1,921
6 (1.5)	10	8	80	54	1.730	21	140	4	388
7 (2.0)	10	9	90	71	1.851	27	183	6	598
8 (1.4)	10	10	100	125	2.097	56	278	19	746
9 (2.5)	11	7	64	91	1.958	45	183	12	403
E. coli (MPN/100g) in mussels									
1 (1.3)	10	6	60	35	0.484	18	70	<18	330
2 (1.3)	11	7	64	39	0.441	21	71	<18	220
3 (1.6)	7	3	43	49	0.932	10	241	<18	5,400
4 (1.1)	10	7	70	34	0.407	19	62	<18	230
5 (0)	10	9	90	70	0.605	30	166	<18	460
6 (1.5)	10	4	40	18	0.033	17	19	<18	20
7 (2.0)	10	5	50	22	0.250	15	31	<18	110
8 (1.4)	10	4	40	35	0.498	17	71	<18	230
9 (2.5)	7	5	71	82	0.560	32	214	<18	460
Norovirus (genogroup I) (copies/g) in mussels									
1 (1.3)	10	10	100	16	1.202	6	44	1	227
2 (1.3)	11	11	100	26	1.409	11	58	5	253
3 (1.6)	7	7	100	14	1.133	4	52	1	201
4 (1.1)	10	7	70	15	1.366	9	63	1	257
5 (0)	10	10	100	83	1.917	39	175	14	405
6 (1.5)	10	9	90	11	1.023	6	18	2	27
7 (2.0)	10	9	90	10	1.003	5	19	2	39
8 (1.4)	10	8	80	15	1.180	7	31	2	59
9 (2.5)	7	4	57	34	1.536	20	60	18	92
Norovirus (genogroup II) (copies/g) in mussels									
1 (1.3)	10	9	90	99	1.994	43	227	15	1,303
2 (1.3)	11	9	82	144	2.157	65	317	14	1,026
3 (1.6)	7	7	100	56	1.745	11	280	1	403
4 (1.1)	10	9	90	69	1.838	29	167	9	901

5 (0)	10	10	100	278	2.444	129	598	16	1,047
6 (1.5)	10	8	80	50	1.695	14	175	1	775
7 (2.0)	10	8	80	62	1.793	23	168	9	1,528
8 (1.4)	10	8	80	69	1.840	26	185	12	1,111
9 (2.5)	7	5	71	44	1.640	15	125	8	204

n - number of samples; n (pos.) - number of NoV positive samples; % (pos.) - percentage of NoV positive samples; GM - geometric mean; St. Dev. - standard deviation; Min. - minimum; Max. - maximum.

Concentrations of *E. coli* were generally below the class A threshold ($\leq$ 230/100 g) on most sampling occasions. Geometric mean *E. coli* concentrations in oysters ranged from 21 in station 6 to 94 in station 9 while GM concentrations in mussels ranged from 22 at station 7 to 82 at station 9 (all MPN/100 g) (Table 16). The highest *E. coli* concentrations were detected in stations positioned away from the LSO (station 9 for oysters and station 3 for mussels). Concentrations of *E. coli* below the limit of detection (LoD) of the MPN method were detected on occasions in all stations.

The lowest percentages of NoV-positive results were detected in shellfish from stations 3 and 9. However, considering the whole dataset, statistical analysis by ANOVA did not provide evidence of differences in $\log_{10}$-transformed concentrations of NoV in oysters between sampling stations. In contrast, evidence was found of differences in $\log_{10}$-transformed concentrations of NoV GI in mussels between station 5 (closest to the LSO) and those in stations 6 and 7 (ANOVA; p = 0.032). In general, and considering all stations, mean concentrations of NoV GII in shellfish were consistently higher than those of NoV GI. In oysters, the differences in mean NoV concentrations sampled from the most and the least contaminated stations were 1 $\log_{10}$ for GI and 0.8 $\log_{10}$ for GII. In mussels, the differences in mean concentrations between the least and the most contaminated site were 0.9 $\log_{10}$ for GI and 0.8 $\log_{10}$ for GII. It should be noted however that these differences may not have a health risk significance because of the high number of NoV results below LoQ.

Pacific oysters had a higher percentage of NoV positive samples than mussels (Table 16). Pacific oysters also had higher maximum concentrations of NoV GI than mussels. However, for NoV GII this pattern was not apparent. Overall and taking the entire dataset, there was no evidence of significant differences in mean concentrations of NoV between these species during the study period.

Table 17 shows summary statistics for *E. coli* and NoV concentrations in native oysters sampled from the estuary (site 13). The table does not include one result for sample collected between stations 6 and 7 (607 NoV GI and 1,182 NoV GII copies/g) and one result for sample collected 260 m from station 3. Sampling bags at these stations were lost during the experiment during storm events.

Table 17 Summary statistics of concentrations of *E. coli* and norovirus in shellfish sampled from the estuary (site 13).

Station (distance from outfall, km)	n	n (pos.)	% (pos.)	GM	Log$_{10}$ St. Dev.	95% confidence interval Lower	Upper	Min.	Max.
E. coli (MPN/100 g)									
1 (2.3)	14	14	100	1,156	0.609	555	2,408	50	5,400
2 (3.5)	9	9	100	401	0.536	179	897	78	3,500
3 (5.9)	9	9	100	519	0.640	198	1,361	70	3,500
4 (11.5)	10	10	100	516	0.714	187	1,430	20	5,400
5 (9.7)	13	12	92	171	0.568	84	348	<20	1,100
6 (15.3)	5	5	100	249	0.760	54	1,154	20	1,700
7 (12.3)	6	4	67	85	0.610	28	262	<20	490
Norovirus (genogroup I) (copies/g)									
1 (2.3)	14	14	100	228	0.731	94	550	4	1,571
2 (3.5)	9	9	100	257	0.690	91	727	8	1,303
3 (5.9)	9	9	100	464	0.393	257	837	54	1,090
4 (11.5)	10	10	100	246	0.558	111	546	45	1,132
5 (9.7)	13	12	92	74	0.493	40	137	10	268
6 (15.3)	5	3	60	9	0.338	4	17	4	19
7 (12.3)	6	6	100	195	0.377	97	391	52	513
Norovirus (genogroup II) (copies/g)									
1 (2.3)	14	14	100	458	0.953	145	1,447	2	4,533
2 (3.5)	9	8	89	779	0.573	329	1,845	88	2,973
3 (5.9)	9	9	100	573	0.853	159	2,070	4	2,452
4 (11.5)	10	10	100	569	0.731	200	1,615	7	2,153
5 (9.7)	13	11	85	161	0.672	70	374	16	1,196
6 (15.3)	5	2	40	16	0.077	14	19	14	18
7 (12.3)	6	6	100	690	0.394	334	1,426	140	1,602

n - number of samples; n (pos.) - number of NoV positive samples; % (pos.) - percentage of NoV positive samples; GM - geometric mean; St. Dev. - standard deviation; Min. - minimum; Max. - maximum.

Concentrations of *E. coli* decreased from 1,156 MPN/100 g at station 1 near the STW outfall to 85 MPN/100 g at station 7 in the estuary mouth (Table 17). All *E. coli* results in oysters from stations 1, 2, 3, 4 and 6 were above the limit of detection of the MPN method. *E. coli* results within the range for class A ($\leq$ 230 MPN/100 g) were occasionally detected at all stations. *E. coli* results within the range for class C ($\leq$ 4,600 MPN/100 g) were detected at stations 1 and 4.

A high percentage of NoV-positive samples were detected at all stations with 100% of samples positive for GI at stations 1–4 and 7, and for GII at stations 1, 3, 4 and 7 (Table 17). GI was more prevalent than GII. Considering the group of stations positioned in the main study channel, mean NoV GI concentrations increased from 228 copies/g at station 1 (nearest the STW outfall) to 464 copies/g at station 3 (6 km from the outfall) and decreased from this station to 74 copies/g (< LoQ) at station 5 (9.7 km from the outfall) near the estuary entrance. For NoV GII, mean levels increased from 458 copies/g at station 1 to 779 copies/g at station 2 and decreased from this station to 161 copies/g at station 5.

The differences in mean levels between stations 1 and 5 correspond to a 0.5 $\log_{10}$ decrease for both NoV genogroups. It is noteworthy that the highest mean NoV concentrations were detected at stations 2 (GII) and 3 (GI) which are positioned in the anticipated path of not only the main STW of the study but also a relatively smaller STW that discharges to a confluent river channel. Evidence was found of significantly higher $\log_{10}$-transformed concentrations of *E. coli* in oysters at station 1 than those in oysters at stations 5/7 (ANOVA; p = 0.006).

The highest maximum concentrations of *E. coli* and NoV were obtained in the station closest to the STW outfall. However, the highest mean NoV concentrations were not detected at this station. The lowest levels of NoV were detected at station 6 positioned in an area of the estuary outside the main study channel. Taking the whole dataset, evidence was found of differences in $\log_{10}$-transformed concentrations of NoV GI in oysters at station 3 and at stations 5 and 6 (ANOVA; p = 0.001) (Figure 17A). In contrast, no evidence was found of statistically significant differences in NoV GII concentrations in oysters between stations (Figure 17B).
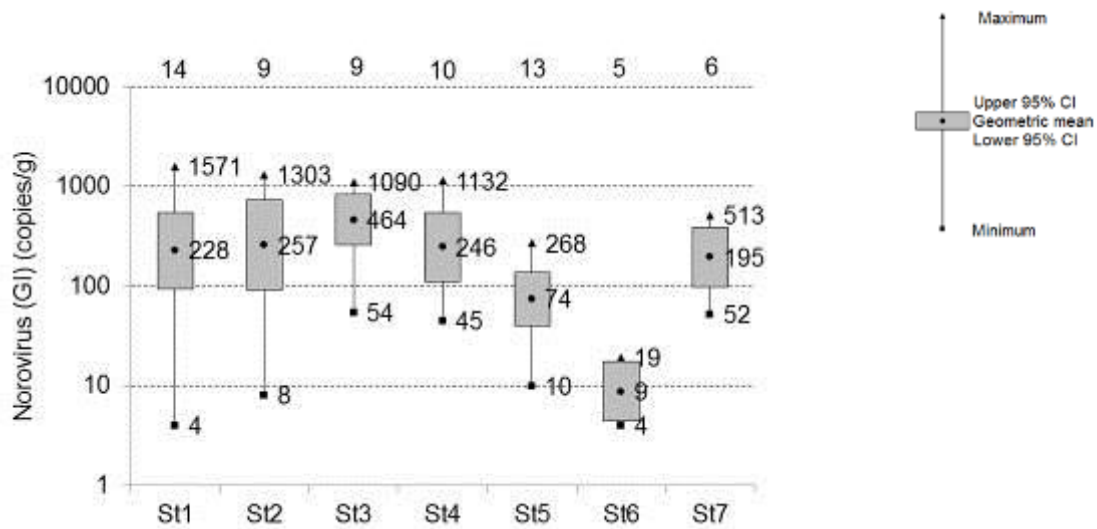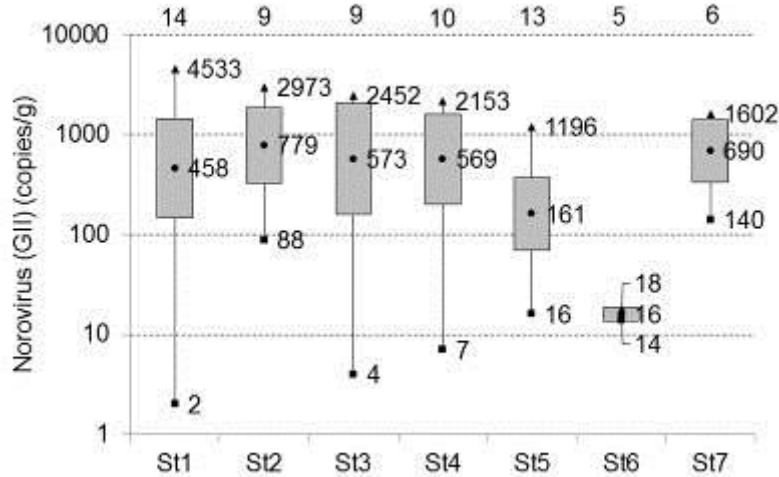
Figure 17 Box and whisker plots of concentrations of norovirus genogroups I (A) and II (B) in oysters sampled from the estuary (site 13).
The numbers of samples with quantitative results are shown above the boxplots. Limit of quantification = 100 copies/g.

The variation of NoV contamination in shellfish in different periods of high virus prevalence in the community was compared. In this analysis, results from all stations and both sites were combined. Samples collected during the period October–March were classified as "high prevalence" as previous research has indicated significantly higher concentrations of NoV in shellfish production areas in the UK (Lowther *et al.*, 2012a) during this period. Figure 18 shows geometric means, 95% CI and ranges of $\log_{10}$-transformed concentrations of NoV in shellfish for the three periods of high prevalence. Mean concentrations of GI in 2012/13 were 0.8 $\log_{10}$ higher than those in 2013/14 and 2014/15. For GII, mean concentrations in 2012/13 were 0.5 $\log_{10}$ higher than those in

2013/14 and 0.8 log$_{10}$ higher than those in 2014/15. For GI and GII, evidence was found of significant differences in geometric mean concentrations between 2012/13 and 2013/14 ($p < 0.001$) and between 2012/13 and 2014/15 ($p < 0.001$), respectively.
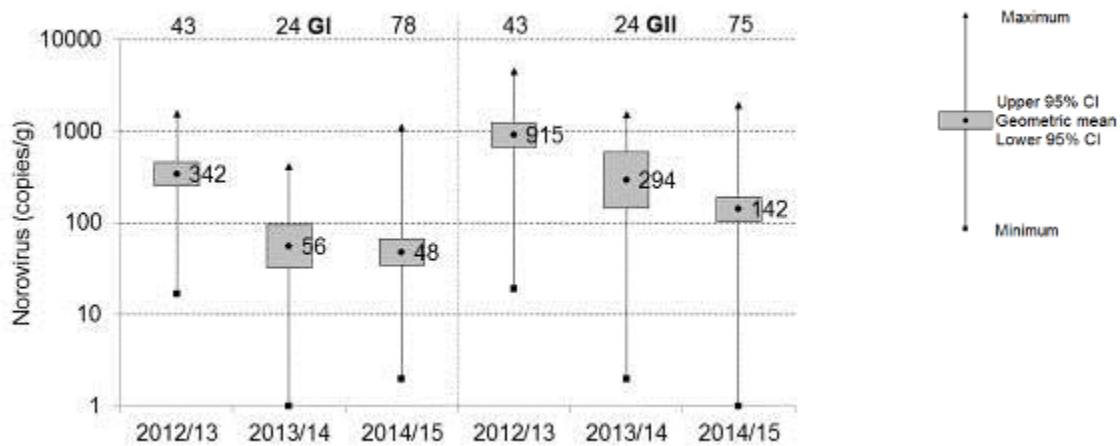
Figure 18 Box and whisker plots of concentrations of norovirus in shellfish samples from the estuary (site 13 and bay (site 32) classified into three periods of high prevalence of the virus.
Data from all stations combined. Data periods: October 2012–March 2013, October 2013–March 2014 and October 2014–March 2015. The numbers of samples with quantitative results are shown above the boxplots. Limit of quantification = 100 copies/g.

4.6.2 Relationships Between Norovirus in Shellfish and in Stormwater

The relationships between concentrations of NoV in stormwater and in shellfish were studied using results obtained in seven wet-weather sampling campaigns conducted in site 13 during the period 5 November 2012–19 March 2013. During this period, rainfall levels in the catchment ranged from 0.2 mm to 30.4 mm and the telemetry alarm of the storm tank at STW A recorded 167 discharge events. The longest discharge persisted continuously for 38 days. This exceeds the 3% total annual duration set out in the EA discharge consenting policy for SWPAs (see Section 1.3). It was also observed that not all discharge events coincided temporally with rainfall events.

Figure 19 shows that the concentrations of NoV GII in oysters sampled from station 1 (nearest from the STW A outfall) increased as GII concentrations in settled storm discharges also increased. NoV in settled storm ranged from 48 to 73,953 copies/ml while concentrations in oysters ranged from 50 to 1,998 copies/g. A similar relationship was not observed for NoV GI. These results provide evidence of an association between

these variables although the relationship is based on a reduced number of data pairs and therefore more data would be needed to confirm this.
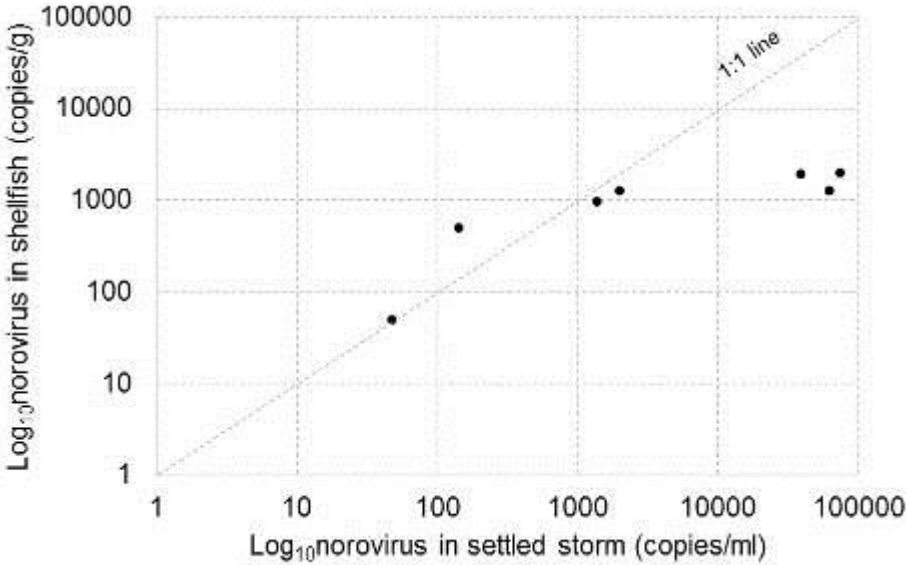
Figure 19 Scatterplot of concentrations of norovirus (GII) in oysters from station 1 and settled storm discharges into the estuary (site 13).
Limit of quantification in shellfish = 100 copies/g.

4.6.3 Effect of UV Disinfection of Stormwater on Microbiological Levels in Shellfish

Results presented in Section 2.5.2 indicate a positive association between NoV concentrations in shellfish and the number of stormwater overflows. During the sampling programme undertaken at site 13, a new UV disinfection plant was installed in the storm tank at STW A to reduce the volume and frequency of untreated settled stormwater spills from the storm tank to the estuary. The UV dose at this plant was set at 20 mJ/cm^2 as required by the EA. This infrastructure change provided an opportunity to obtain quantitative information on NoV levels in the oysters following the installation of the UV disinfection plant at this experimental site.

Table 18 summarises the concentrations of *E. coli* and NoV in oysters collected from three stations in the main estuary channel before and after the installation of the UV plant. The two sets of samples were collected under similar environmental conditions with respect to water temperature, number and duration of settled storm discharges and rainfall and are therefore comparable. The flows in the river measured upstream of the sewage outfall on these sampling occasions were 2.002 m^3/s on 29/01/13 and 1.540

m³/s on 20/01/15 which indicate that the samples were collected during high-flow conditions (mean flow in the river is 0.312m³/s).

Concentrations of NoV in oysters sampled before the UV plant (29/01/13) were 1 $\log_{10}$ higher than those sampled after the UV plant (20/01/15). The differences in *E. coli* concentrations in oysters between the two sampling occasions were of lower magnitude (0.3–0.5 $\log_{10}$) than those detected for NoV. It is possible that these differences were attributed to the effect of the new UV disinfection plant and/or to the higher prevalence of NoV in the community in the winter of 2012/13 relative to that in the winter of 2014/15 (Public Health England, 2015). It was not possible to investigate this further because no further shellfish sampling was conducted in this site after 20/01/15.

Table 18 Concentrations of *E. coli* and norovirus in oysters, average water temperature, total rainfall and number and duration of sewage spills before and after the installation of a UV disinfection plant in the storm tank at the sewage treatment works A.

Station number	*E. coli* (MPN/100 g)	NoV GI (copies/g)	NoV GII (copies/g)	Water temp. (°C)[a]	Sewage spill events in preceding week (no/duration)[b]	Total rainfall in preceding week (mm)[c]
Collection date: 29/01/2013 – before the installation of the UV disinfection plant						
1	1,700	457	1,855	6.6	21 (2,067 min.)	35.8
2	1,700	721	2,655	6.4		
3	1,100	350	523	6.7		
Collection date: 20/01/2015 – after the installation of the UV disinfection plant						
1	790	15 (<LoQ)	78 (<LoQ)	5.6	18 (2,315 min.)	32.8
2	490	61 (<LoQ)	185	5.8		
3	490	18 (<LoQ)	88 (<LoQ)	7.0		

[a] Water temperature measured at the time of sampling. [b] Sewage spill events recorded by telemetry at STW A. [c] Rainfall recorded by tipping bucket station. NoV - norovirus; GI - genogroup I; GII - genogroup II; temp. - temperature; no - number; min. - minutes; LoQ - limit of quantification.

4.7 Discussion

The microbiological impacts of sewage effluent on shellfish were studied in the experimental sites. These sites were a shallow tidal estuary impacted at the head by a small river and continuous and intermittent sewage discharges (site 13), and in a deep open coastal embayment mainly impacted by a LSO (site 32). The experimental protocol involved placing bags with native oysters for sampling at 7 stations in the estuary and cages with mussels and Pacific oysters at 9 stations in the bay. Concentrations of *E. coli*

and NoV were monitored in the shellfish to understand how levels of these microbiological contaminants vary with distance between the sampling stations and the sewage discharges.

The results showed high NoV positivity in the three species monitored with more than 80% of samples positive for both genogroups at most stations and in both sites. This high prevalence of NoV is consistent with results from previous investigations in the UK (Lowther, Henshilwood and Lees, 2008; Lowther *et al.*, 2012a) and suggests that the virus, as indicated by PCR, may be ubiquitous in polluted coastal waters during the monitoring period. From a policy development perspective, this information is important because it indicates that control measures based on qualitative (presence/absence) criteria could have high impact on SPAs in the UK.

Norovirus GI was more frequently detected than GII. However, in terms of absolute quantities, the shellfish were consistently more contaminated with GII than with GI. This is also in agreement with other studies conducted both in the UK (Henshilwood, 2002; Lowther *et al.*, 2012a) and other parts of Europe (Pavoni *et al.*, 2013) and reflect the predominance of GII strains in sporadic cases and outbreaks of human illness worldwide (Koopmans, 2008; Hall *et al.*, 2011). Manso and Romalde (2013) reported higher frequency of occurrence of GII in mussels from Galicia (Spain). Associations between both genogroups and cases of human illness have been reported (Gallimore *et al.*, 2005; Le Guyader *et al.*, 2008). Therefore, it is important to detect and quantify both human genogroups for risk assessment purposes. The EFSA Panel on Biological Hazards has suggested that quantitative values for both genogroups should be added to give an overall measure of NoV titre for risk assessment (EFSA, 2012). This approach has been used in this thesis to study the relationship between NoV contamination and sewage dilution and to assess the impact of NoV limits for SPAs as presented in Chapter 6 below.

The NoV concentrations in shellfish obtained in this study were similar to those found in mussels grown in floating rafts and wild shellfish (mussels, clams and cockles) in Galicia (Spain) (10^2–10^3 copies/g for GI and from 10^1–10^4 copies/g for GII) (Vilariño *et al.*, 2009). Using human volunteer studies, Teunis *et al.* (2008) found that the probability of an infected subject expressing NoV symptoms at a dose of 10^3 genome copies of NoV GI is about 10%. Therefore, consumption of raw oysters from site 13 would present at least

a 10% risk of human illness. The maximum concentration detected in this study (4,533 copies/g NoV GII) was lower than that found by Lowther *et al.* (2012a) in the NoV surveillance study conducted in 39 oyster production areas in the UK (6 samples were found with GII concentrations in excess of 10,000 copies/g). This may be associated with higher NoV prevalence in human populations in the winter of 2009/10 (first year of the surveillance study) than in the winters of 2013/14 and 2014/15 as suggested by the laboratory reports of NoV outbreaks in E&W published by Public Health England (2015). This could also be due to site-specific differences within the wide range of sites in the surveillance study. Evidence was also found in this study of significant differences in mean concentrations of NoV between typical periods of high prevalence (2012/13 and 2013/14 for GI and 2012/13 and 2014/15 for GII). These differences were again consistent with the inter-annual differences in laboratory outbreak reports indicating that NoV epidemiology is rapidly reflected in the prevalence of NoV in shellfish and this should be considered when evaluating the virological quality of SPAs.

Large variations were found in NoV concentrations at both study sites. In the estuary (site 13), the largest differences in native oysters were 3.0 $\log_{10}$ for GI and GII while in the bay (site 32) the differences were detected in Pacific oysters (3 $\log_{10}$ for GI and 2 $\log_{10}$ for GII) while in mussels the differences were 1.5 $\log_{10}$ for GI and 2.2 $\log_{10}$ for GII. However, the characteristics of NoV contamination in the study sites were substantially different. In the estuary, NoV contamination was generally characterised by a high percentage of positive samples with concentrations above LoQ. This is a similar pattern of NoV contamination to that found by Lowther *et al.* (2012a) in a large number of estuarine systems across the UK. Le Guyader *et al.* (2000) compared levels of NoV contamination in shellfish from different geographical areas in France and, as expected, found higher frequency of NoV-positive samples at sites frequently impacted by sewage pollution events than those that were not. In this study, the estuary was impacted by a high number of sewage spill events during the monitoring period. The highest mean concentrations of NoV were found in oysters from stations positioned in the main estuary channel near the STW A outfall. The increase in mean concentrations of NoV between stations 1 and 3 is probably associated with the combined effect of sewage effluents from STW A and B impacting the upper reaches of the channel. At station 2, the GM NoV GII concentration (779 copies/g) exceeded the mean concentration found in

oyster samples associated with NoV outbreaks (652 copies/g) by Lowther *et al.* (2012). Downstream from station 3, mean NoV concentrations decreased as distance from STW A outfall increased. However, GII concentrations in oysters at stations 1, 2, 3 and 4 often exceeded 1,000 copies/g and the difference in mean levels between the most and the least contaminated stations was only 0.5 $\log_{10}$. Studies on NoV contamination in Sydney Rock Oysters undertaken in Waikare Inlet (New Zealand) (2.3 m depth on spring tides) detected NoV GII at a distance of 8.5 km from the sewage source on the 21st day after an overflow of 3.3 x 10^6 litres of untreated sewage (Brake *et al.*, 2011). These results illustrate the large geographical extent of NoV contamination in shellfish typically observed in estuarine environments characterised by poor flushing/long residence times of the water.

In the bay, NoV contamination was generally characterised by high percentage of samples below LoQ with the highest mean concentrations in both oysters and mussels generally detected at station 5 (STW D LSO location). However, statistically significant differences were only found for GI in mussels between stations 5 and 6/7. Occasionally, maximum NoV concentrations in excess of 1,000 copies/g were detected, particularly in mussels at stations positioned on the eastern part of the bay. Geometric mean concentrations of NoV above LoQ were detected at stations 5 (LSO), 2 (eastern part of the bay) and 8 (mussel farm). In contrast, the highest mean concentrations of *E. coli* were obtained at station 9, an inshore station positioned on the western part of the bay, approximately 2.5 km away from the LSO. These results indicate that while STW D is the most significant input of NoV into the bay other shoreline and/or local sources of *E. coli* contamination (e.g. seabirds) probably impacted the western part of the bay.

In a study of the spatial distribution of NoV contamination in mussels placed in cages suspended at 1 m depth around a LSO in Kinmel Bay (Wales), Winterbourn (2014) found NoV differences of approximately 0.5 $\log_{10}$ for GI and GII between the most eastern and western parts of the sampling grid (representing alongshore transport). The differences in NoV levels between the most northern and southern cages (representing onshore transport) were 0.1 $\log_{10}$ for GI and 2.6 $\log_{10}$ for GII. The highest NoV concentration detected was 9,958 copies/g (GII) in shellfish positioned 2 km to the east of the LSO. Kinmel Bay is shallower than site 32 and the LSO discharges secondary-treated effluent at 7 m depth. Furthermore, the cages in the Welsh study were placed 1

km apart which represents a relatively finer grid than that used in this study. In Dunedin-Otago Peninsula (New Zealand), Greening and Lewis (2007) found that mean levels of NoV in mussels decreased from 3.0 $\log_{10}$ to approximately 2.0 $\log_{10}$ over a distance of 9 km from the STW outfall. The study was conducted in an open coast environment where the sewage plume travels close to the coast and is poorly diluted by tidal currents which is similar to site 32. It has long been assumed that LSOs with effective diffusers discharging into moderately deep waters substantially reduce the health impacts because of the high dilution and efficient dispersion of the sewage effluent. The results of this study indicate that whilst this is valid for FIOs it cannot be assumed for NoV because of the long persistence of the virus in the marine environment. Models that relate NoV input concentrations to concentrations in shellfish are therefore fundamental to risk management. Using these models, regulators can calculate limits on emissions for any sewage discharge and assess their impacts on SWPAs. Ideally, these models would also incorporate information on sewage effluent dilution at several locations in the growing waters because the uptake and accumulation of NoV depends upon local hydrodynamic conditions.

To date, few studies have investigated NoV contamination levels in co-located species (i.e. exposed to the same environmental conditions). These studies are important because they can help identify appropriate monitoring strategies for SPAs with multiple species. In some EU Member States, shellfish hygiene regulators have monitored a single "sentinel" species to indicate the microbiological risk for a larger group of species, rather than monitoring all species in the SPA (EU Working Group on Microbiological Monitoring of Bivalve Mollusc Harvesting Areas, 2005). To be effective in protecting public health, the indicator species should show an equivalent or higher level of contamination than the species it represents. Historically in E&W, all species have been classified on an individual basis. However, the potential use of an indicator species has been under consideration by the FSA (Younger, 2014). In this study, no significant differences were found in mean NoV concentrations between co-located oysters and mussels in the bay. A similar study conducted in two production areas in Italy also did not find differences in NoV contamination between clams, mussels and oysters grown using different cultivation methods (Suffredini *et al.*, 2012). The authors noted however higher NoV recovery efficiency and higher NoV positivity in mussels. Based on these

results, the authors concluded that mussels could be used to represent clams and oysters in relation to NoV contamination. However, it should be noted that, when exposed to sewage contamination and depurated under controlled conditions, oysters take longer to eliminate viruses (F+ bacteriophage, a surrogate for NoV) than mussels (Doré and Lees, 1995). As Younger (2014) concludes, the use of mussels as an indicator species to reflect the NoV risk of oysters following sewage discharges should be approached with caution since it may not be sufficiently protective of public health.

For the first time, evidence of a positive association was found between levels of NoV GII in discharges of settled stormwater and levels of the virus in shellfish. These results highlight the importance of SOs in NoV contamination of shellfish. The experimental STW has known ground water infiltration problems which cause the storm tank to discharge frequently. The high number of storm tank spills has been considered unusual by the EA and enforcement action was taken during the sampling programme. To address this problem, a new UV disinfection plant was installed at these works. The monitoring undertaken at this site indicated lower levels of microbiological contamination in the oysters following the installation of the UV plant. These results suggest the UV disinfection could be an effective treatment option for sites impacted by stormwater. Currently, disinfection of stormwater is not common practice in E&W. Studies have indicated that UV disinfection could achieve considerable reductions in NoV levels. Lee, Zoh and Ko (2008) found that UV doses of 10, 20 and 25 mJ/cm^2 cause murine NoV1 to reduce 1, 2.8 and 3.3 $\log_{10}$, respectively. In these experiments, the authors inoculated murine NoV into Phosphate buffered saline solution and therefore the results reflect isotonic, non-toxic conditions. The UV apparatus used low pressure (254 nm UV lamps) and the UV irradiance was measured using a radiometer. Low pressure lamps are commonly used by UK water companies in UV disinfection plants. These results are similar to those obtained by Bravo (2011) who detected a 4 $\log_{10}$ inactivation at a UV fluence of approximately 30 mJ/cm^2. In this study, no differences were found between low pressure and polychromatic medium pressure UV lamps. However, the levels of *E. coli* and NoV contamination identified at this site prior to the installation of the UV plant demonstrate the need to develop a policy to reduce the frequency and duration of stormwater discharges into SPAs. This has been recommended previously by the ACMSF (ACMSF, 1998, 2015) and will be more relevant

if standards for NoV in shellfish are introduced in the food hygiene legislation in the future.

To conclude, the monitoring programme undertaken in the experimental sites evidenced large areas of NoV contamination (as indicated by PCR) on a presence/absence basis, consistent with previous similar studies undertaken in the UK and elsewhere (Greening and Lewis, 2007; Winterbourn, 2014). However, on a quantitative basis, the spatial variation of NoV contamination reflected the different hydrographic and pollution source characteristics of these sites. In the shallow and poorly flushed estuary (site classified at class C in the main channel and class B in adjacent channels under Regulation (EC) No 854/2004), NoV contamination showed a decreasing gradient in the main estuary channel over > 10 km from the STW A outfall. However, NoV GII concentrations in excess of 1,000 copies/g were still detected at the mouth of the estuary (12 km from the STW A outfall) indicating potential influence of virus contamination from other known sewage-related sources in the catchment. In the deep and well-flushed open coast embayment, oyster samples were 100% positive for NoV GI and GII at most stations. Under the *E. coli* classification criteria of Regulation (EC) No 854/2004, these sites would be class A. Based on these results, detailed hydrodynamic studies were undertaken to establish (a) whether NoV contamination could be predicted based on the knowledge of the transport and dilution of the sewage effluent and (b) the feasibility of implementing a "buffer zone" approach as control measure for NoV at these sites. The results of these studies are detailed in Chapters 5 and 6 below.

Chapter 5

Drogue Tracking, Current Profiling and Dye Tracing Studies in Shellfish Production Areas

"The main advantage of a tracer simulation test is that the tracer imitates exactly the movement of a solute injected at a given location. The tracer responds to the hydraulics of the tidal system without the necessity of making extensive hydraulic measurements." Kilpatrick and Cummings (1972, p. 27)

This chapter describes work conducted to characterise the surface water movements and the time of travel, dispersion and dilution of sewage effluent in the experimental sites 13 and 32. As noted in Chapter 1, knowledge of the characteristics of circulation of microbiological contaminants in SPAs is important to contextualise the information on pollution sources obtained in the sanitary survey as required by Regulation (EC) No 854/2004. The assumption here was that if there is an association between the dispersive and dilution characteristics of the sewage effluent and the microbiological impacts evidenced by the field studies, then it should be possible to develop control measures for these contaminants in production areas, including the identification of the conditions where harvesting closures may or may not be necessary due to NoV contamination.

Initial consultation with the EA and water companies indicated that hydrodynamic models were not available for the study sites. These models require specialist modellers which were not available in the scope of this research project. Furthermore, hydrodynamic models do not usually perform well in shallow estuaries with limited tidal exchange or large drying areas, unless extensive calibration exercises are undertaken (Boye *et al.*, 2015; Gao *et al.*, 2015). In deep, open coast environments with weak tidal currents such as the experimental site 32, stratification of the water column can have a significant effect in modifying the fate of sewage plumes as determined by tidal currents (Sherwin and Jonas, 1994). The alternative approach was to undertake dye tracing studies to obtain dilution estimates and simulate the movements of sewage contamination in the study sites. In the bay, a drogue tracking exercise was also undertaken because wind and density-driven currents could have a significant effect on the circulation of contaminants on the coast. These tools have been increasingly used by

shellfish safety authorities to determine the impacts of raw sewage discharges on production areas following sewage treatment failures (Goblick *et al.,* 2011; USFDA, 2013).

Author contributions: the author of this thesis designed the drogue tracking studies, liaised with external organisations, assisted with the deployment of the current profiler, carried out the dye tracing studies and drafted the manuscript. Colleagues at USFDA assisted with the tracing studies and data analyses. Colleagues at Cefas carried out the microbiological testing and assisted with the tracing studies. Colleagues at Cefas and USFDA gave final approval for publication of the manuscript.

5.1 Drogue Tracking Study

Satellite-enabled drogues were used to obtain an accurate record of surface water movements in the bay (site 32) on 5 May 2014. Nine drogues were built using Globalstar satellite modems and a 64 channel global positioning system (GPS) positioned just under the top waterproof cover alongside the satellite modem antenna (Figure 20A). These sophisticated drogues were used because the bay covers a large geographical area and simpler drogues would not be visible from a fixed point on the shoreline. Each drogue was 1.5 m in length and 110 mm in diameter (Figure 20B) to create a spar buoy with very low reserve buoyancy. The drogues were ballasted by the battery packs (4 parallel banks of 6 batteries providing 9 v at 72 Ah) and by lead weights to make them slightly positively buoyant. In calm waters, approximately 200 mm of the drogue was above the water surface (Figure 20C) to allow the GPS and the Globalstar modem to record the information. The drogues were set up to record positional data every 10 minutes for a period of up to 48 h. The positional data were transferred in real-time to a dedicated website hosted on Triskel Marine's server.

The drogues were released as close as possible from the shellfish sampling cages from a vessel. The releases took place on the flood tide and under poor weather conditions with a high percentage of cloud cover (88–100%), rain (0.3–3.2 mm/3h) and gentle to fresh breeze from south-south west (8–18 knots).

Figure 20 Satellite-tracked drogues used in the bay (site 32) showing electronics prior to building into the drogue casings (A), drogues ready for deployment (B) and ballasted drogue in the water (C).

5.2 Acoustic Doppler Current Profiling

A Nortek Aquadopp acoustic Doppler current profiler (ADCP) was deployed in the bay (site 32) near the STW D LSO at station 5 on 12 July 2014 to measure water current speed and direction. Prior to deployment, the profiler was calibrated, mounted in a cage and attached to an aluminium frame. A pinger was attached to the frame as a recovery system. The system was deployed from a vessel on the seabed and set up to record water velocity and water depth until 10 August 2014.

5.3 Dye Tracing Studies

5.3.1 Determination of Dye Quantities and Deployment of Fluorometers

The appropriate marine licences for conducting the work were granted by the Marine Management Organisation (MMO)[3] and the appropriate health and safety protocols and communication procedures were discussed and agreed with the water companies prior to the studies. Rhodamine WT was selected as water tracer because this substance is water soluble, highly detectable, harmless in low concentrations and stable in aquatic environments (Wilson, Cobb and Kilpatrick, 1986). The use of a conservative tracer was considered an appropriate option because of the long environmental persistence of NoV (Seitz *et al.*, 2011). The tracer was introduced in the final effluent chambers at STW A (site 13) and STW D (site 32) prior to discharge in the receiving waters. The Rhodamine WT dye was injected over 12.4 h (half of the semi-diurnal tidal cycle) and the movements of the dye-tagged effluent were monitored in the receiving waters following the procedures described by Goblick *et al.* (2011). The amounts of dye required for the studies were determined based on STW flow data provided by the water companies (see Section 3.3). A set of fluorometers were calibrated in the laboratory prior to the studies. In the field, fluorescence readings were taken before, during and after the dye injections. The quantities of dye for injection were determined using a calculation spreadsheet developed by the USFDA kindly supplied by Greg Goblick. The STW flows, calculated dye concentrations in the final effluent and expected dye fluorescence concentrations in the receiving waters for four dilution ratios are shown in Appendix IV.

A stock solution (160 l) of Rhodamine WT dye and deionised water were used to prepare 9 dilution standards (100,000,000–0.1 parts per billion; ppb). These standards were used to calibrate submersible WetLabs-FLRHB ECO fluorometers with internal batteries (4 fluorometers for site 13 and 8 fluorometers for site 32) and a further 2 submersible Wetlabs-FLRHRT fluorometers powered by an external source of energy. The fluorometers were calibrated in the range of 0.1 ppb to 100 ppb at the instrument

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[3] The tracer used in this study (Rhodamine WT) is included in the list of approved tracers one of the approved tracers and is therefore now exempt from the requirements to obtain a marine licence from October 2015.

limit of detection of 0.01 ppb. The FLRHB fluorometers were attached to the shellfish cages and recorded dye fluorescence in the fixed sampling stations during the day of the dye injection and subsequent 7 days. The FLRHRT fluorometers were towed from vessels to record background fluorescence on the days before the dye injections and on the days of the dye injections during daylight hours.

The background fluorescence readings were subtracted from those obtained during the dye injection and subsequent days to determine the concentrations of dye-tagged sewage effluent in the estuary and bay. In the estuary (site 13), submersible fluorometers were attached to shellfish cages at stations 1, 2, 3 and 5 to characterise the dispersion and dilution of sewage effluent from STW A outfall in the main estuary channel. These instruments collected data from 19/01/15 (approximately 24 h before the beginning of the dye injection) to 27/01/15. In the bay (site 32), submersible WetLabs-FLRHB ECO fluorometers were attached to stations 1, 2, 4, 5, 6, 7, 8 and 9 (the cage at station 3 was lost due to storm events). These fluorometers recorded data from 15/06/15 (approximately 24 h before the beginning of the dye injection) to 23/06/15. The fluorometers were programmed to collect data while submerged and were kept sealed and stored away from direct light interference with the fluorescence readings. The towed fluorometers were placed inside a protective unit made of PVC (Figure 21C) prior to use to prevent exposure to light and to reduce cavitation effects during data collection periods.

5.3.2 Dye Injection and Monitoring

At STW A, the Rhodamine WT dye injections commenced on 20/01/15 at 04:00 (around low water) and concluded on the same day at 14:30 (around mid-ebb tide). The dye was injected at a constant rate (457 ml/minute; 199 revolutions/minute) from a polyethylene container using a Masterflex 7553-20 variable speed peristaltic pump (Cole Palmer, IL) and Masterflex Tygon tubing into the final effluent chamber at the weir to ensure complete mixing of the dye mixture with the sewage effluent (Figure 21A). At this site, the dye mixture (160 litres) was made of 80 litres of deionised water and 80 litres of dye from the stock solution. Deionised water was used to produce the dye

mixture to better represent the density characteristics of the final effluent. During the dye injection, the final effluent was a combination of flows from the stormwater tank and from the UV disinfection plant (Figure 21B) and therefore both discharges were labelled by the dye mixture. The initial concentration of the dye at this site was 2,258 ppb, based on the flow records and the flow from the dosing container. In the estuary, the dye was tracked using a Wetlabs-FLRHRT fluorometer towed from a vessel (Figure 21C–D) interfaced with RAFT-MAP GIS software. Dye concentrations were measured within the sewage plume and around the edges of the plume at the surface as indicated by the software readings in real-time. The dye tracking at this site took place during daylight hours on 20/01/15 from 10:57 to 16:37. The weather conditions on the day were light to moderate breeze from E-NE in the morning turning to SW in the afternoon; the air temperature varied from 1 °C to 6 °C and the weather was dry.

The dye injection at STW D commenced on 16/06/15 at 05:03 (around peak HW) and concluded on the same day at 17:03. The pump head Easyload model 7518-00 was set to a constant pumping rate of 183 ml/minute and maintained at a calibrated 209 revolutions/minute. At this site, 140 litres of dye mixture (70 litres of deionised water added to 70 litres of dye) were used. The dye mixture was injected into the final effluent chamber at the weir prior to effluent discharge into the bay via the LSO. The initial concentration of the dye in the effluent was 3,792 ppb based on average flow data at the STW, the injection rate, the initial concentration of the dye in the dosing container (100,000,000 ppb) and the dye mixture flow from the container. The dispersion and dilution of the dye were tracked in the bay as described above on 16/06/15 from 09:58 to 13:12 and on 17/06/15 from 07:48 to 13:09. On 16/06/15, the weather conditions were light to gentle breeze from SW in the morning turning to NW in the afternoon, the air temperature varied from 13 °C to 16 °C and the weather was dry. On 17/06/15, the weather was gentle to moderate breeze from the W, the air temperature varied from 15 °C to 16 °C and the weather was also dry.

Figure 21 Dye tracing study showing set up for dye injection (A), dye injection into the final effluent chamber (B) and dye tracking using towed fluorometer (C, D) in the estuary (site 13).

5.3.3 Determination of Steady-State Sewage Dilution

The dye fluorescence concentrations were used to determine the overall steady-state dilution of sewage effluent in the study sites using the superposition method described by Kilpatrick (1993) and Kilpatrick and Cobb (1985) and modified by Goblick *et al*. (2011). In summary, dye injected at constant rate for 12.4 h builds-up until it reaches a plateau level and produces a dye concentration peak in the receiving waters on a concentration-time curve. For a semi-diurnal tidal regime, the rise and fall of dye concentrations measured in the waters are the product of the quantity of dye injected and the mixing due to tidal effects. In general, higher dye concentrations typically occur around LW; conversely, lower concentrations are found around HW. Tidal cycles can therefore represent a quasi-steady-state period. The ultimate dye concentration at any given location in the receiving waters was obtained by adding the resultant concentration-time curves for various half-tide cycle intervals (Kilpatrick and Cummings, 1972; Yotsukura, Cory and Murakami, 1972). The main advantage of this method is that it reduces the volume of dye and therefore the injection time from several days to ½ tidal day (12.4 h) required to characterise the build-up of sewage dilution in estuarine systems (Goblick *et al.*, 2011). In this study, two measures of steady-state condition were determined:

(a) average dye concentrations per day (using all dye concentration data); and

(b) peak 1 h average concentration on each day.

This peak 1 h concentration represents an hour when, at steady state, the shellfish are exposure to the greatest quantity of sewage effluent and therefore is the period of greatest accumulation of microbiological contaminants.

A five-point moving average was applied to the raw dye fluorescence readings as a smoothing factor to reduce the effect of measurements that reflected occasional interference of the fluorometers with the shellfish cages. The dilution of dye-tagged sewage effluent was calculated by dividing the initial concentration of dye in the final effluent by the final (five-point moving average) concentrations.

5.4 Results

5.4.1 Drogue Tracking Study

The drogues were released on the ebb tide sequentially at each of the nine sampling station locations as follows: 7–9–8–6–3–5–4–1–2. The first drogue was released at LW + 0.5 h. Wind speed and direction recorded on the day of the drogue releases ranged from 8.3 knots recorded near station 7 to 18.1 knots recorded near station 5 (STW D LSO) (gentle to fresh breeze) from the S-SW. The water temperature recorded at the release points ranged from 13.8 °C near stations 2 and 6 to 14.6 °C near station 7. Most of the drogues followed a mean eastward circulation pattern normal to the S-SW wind (Figure 22).
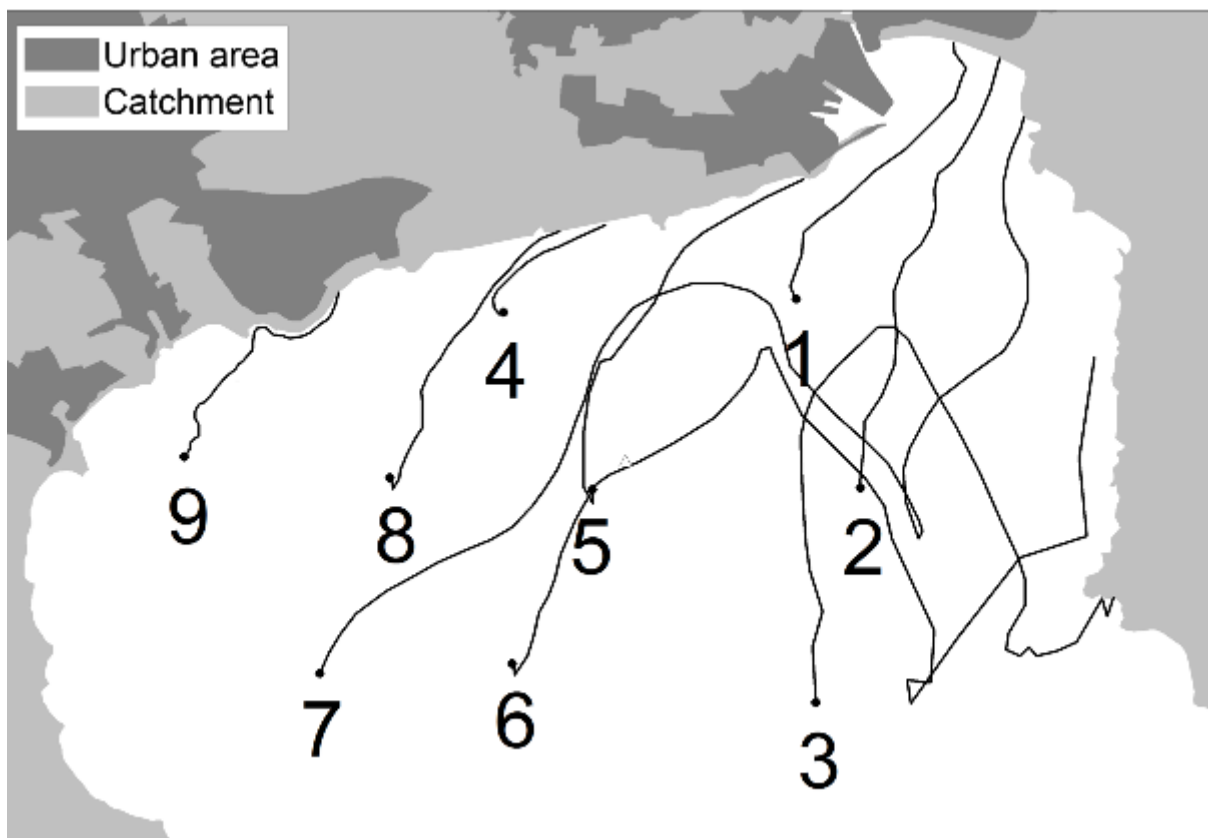
Figure 22 Drogue tracks in the bay (site 32) on 5 May 2014.

Within 8 h of deployment, six drogues (from stations 5, 4, 9, 8, 1 and 2) reached the shoreline (Table 19). These drogues travelled at 0.067–0.149 m/s. Considering that

wind speed at the time was about 8 m/s, it was estimated that the drogues moved at 1–2% of the wind speed. These travel speeds are consistent with mean currents measured by the ADCP deployed at station 5 near the STW D LSO (Appendix V). The remaining drogues continued to move eastward around the bay in a clockwise circulation pattern at 0.09–0.1 m/s (Figure 22). The change in direction detected by the satellite trackers for these drogues coincided with LW (04:35) when wind speed dropped. These drogues continued to move eastward until they reached the shoreline in the morning of 06/05/14. Temperature-depth profiles measured before and after the drogue tracking study do not evidence thermal stratification in the water column (Appendix VI). Therefore, it was assumed that the movement of the drogues reflected wind-driven currents at the surface in a well-mixed water column.

Table 19 Distances travelled and average speeds of nine drogues tracked in the bay (site 32) on 5 May 2014.

Station number	Total distance travelled (m)	Time taken to reach foreshore (hh:mm:ss)	Number of recorded positions	Minimum speed (m/s)	Maximum speed (m/s)	Average speed (m/s)
1	535.29	01:17:03	6	0.068	0.287	0.149
2	1667.92	03:28:14	21	0.081	0.226	0.132
3	5315.17	16:33:46	100	0.009	0.204	0.089
4	565.4	02:19:29	14	0.03	0.092	0.067
5	1573.81	04:08:59	25	0.04	0.167	0.105
6	7058.94	20:20:33	119	0.008	0.357	0.099
7	6371.7	17:41:45	103	0.013	0.429	0.103
8	1267.19	04:48:57	27	0.03	0.119	0.078
9	792.52	02:10:37	13	0.05	0.161	0.102

5.4.2 Dye Tracing Studies

Average background levels of fluorescence recorded by the fixed fluorometers on the day before the dye injection (19/01/2015 in site 13; 15–16/06/2015 in site 32) were similar to those obtained by the USFDA in US coastal waters (USFDA, 2013, 2013a) (Appendix VII).

Fluorescence data from seven stations were used to determine the distribution and concentration of dye tagged effluent in the bay (site 32). The fluorescence data recorded at station 6 were extremely erratic varying several log orders of magnitude possibly due to interference with the sensors and were therefore excluded from the dilution calculations. Figure 23 shows dye concentrations based on the superposition method and calculated dilutions for individual stations in the bay (site 32). At station 5 (STW D LSO), the peak concentration of dye tagged effluent (7.26 ppb) was detected around high water (HW) approximately 5 h after the beginning of the dye injection on 16/06/15 (Figure 23D). A larger mass of dye-tagged effluent of lower concentrations was detected in this station over a longer period of time until dye concentrations reduced to background levels on 16/06/15 at 5:30 (around HW). This suggests that the mass of dye-tagged effluent was rapidly flushed away from the LSO. Smaller quantities of dye impacted the area around the LSO on the following tidal cycles with minute traces of dye (< 0.9 ppb above background) detected until the recovery of the fluorometers on 23/06/15 (7 days after the dye injection). A degree of dye build-up occurred at this station from 16/06/15 (beginning of the dye injection) to 19/06/15. The average dilution for the steady-state condition (plateau level in the concentration-time curve) at this station was 5,867:1 and the steady-state peak 1 h average dilution was 1,661:1. The maximum dilution obtained based on the peak dye concentration at station 5 was 549:1.

The mass of dye-tagged effluent moved in a clockwise circulation pattern away from the STW outfall and impacted station 1 on the next flood tide on the day of the dye injection (16/06/15) (Figure 23A). On 16/06/15 at LW - 2 h, no dye was recorded by the fluorometer. The peak dye concentration obtained at this station (0.85 ppb) was recorded on the mid-flood tide on 18/06/15 at 15:50. Small amounts of dye were recorded in the following days and therefore little build-up occurred as indicated by the superposition curves in Figure 23A.

In the following hours, the plume of dye tagged effluent continued to travel offshore towards station 2 (Figure 23B). The leading edge of the sewage plume reached this station on the day of the dye injection (16/06/15) at approximately 13:30 (around HW). A peak dye concentration (6.74 ppb) was detected around HW + 1.25 h on the day of the dye injection. Similar to the pattern detected at station 1, the dye concentrations reduced to background levels very quickly at around 23 h (LW-1 h) on 16/06/15. In

subsequent days, this station was impacted by low quantities of dye. Pollutant build-up was detected at this station as some dye remained in this area of the bay for several days after the termination of the dye injection.

The three most inshore stations (numbers 4, 8 and 9) in the central and western parts of the bay were impacted by relatively lower quantities of dye-tagged effluent than the stations on the eastern part (Figure 23C, F, G). Dye concentrations at station 4 were lower than those anticipated considering the proximity of this station to the STW D LSO (1.18 km). At this station, peak levels of dye were detected on consecutive days (17, 18, 19 and 21/06/15). However, these peak concentrations returned quickly (< 30 min.) to background levels. The maximum concentration of dye (1.15 ppb) was recorded on 22/06/15, 6 days after the beginning of the dye injection. Pollutant build-up occurred in this area of the bay during the monitoring period. The station positioned on the edge of the mussel farm (station 7) was impacted by low quantities of dye (< 0.2 ppb) over long periods of time (Figure 23E). The maximum concentration (2.31 ppb) was detected on 19/06/15 at 14:20 (LW + 0.5). At station 8, pollutant build-up was observed during 5 days (Figure 23F). The peak dye concentration recorded at this station was 2.98 ppb on 18/06/15 at 16:26 (LW + 3 h) (2 days after the beginning of the dye injection). Residual levels of dye (occasionally > 0.2 ppb) were detected both before and after this peak indicating that this part of the bay was not heavily impacted by substantial amounts of effluent. After 5 days, the fluorometer measurements suggested that the dye was flushed away from this area of the bay. Station 9 was impacted by a small quantity of dye on 19/06/15 (3 days after the beginning of the dye injection) at 16:30 (LW + 2.7 h). The maximum dye concentration detected at this station was 2 ppb. This dye was also rapidly flushed away from this station.

A – Station 1
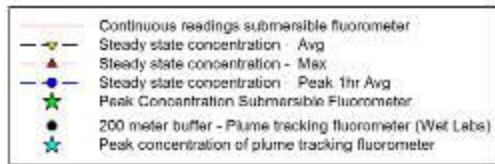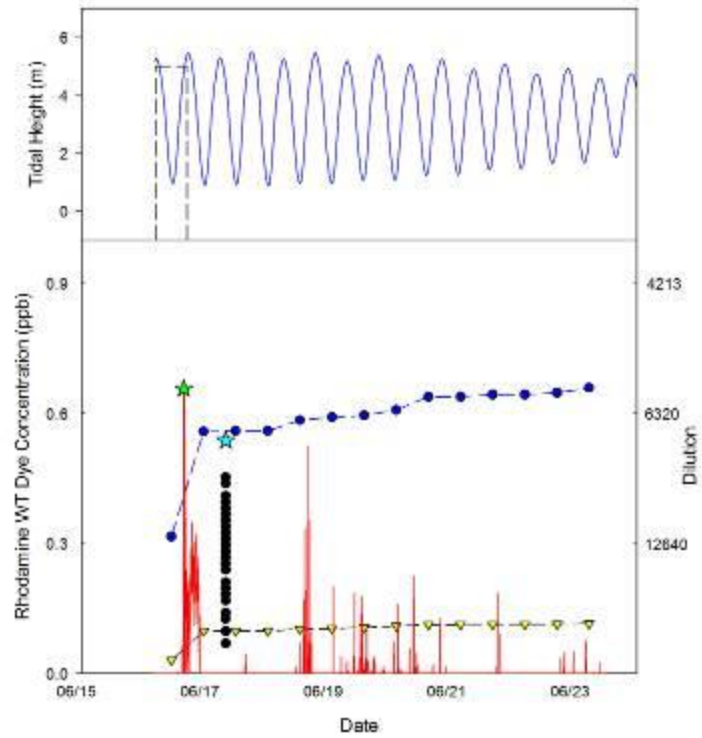
B – Station 2
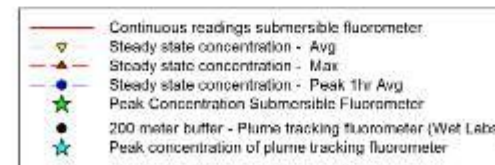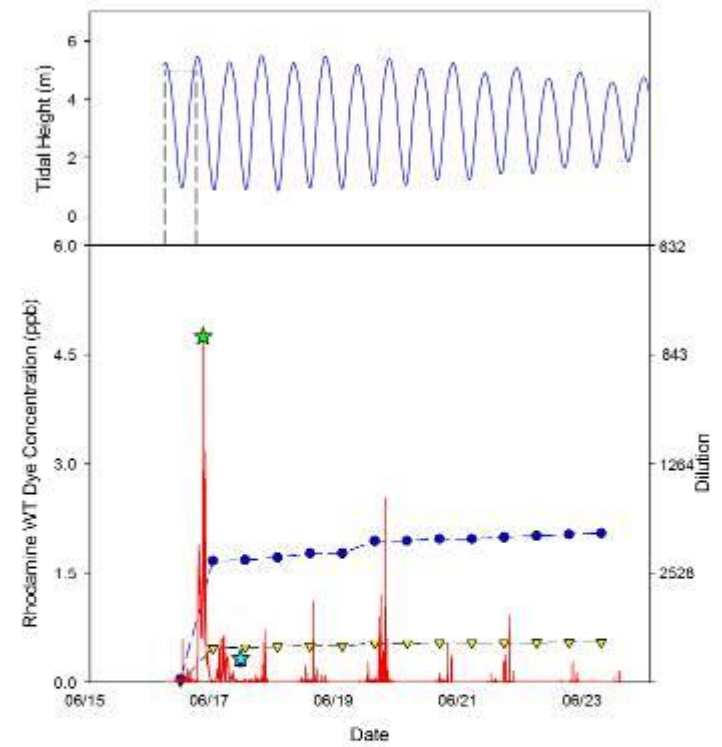
C – Station 4

D – Station 5

99
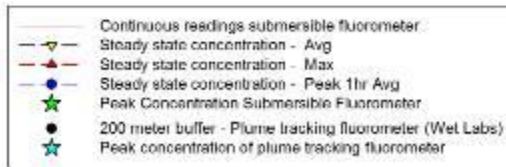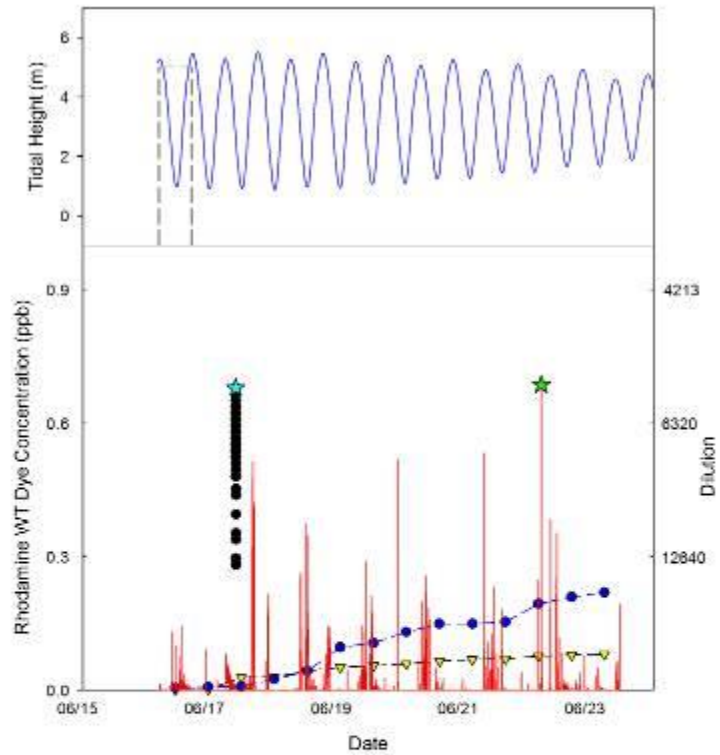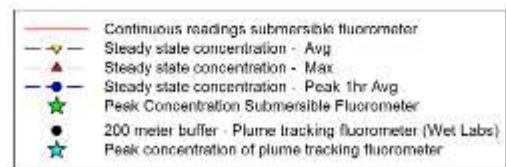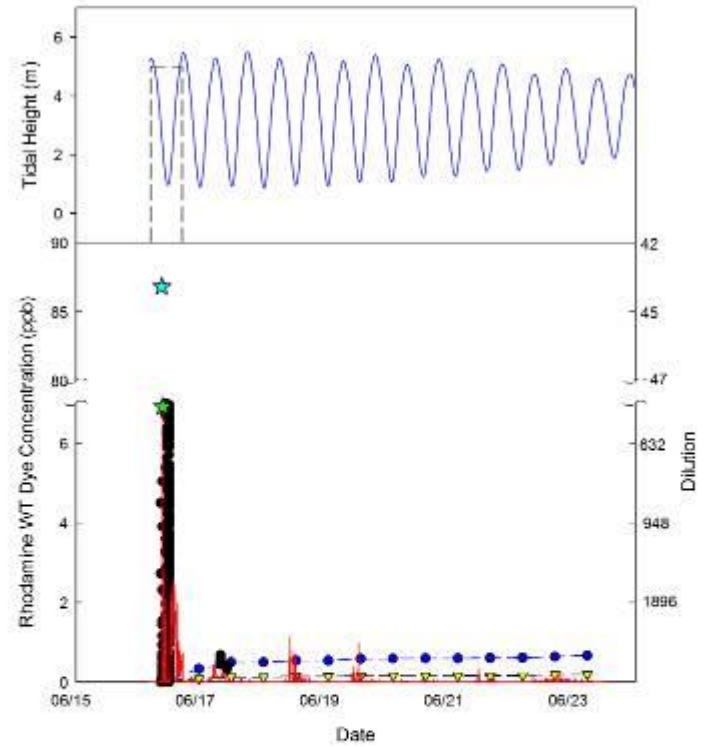
E – Station 7

F – Station 8

100
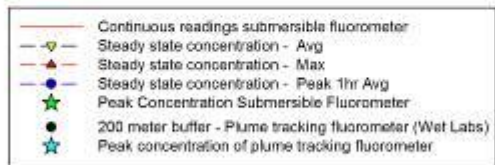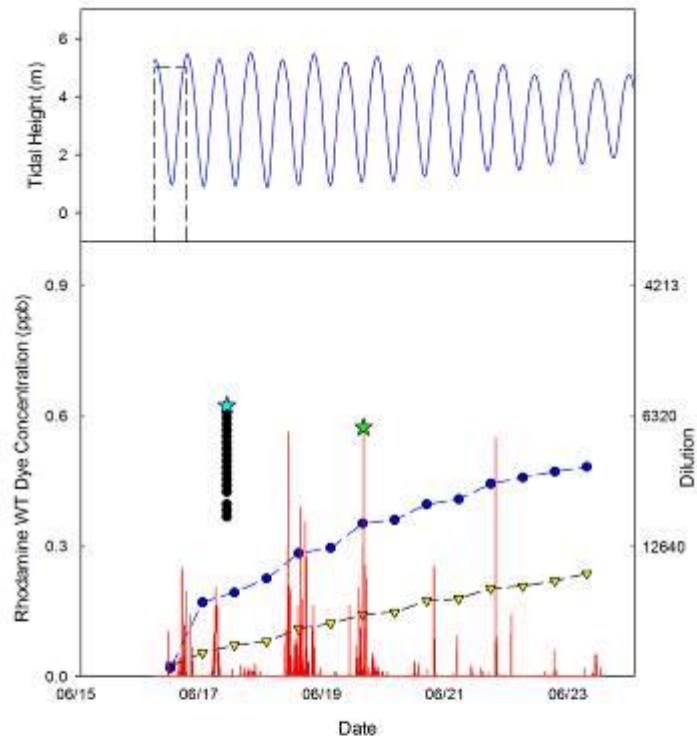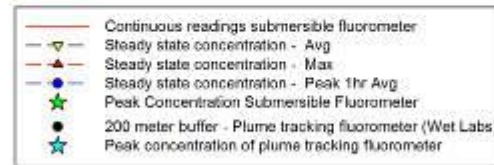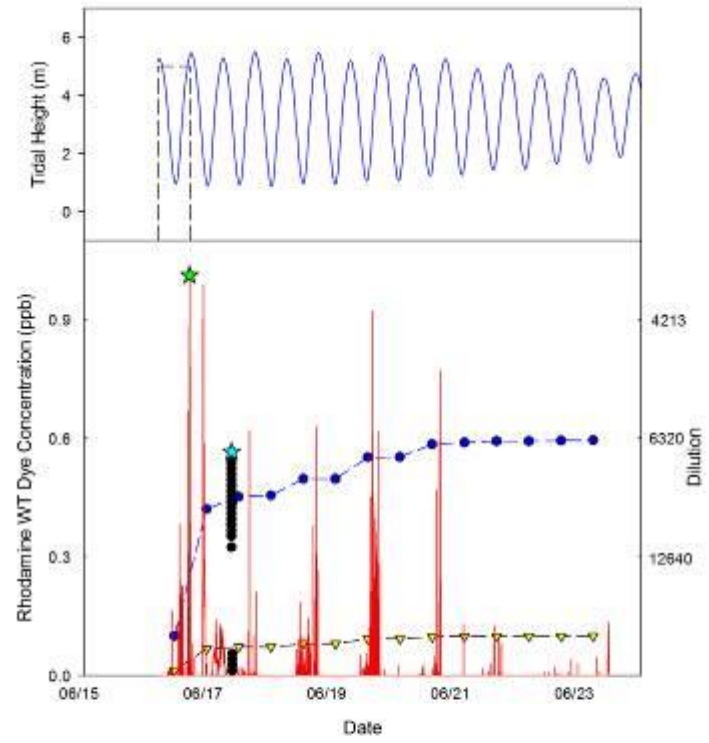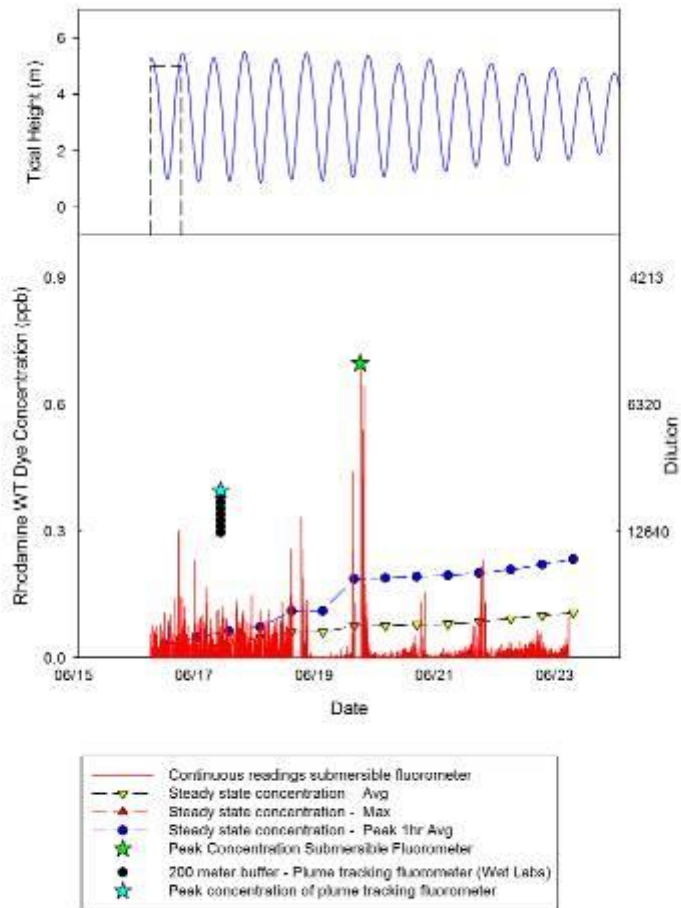
G – Station 9

Figure 23 Time series of dye concentrations measured by the stationary fluorometers with calculated dilution curves in the bay (site 32).

The period of dye injection is marked as dashed line on the tidal heights above the dye readings. The continuous red line represents continuous dye measurements. The dashed blue line with blue circles represents average peak 1 h steady state dye concentrations. This peak 1 h is the average dye concentration calculated using data collected 30 min. before and 30 min. after the peak concentration.

The dashed black line with yellow triangles represents average steady stage dye concentrations. The black dots represent plume tracking fluorescence measurements taken within a 100 m buffer. The blue stars represent peak dye concentration measured by tracking fluorometer. The green star represents peak dye concentration measured by fixed fluorometer. The fluorometer placed at station 6 recorded erroneous fluorescence readings and these data were not included in the analysis. The shellfish cage at station 3 was lost prior to the dye study.

Figure 24 shows Rhodamine dye concentrations and calculated dilution curves for individual stations in the estuary (site 13). Overall, substantial pollutant build-up was detected at all stations as indicated by the average and peak 1 h superposition curves. At station 1, the fluorometer detected the maximum dye concentration (27.4 ppb) on 20/01/15 at 17:00 (LW - 0.25 h), 11 h after the beginning of the dye injection (Figure 24A). Dye concentrations reduced to background levels on the same day at 22:45 (HW - 1 h). This indicates that the dye-tagged effluent was pushed away from the upper reaches of the channel during the ebb tide. Peak levels of dye were recorded on 21/01/15 at 05:30 (LW + 0.5 h; 9 ppb), 21/01/15 at 16:00 (LW - 1.5 h) (4 ppb) and 22/01/15 (05:10) (LW - 0.8 h; 2.7 ppb). All the dye was flushed away from the area represented by station 1 on 26/01/15 (6 days after the day injection) at 14:00 (HW - 2 h).

At station 2, the maximum concentration of dye (11.56 ppb) was detected on 20/01/15 at 17:10 (LW + 0.5 h) (Figure 24B). The temporal pattern of dye-tagged effluent observed in this station was similar to that observed at station 1, although with lower amounts of dye detected on the first day. Peak levels of dye were detected on 21/01/15 at 05:45 (LW + 0.75 h) (9 ppb), 21/01/15 at 16:20 (LW - 1.25 h) (4 ppb) and 22/01/15 at 06:50 (LW + 1 h) (2.20 ppb). These peak levels were followed by peaks of lower magnitude in subsequent days. This station approached steady state conditions on 26/01/15 at 15:10 (HW + 1 h) when dye concentrations reached background levels.

A – Station 1
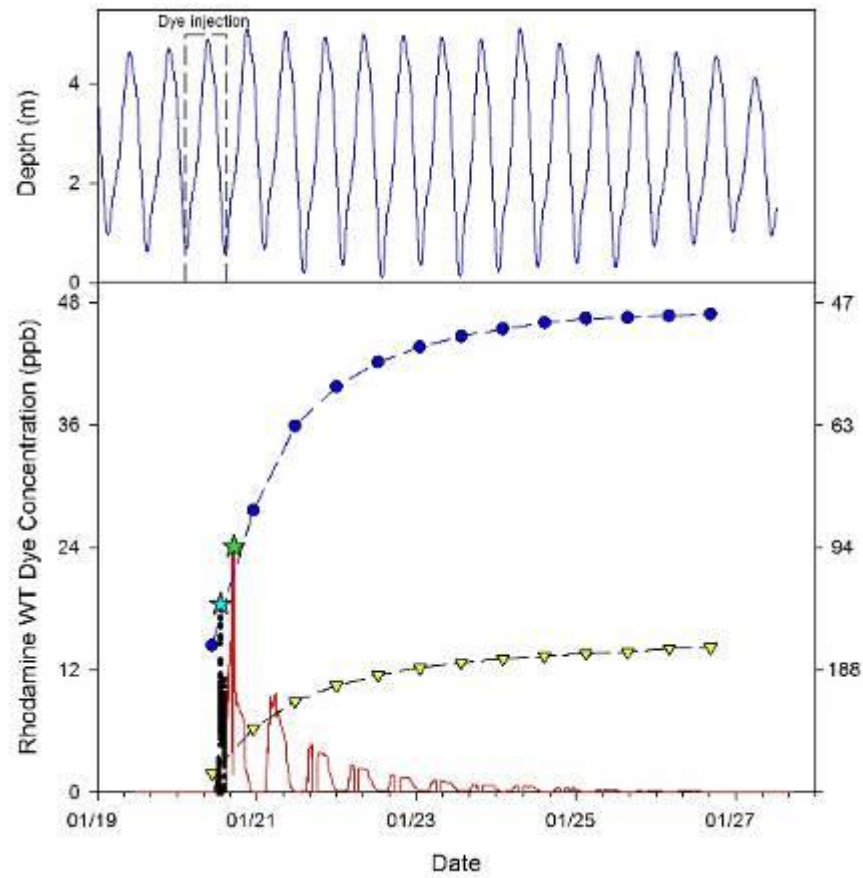
B – Station 2
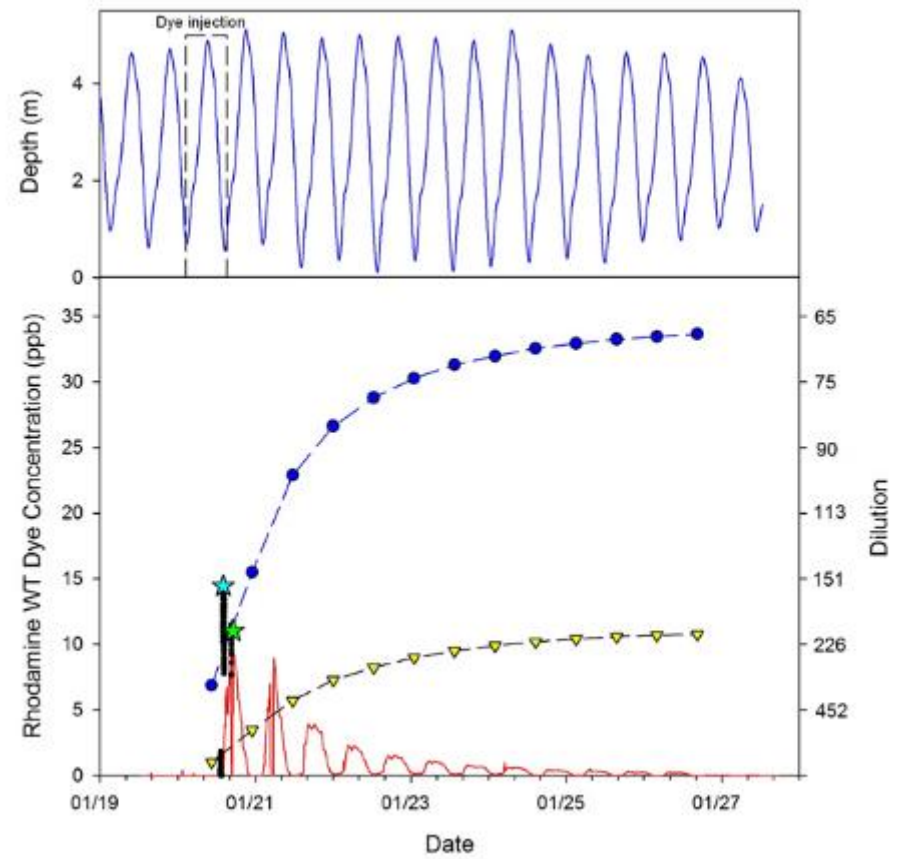
C – Station 3
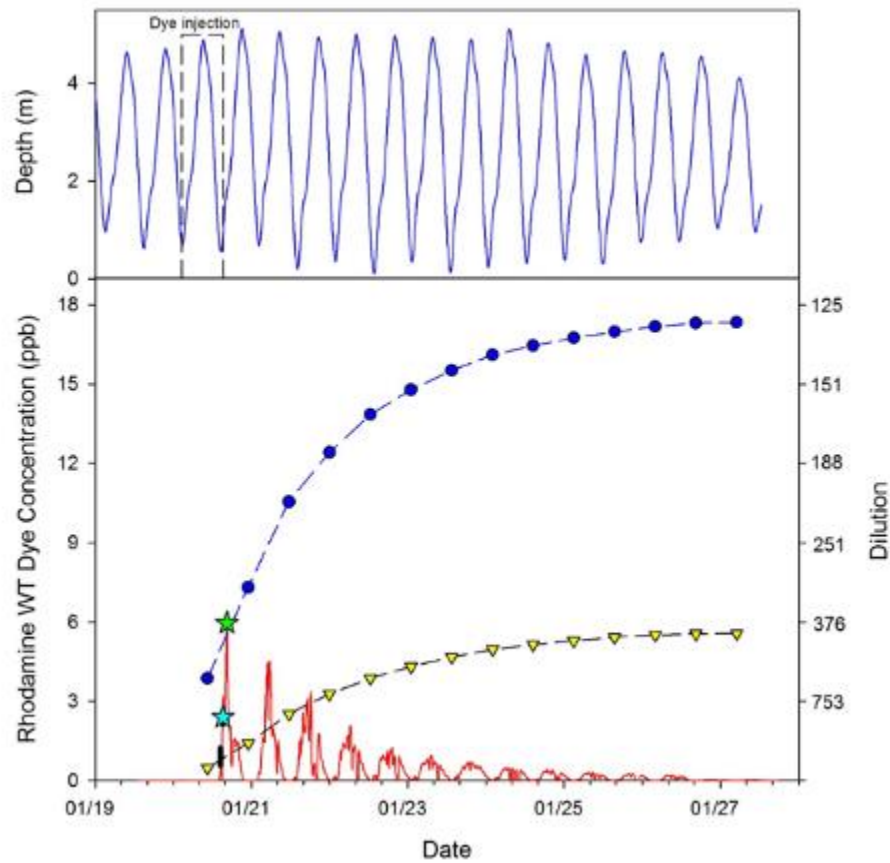
D – Station 5
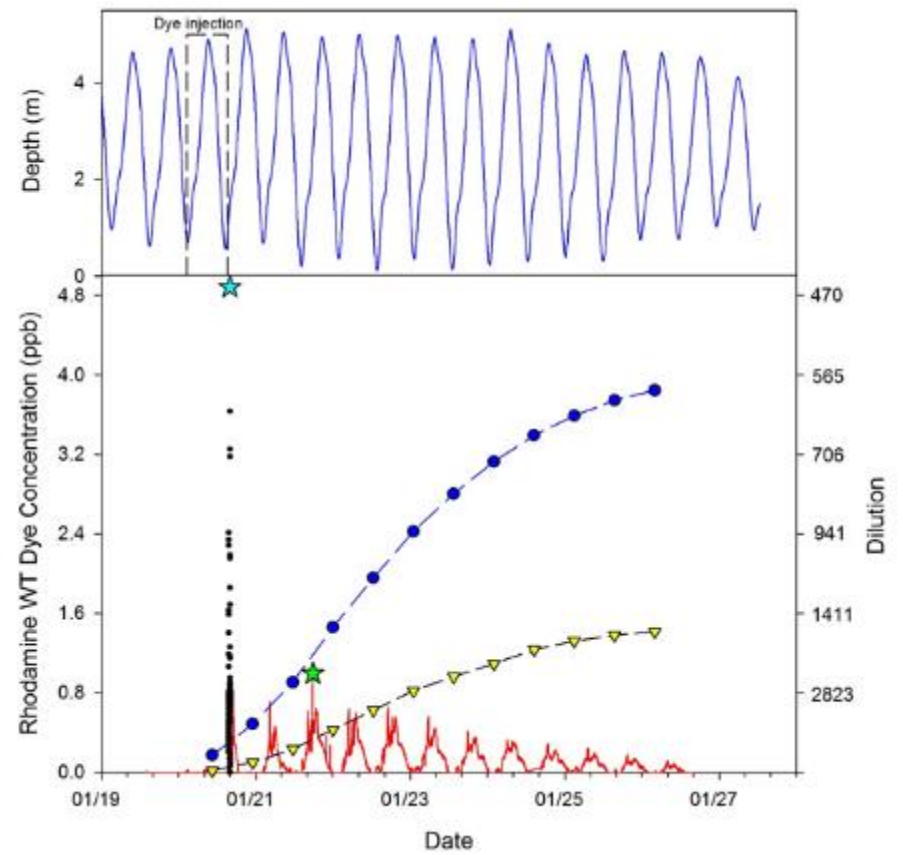
Figure 24 Time series of dye concentrations measured by the stationary fluorometers with calculated dilution curves in the estuary (site 13).
The period of dye injection is marked as dashed line on the tidal heights above the dye readings. The continuous red line represents continuous dye measurements. The dashed blue line with blue circles represents average peak 1h steady state dye concentrations. The dashed black line with yellow triangles represents average steady stage dye concentrations. The black dots represent plume tracking fluorescence measurements taken within a 100 m buffer. The blue stars represent peak dye concentration measured by tracking fluorometer. The green star represents peak dye concentration measured by fixed fluorometer.

Dye readings at station 3 were lower than those at stations 1 and 2 (Figure 24C). The peak concentration detected at this station was 5.99 ppb on 20/01/15 at 16:30 (LW - 0.17 h). Dye concentrations reduced to background levels on the same day at 21:30 on the flood tide (HW – 2 h). Peak levels of dye were subsequently recorded on 21/01/15 at 04:40 (LW - 0.5 h) (4.5 ppb), 21/01/15 at 18:10 (3.3 ppb) (LW + 0.7 h), 21/01/15 at 05:15 (2.3 ppb) (LW), 22/01/15 at 06:30 (2 ppb) (LW + 0.7 h) and 22/01/15 at 19:05 (1.12 ppb) (LW + 0.7 h). These peak concentrations were followed by peaks of lower magnitude (< 1 ppb) in subsequent days. Only traces of dye were recorded at this station on the flood tide 7 days after the dye injection. At station 5, the maximum concentration of dye (1.31 ppb) was detected on 21/01/15 at 22:00 on the flood tide (HW - 2.5 h), after two peak levels of dye on 20/01/15 at 17:00 (0.82 ppb) (LW + 0.5 h) and on 21/01/15 at 04:20 (0.71 ppb) (LW - 0.75 h) (Figure 24D). Peak dye concentrations of decreasing magnitude were recorded in the following days with only traces of dye (< 0.1 ppb) detected 7 days after the dye injection. Table 20 summarises the maximum instantaneous dye concentrations (ppb) and the corresponding minimum effluent dilutions obtained in the study sites.

Table 20 Maximum fluorescence concentrations recorded by the stationary fluorometers and corresponding minimum dilutions of sewage effluent in the estuary (site 13) and bay (site 32).

Study site	Station number								
	1	2	3	4	5	6	7	8	9
13	27.39 (82)	11.56 (195)	6.26 (361)	-	1.31 (1,719)	-	-	-	-
32	0.85 (4,340)	6.74 (558)	-[a]	1.15 (3,233)	7.26 (514)	-[b]	2.31 (1,590)	2.98 (1,200)	2.00 (1,900)

All fluorescence concentrations in ppb. [a] This shellfish station was lost in the week prior to the dye study during stormy weather. [b] The fluorescence data recorded at this station were considered erroneous and were excluded from the analyses.

In the estuary, the ebb tide on the day of the dye injection was at 10:35. The leading edge of the dye-tagged sewage effluent was tracked past station 5 at 17:02. The dye was firstly detected at station 1 at 13:55 and at station 2 at 14:38. Based on these results, the estimated average time of travel of dye-tagged effluent in the main channel was 1.57 km/h. This time of travel provides an indication of the time available to shellfish authorities for closure of shellfish harvesting activities in the event of a sewage spill.

Figure 25 shows the results of Rhodamine WT dye concentrations and corresponding dilutions obtained by the boat-towed fluorometers in the study sites. Boat tracking was only conducted during daylight hours and therefore, for the purposes of assessing the patterns of sewage effluent dispersion and dilution, these results should be analysed in conjunction with the data obtained by the fixed fluorometers. These maps are however detailed illustrations of the shape and the edges of the dye-tagged sewage plumes as they travelled away from the STW outfalls.

In the estuary (site 13), significant concentrations of dye-tagged effluent (10–50 ppb) travelled down the main channel of the estuary and impacted stations 1 and 2 during the ebb tide on the day of the dye injection (Figure 25A). The leading edge of the plume was identified at the end of the tracking period just past station 4 (1–5 ppb) where more dilution was available due to the larger intertidal area and deeper waters. These results are in agreement with the results obtained by the fixed fluorometers and confirm that the shellfish cages were adequately placed to represent the range of dilutions and dye concentrations in the main estuary channel.

In the bay (site 32), dye concentrations in excess of 50 ppb were detected around station 5 (nearest the STW D LSO) in the first hours after the beginning of the dye injection (Figure 25B). The area impacted by dye concentrations in the range 1–5 ppb was detected mainly within a 1 km radius from this station. The edges of the dye-tagged effluent plume correspond to concentrations within the range 0.5–1 ppb. It can be observed that the plume impacted mainly the eastern part of the bay. This is again consistent with the data obtained by the fixed fluorometers which indicated a clockwise circulation pattern inside the bay. However, the map also shows a large area impacted by the plume on the southwestern part of the bay and indicates offshore advection of the dye-tagged effluent on the second ebb tide. On 17/06/15, the edge of the plume was detected outside the bay (5 km from the LSO). This agrees with the results of the fixed fluorometers which indicated that the inshore stations were impacted by low quantities of dye (< 0.5 ppb). Therefore, under the conditions of the dye study, the sewage plume from the LSO is unlikely to impact these areas of the bay.

A

Legend

Outfall location	Shellfish Station

Concentration (ppb) **Dilutions**

0.00 - 0.01	>=225,800
0.01 - 0.05	225,800 to 45,160
0.05 - 0.10	45,160 to 22,580
0.10 - 0.50	22,580 to 4,516
0.50 - 1.00	4,516 to 2,258
1.00 - 5.00	2,258 to 452
5.00 - 10.00	452 to 226
10.00 - 50.00	226 to 45
50.00 - 151.00	<=45

B

Legend

Outfall Location	Station Locations

Concentration (ppb) **Dilutions**

<= 0.01	>= 379,200
0.01 to 0.05	75,840 to 379,200
0.05 to 0.10	37,920 to 75,840
0.10 to 0.50	7,584 to 37,920
0.50 to 1.0	3,792 to 7,584
1.0 to 5.0	758 to 3,792
5.0 to 10	379 to 758
10 to 50	76 to 379
> 50	<= 76

Figure 25 Dye concentrations and dilutions recorded by the towed fluorometers in the estuary (site 13; A) and bay (site 32; B).
The dilutions were determined by dividing the initial dye concentrations in the STW effluent by the final dye concentrations measured in the estuary and bay.

5.4.3 Relationships Between Norovirus in Shellfish and Sewage Dilution

Following detailed characterisation of water movements and the dispersion and dilution of sewage effluent in the experimental sites, the study focused on the relationship between the NoV concentrations detected in shellfish (reported in Chapter 5) and the sewage effluent dilutions obtained in the dye studies. The assumption here was that if NoV levels decrease with distance from the STW outfalls, as suggested by the results of microbiological monitoring, then there should be an association between dilution ratios of estuarine water to treated effluent and NoV contamination in shellfish. This relationship could be a useful tool for permitting regimes for sewage discharges and for the identification of buffer zones in SPAs to reduce human exposure to NoV.

Linear regression was used to study the relationships between GM concentrations of NoV in oysters and sewage effluent dilution using Rhodamine WT dye concentrations measured by the stationary fluorometers at both study sites. In this analysis, GM were calculated using NoV data for the period of high prevalence of the virus (October–March); GI and GII concentrations were summed to give an overall indication of the total NoV load at each station as recommended by EFSA (2012). Figure 26 shows an inverse relationship between decreasing concentrations of NoV and increasing levels of effluent dilution. The ranges in NoV levels obtained in the experimental sites overlap to give a full spectrum of contamination levels. The model is statistically significant and the R^2 (adjusted for degrees of freedom) is high and similar to the coefficient ($R^2 = 74\%$) obtained in the model describing the relationship between NoV and the number of sewage spills reported in Chapter 2. The GM and confidence intervals of concentrations of total NoV for four dilution ratios predicted by the linear model are summarised in Table 21. In the USA, the 1,000:1 dilution ratio is the minimum required for shellfish growing waters adjacent to STW outfalls classified as "conditionally approved" and "restricted" (Goblick *et al.*, 2011).
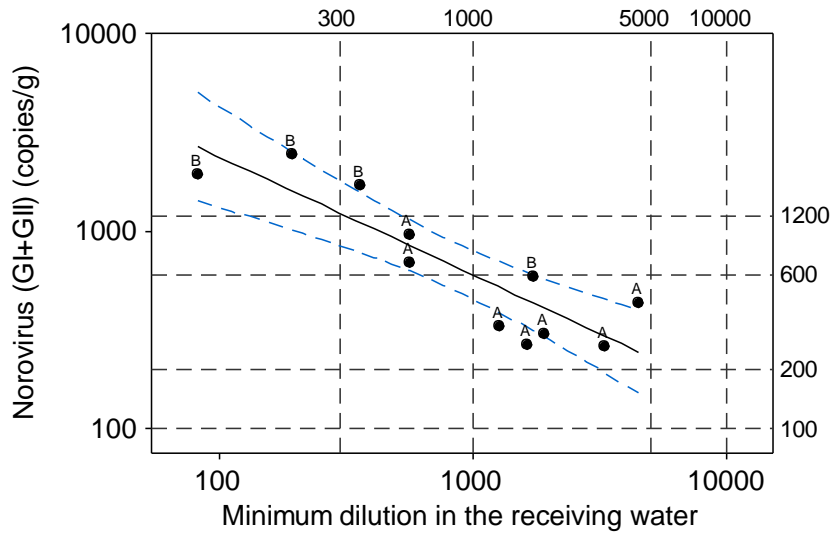
Figure 26 Linear regression of geometric mean concentrations of total norovirus (GI + GII) in oysters as a function of the dilution of sewage effluent in the estuary (site 13; B) and bay (site 32; A).
Minimum dilution was used to represent the worst-case scenario of shellfish contamination. Data period: October–March. The identification of the study sites is shown above the data points. The 95% confidence intervals for the regression line are shown as dashed blue line. Limit of quantification = 100 copies/g.
Linear model: $\log_{10}$norovirus = 4.567 - 0.5974*$\log_{10}$dilution; R^2 (adjusted) = 75.4%; p = 0.000.

Table 21 Predicted mean concentrations of total norovirus (GI + GII) in shellfish corresponding to four dilutions of sewage effluent in the study sites.

Dilution of estuarine water to treated effluent	Total norovirus (GI + GII) (copies/g)		
	Lower 95% CI	Geometric mean	Upper 95% CI
300:1	700	1,200	2,030
1,000:1	300	600	1,260
5,000:1	90	200	650
10,000:1	50	100	500

5.5 Discussion

This chapter described the results of drogue tracking and dye tracing studies to better understand the surface water circulation and the time of travel, dispersion and dilution of sewage effluent in the experimental sites. The approach used in the tracer studies combined dye fluorescence measurements and corresponding calculated dilutions through sewage plume tracking using boat-towed fluorometers and measurement at fixed locations using fluorometers attached to the shellfish cages. The plume tracking measurements allowed identification of the edges of the sewage plumes while the measurements at fixed locations allowed determination of the variation of dye fluorescence over 7 days after the dye injections. This approach was, for the first time, applied in the context of UK shellfisheries and successfully demonstrated physical

linkage between the injection sites (STW A and D) and the shellfish sampling points. Overall, the data obtained by plume tracking matched well with the data collected by the fixed fluorometers and both sets of information should be considered when describing the patterns of sewage effluent advection, dispersion and dilution.

Both experimental sites are characterised by weak tidal currents. The bay (site 32) is more exposed to wave action than the estuary (site 13) (Welsby and Motyka, 1989, 1989a). The results of the satellite drogue-tracking study conducted under gentle to fresh breeze from the S-SW indicated average surface water currents in a clockwise circulation pattern at 0.08–0.15 m/s. As a result of these weak tidal currents, the estimated tidal excursion inside the bay is only approximately 340 m along the coast and 90 m onshore/offshore. In the estuary (site 13), tidal currents in the upper reaches of the main channel on the ebb tide vary from 0.5 m/s on neaps to 1.2 m on springs. The estimated tidal excursion is approximately 10 km on spring tides and 5 km on neap tides.

The dye fluorescence data recorded at the sampling stations and the calculated steady-state dilutions using the super-position method clearly demonstrated the potential for substantial build-up of sewage effluent in the estuary and, to a lesser degree, in the bay. The results indicate that not all the sewage effluent discharged is diluted and dispersed by the tides. The proportion of effluent that remains in the waters mixes with the effluent entering the system thus causing concentrations to increase and build to a steady state maximum. This is common in estuaries (Committee on Wastewater Management for Coastal Urban Areas and National Research Council, 1993). Therefore, the microbiological impacts discussed below may not be representative of episodic sewage spill events from a SO, for example.

In the estuary (site 13), dye concentrations reached maximum levels at station 1 near the STW A outfall around LW. The peak levels of dye were detected approximately 11 h after the beginning of the dye injection. Dye concentrations decreased as distance from the outfall increased and dilution ratios ranged from 82:1 at station 1 to 1,719:1 at station 5. It was estimated that, during the ebb tide, sewage effluent travelled about 9 km in the estuary at 1.75 km/h. All stations in the main channel approached steady-state 7 days after the dye injection. This indicates that the estuary is slowly flushed and is

unlikely to lose a significant fraction of NoV contamination during ebb tides following a discharge event. Consequently, the results of the dye study were consistent with the spatial pattern of NoV contamination in shellfish and explain the frequent high levels of NoV in shellfish sampled from the main estuary channel over sequential tidal cycles.

In the bay (site 32), station 5 positioned near the LSO was the most impacted by dye-tagged effluent. Peak dye concentrations at this station were obtained around HW, approximately 5 h after the beginning of the dye injection. This is the period of time during which sewage effluent travels from the final effluent chamber through the LSO and becomes visible in the bay at the surface. Most of the dye was quickly flushed away from the central area of the bay and moved on a clockwise circulation pattern impacting mainly the eastern part of the bay on the food tide. Maximum dilutions in this part of the bay ranged from 559:1 at station 5 to 6,168:1 at station 1. Comparatively, stations on the western part were impacted by lower quantities of dye indicating that, under the conditions of the study, the effluent plume is unlikely to impact the western part of the bay. This is also consistent with the significantly higher concentrations of NoV GI detected in mussels at station 5 (STW D LSO) than those at stations 6 and 7. Most concentrations were however low and below LoQ and therefore these differences may not represent an actual health risk. However, the results of the tracer study do not explain the higher mean levels of *E. coli* obtained at station 9 suggesting that the STW D outfall may not be the main source of bacterial contamination impacting the bay. Using the boat-towed fluorometer, it was also possible to identify offshore advection of the plume at the surface in a south-westerly direction on the second ebb tide. Overall, the microbiological impacts observed at this site indicate that the LSO with a multiport diffuser effectively reduces the health risks associated with NoV arising from a buoyant effluent plume as affected by the tidal currents and wind-driven currents studied.

The dye dilution estimates were used to establish the relationships between GM concentrations of NoV in oysters and sewage dilution in the study sites. This relationship was modelled by linear regression using data from both study sites during the period of high NoV prevalence in the UK (October–March). The levels of explained variance of the linear model were high (> 75%) (Figure 26) and the regressions were statistically significant, indicating that conducting tracing studies such as those reported here to represent the spatial and temporal variability of dye-tagged effluent and use

dilution levels at compliance sites can be used to predict the NoV risk in commercial shellfisheries. A similar study conducted by Goblick *et al.* (2011) also indicated that the bio-accumulation of male-specific coliphage, NoV GII, faecal coliforms and *E. coli* in oysters were directly related to dye concentrations measured at four stations in Mobile Bay (USA). The results presented in this study support the approach taken in the USA on the use of dilution ratios of estuarine water to sewage effluent for the purposes of managing microbiological risks in shellfisheries (Goblick, 2015) and demonstrate that a similar approach could be used in the UK. Different dilution ratios could be used by regulators to manage SPAs with different risks. The experimental sites studied here are contrasting in relation to pollution source impacts and therefore the results can be applied with confidence to other sites with similar characteristics. In the USA, an effluent dilution of 1,000:1 is an absolute requirement for the establishment of a prohibited NoV buffer zone around a STW discharge impacting a shellfish production area classified as "conditionally approved" or "restricted". In the sites studied here, the daily average dilution estimates indicated that, in the estuary (site 13), the 1,000:1 dilution would occur at 7.5 km from the STW A outfall while in the bay this dilution ratio would occur at 0.7 km from STW D LSO. The predicted mean concentration of total NoV corresponding to this dilution was 600 copies/g. In a comparison of NoV concentrations in oyster samples linked to NoV illness with concentrations found in oysters from SPAs, Lowther *et al.* (2012) found that the GM concentration in outbreak samples was 1,048 copies/g. From a policy development perspective, this work represents a significant progress in this area and demonstrates that the implementation of a minimum 1,000:1 dilution ratio for class B production areas in the UK would contribute to reduce human exposure to NoV. However, it should be noted that the relationship between NoV contamination and sewage dilution was established using a measure of central tendency (GM) for microbiological data and therefore reflects "average" conditions in the study sites. The 95% confidence interval for the 1,000:1 dilution ratio is relatively large (300–1,260 copies/g) and this should be considered when using these results to establish buffer zones in other sites. To achieve higher safety levels, it is appropriate to consider additional control measures at primary production and/or end product. This is further explored in Chapter 6.

Chapter 6

Measures to Control Norovirus Contamination in Shellfish Production Areas

"(2) Removal of mussel beds to a safe distance. – In certain fisheries, where the beds consist of planted mussels, abandonment of ground subject to sewage pollution, and planting on safe ground might be practised, if such could be found. Or, again, what amounts, in effect, to the same thing, new beds might be established in clean areas, being stocked, if necessary, from polluted beds." In (Dodgson, 1928, p. 63–64)

This chapter presents the results of an assessment of measures to control the risk of NoV contamination in SPAs to address objective 4 of this thesis. The control measures focus on the establishment of NoV limits for production areas and buffer zones around sewage outfalls and other point sources of sewage pollution. This assessment is informed by the empirical data reported in Chapters 3–5 using the experimental sites 13 and 32 as case studies. For the purposes of identifying buffer zones in the study sites, consideration was given to the guidance set out in the US NSSP because EU Food Hygiene Regulations do not contain requirements for the establishment of these zones. Therefore, the purpose of this assessment was to illustrate an approach that could be taken to delineate buffer zones and their impacts on the hygiene status of SPAs in the UK. The assessment also includes a validation exercise in which buffer zone distances were identified for a selection of commercial SPAs using published and modelled NoV data. These control measures are considered additional public health measures to the protection afforded by the existing *E. coli* monitoring and post-harvest treatments described in Chapter 1. There is however much uncertainty as to how the *E. coli* monitoring data should be used to indicate the viral risk and this is also considered in this assessment.

6.1 Relationships Between *E. coli* and Norovirus and Limits for Production Areas

Regulatory standards for SPAs based on *E. coli* monitoring have been in place in the EU for many years and have contributed to control the incidence of illnesses of bacterial origin (see Figure 2). However, there is growing awareness that *E. coli* monitoring, in

isolation, and the application of depuration, are not effective in controlling the health risks from enteric viruses. On an individual sample basis, many studies have found poor correlations between *E. coli* and NoV (Serracca *et al.*, 2010; Lowther *et al.*, 2012a). In the marine environment, a linear correlation between *E. coli* and NoV is improbable because the biological differences between viruses and bacteria (*E. coli* presence is much less episodic than NoV) which determine different dilution, transport and inactivation rates outside the human host. Therefore, single or small numbers of regulatory *E. coli* results do not provide a reliable indication of the possible risk by viral pathogens (Cefas, 2014). This risk is assessed with more confidence if based on a time series of *E. coli* data (Lowther *et al.*, 2012a). It is often assumed that failure to detect *E. coli* in surface waters indicates that the detection of viral pathogens is unlikely. Conversely, when FIOs are detected, the likelihood of detecting NoV increases significantly. This concept is illustrated in Figure 27 below. An exponential decay model fitted to the monitoring data obtained in the bay (site 32) shows that the GM of NoV approaches LoQ (100 copies/g) as the percentage of *E. coli* results below LoD increases from 0 to 60%. The spread of data points in the lower range of percentage of *E. coli* results do not fall close to the curved fitted line causing the standard error of the regression (S) to be relatively high.
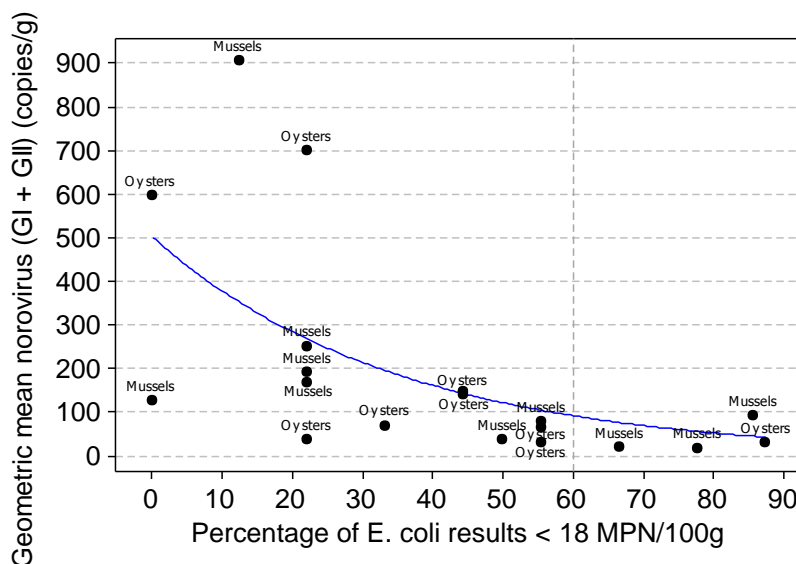
Figure 27 Relationship between geometric mean concentrations of total norovirus (GI + GII) and the percentage of *E. coli* results below the limit of detection of the MPN method in shellfish from the bay (site 32). Limit of quantification = 100 copies/g. Models: geometric mean norovirus (GI + GII) = 500.811*exp(-0.028465*% < 18 *E. coli*) (S = 5.397).

Summary statistics of *E. coli* concentrations were calculated for six ranges of NoV contamination. All data from both experimental sites were used in this analysis. Table 22 shows that mean concentrations of the faecal indicator organism increased from 38 to 297 as NoV contamination levels also increased from < 100 to > 1,000 copies/g. However, *E. coli* results below LoD were detected in all NoV contamination ranges. The production areas would be class B under Regulation (EC) No. 854/2004 irrespective of the level of NoV contamination detected in the shellfish. This indicates that mean levels of *E. coli* would be a more appropriate measure than absolute limits for management of NoV risks in SPAs.

Table 22 Means and ranges of *E. coli* concentrations corresponding to six ranges of paired total norovirus (GI + GII) concentrations in shellfish from the estuary (site 13) and bay (site 32).

Norovirus (copies/g)		*E. coli* (MPN/100 g)				
Range	n (%)[a]	n[b]	Minimum	Maximum	Geometric mean[c]	Class[d]
Not detected	13 (5.2)	13	< 18	170	38	A
< 100	96 (38.6)	95	< 18	5,400	43	B
100–200	22 (8.8)	22	< 18	1,300	43	B
200–500	42 (16.9)	39	< 18	5,400	86	B
500–1000	31 (12.4)	26	< 18	1,300	92	B
1,000–10,000	45 (18.1)	44	< 18	5,400	297	B

[a] Number and percentage of norovirus results falling into each of the six concentration ranges. [b] number of *E. coli* results falling into each of the six concentration ranges. [c] *E. coli* results at the lower limit of quantification of the MPN method were adjusted to 19 MPN/100 g (USFDA and ISSC, 2013). [d] Classification that would be given to the production area based on the *E. coli* criteria of Regulation (EC) No. 854/2004.

The establishment of NoV standards for shellfish in the EU Food Hygiene Regulations has been suggested as an alternative measure to reduce the NoV risk. In 2012, the EFSA Panel on Biological Hazards published an opinion on additional control measures for oysters (high risk species) which considered options for NoV limits in shellfish (EFSA, 2012). Using NoV monitoring data from Ireland, UK and France, the panel evaluated compliance impact scenarios for five potential NoV limits for the period of high prevalence (January–March 2010). The report concluded that a NoV limit at the LoQ (100 copies/g) would result in a high percentage of non-compliant samples while intermediate limits (e.g. 200, 500 or greater copies/g) would have an intermediate impact on sample non-compliance. A limit established at the upper level of contamination (e.g. 10,000 copies/g) would correspond to 100% compliance.

A similar compliance impact assessment was conducted using the NoV monitoring data collected in this study considering a longer period (October–March) than that considered in the EFSA assessment. In this assessment, it is possible to compare levels of compliance in shellfish from two production areas impacted by different levels of sewage pollution and, for the first time, to compare levels of compliance in co-located shellfish. The results of this assessment are summarised in Table 23.

Table 23 Percentage of shellfish samples non-compliant with five possible total norovirus (GI+GII) limits during the period of high prevalence of the virus in the estuary (site 13) and bay (site 32).

Study site (species)	Data periods	n*	Limit (copies/g)				
			100	200	500	1,000	10,000
13 (oysters)	October 2012–January 2015	43	86.0	81.4	67.4	51.2	0.0
32 (mussels)	March 2014–March 2015	42	78.6	64.3	26.2	11.9	0.0
32 (oysters)	March 2014–March 2015	43	88.4	72.1	41.9	11.6	0.0

n = total number of samples. * Includes results from all stations in the shellfish production area.

A high percentage (78.6–88.4%) of samples would be non-compliant with a NoV limit at the low end of the possible range (100 copies/g) indicating that a limit established at LoQ would have a high management and compliance impact on both study sites. This is consistent with the EFSA assessment. By comparison, a NoV limit established at 1,000 copies/g would result in substantially different compliance scenarios for the study sites with 51% non-compliant samples in the estuary (site 13) and only 12% of samples non-compliant in the bay (site 32). Also of note was the higher percentage of non-compliant samples in Pacific oysters (42%) than in mussels (26%) sampled from site 32 for a NoV limit established at 500 copies/g suggesting that the compliance profile would be dependent on the species monitored, to a certain extent.

6.2 Buffer Zones Around Sewage Outfalls and Other Sources of Sewage Contamination

6.2.1 Assessment of Norovirus Contamination and the Classification Status of the Study Sites in Relation to US NSSP Guidance

Study site 13

At the time of the dye tracing study, the STW A was operating outside of typical "dry weather" operating conditions and would be considered malfunctioning under the US NSSP. The sewerage network which includes these treatment works has known groundwater infiltration problems and the flows into the STW are greater than the capacity of the works to treat sewage influent fully causing the storm tank to discharge untreated settled sewage into the estuary for long periods of time. The continuous discharge of treated effluent from STW A has been subject to UV disinfection since 2008 and a second UV disinfection plant was installed at the storm tank in 2014. The results in Section 4.6.3 suggest that this latter treatment upgrade may have improved the microbiological quality of the water in the estuary although more data would be needed to confirm this. In the absence of these data, the SPA would be recommended for "conditionally restricted" management classification[4] due to the high concentrations of NoV discharged into the SPA (Figure 9) and the low dilution and long residence times of the water in the estuary (see Section 5.5). Under the NSSP, the management options that would be available to the "conditionally restricted" area would be:

- Long-term relaying of shellfish stocks to "approved" waters[5] (typically > 6 months);
- Shellfish growing waters used for long-term relaying must meet the "approved" classification status at all times; and

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[4] A "restricted" classification category under the NSSP guidance is broadly equivalent to class B under Regulation (EC) No 854/2004 in the EU. Further detail on equivalence between the US and EU classification systems is given by Lees (2000). In the USA, SPA that are intermittently affected by pollution (classified as "conditionally approved") are required to have a written management plan based on STW performance which is designated Conditional Area Management Plan (CAMP).

[5] An "approved" classification category under the NSSP guidance is broadly equivalent to class A under Regulation (EC) No 854/2004 in the EU.

- Harvesting of long-term relayed shellfish from "approved" waters can only occur when the viral quality of the shellfish meets a male-specific coliphage (MSC) standard of 50 pfu/100g (USFDA and ISSC, 2013)[6].

Given the microbiological impacts detected in the estuary reported in Chapter 4, it is unlikely that a production area would meet the NSSP "approved" status. If this where the case and long-term relaying of shellfish was not practical, then the most appropriate option under the NSSP requirements could be the use of seed for grow out in another production area. Further monitoring data post-sewerage improvements, indicating a reduction in NoV contamination levels to confirm the results reported in Section 4.6.3 and return of STW A to typical "dry weather" operating conditions, would result in a recommendation for a "conditionally approved" classification. This classification would require the identification of a minimum 1,000:1 dilution as shown in Figure 28. From the linear model of NoV levels versus sewage dilution shown in Section 5.4.3, the predicted GM concentration of NoV for a dilution ratio 1,000:1 would be 600 copies/g. This concentration is within the range of concentrations (> 500 copies/g) associated with higher risk of NoV illness found by Lowther *et al.* (2012).

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[6] MSC is considered a good indicator for NoV (Flannery, Keaveney and Doré, 2009; Doré, Henshilwood and Lees, 2000). Goblick (2015) conducted a review of sewage effluent dilution and MSC data obtained as part of studies undertaken by the USFDA during the period 2008–2015 and observed that, at sites impacted by STW operating as per consent conditions, all shellfish harvested from areas with dilution greater than 1,000:1 had MSC concentrations below 50 pfu/100 g.

Figure 28 Rhodamine dye concentrations and sewage effluent dilutions recorded by the towed fluorometer in the estuary (site 13) on 20 January 2015.
Dilutions of less than 1,000:1 are highlighted in purple.

Under the "conditionally approved" classification, the management plan could consider information on time of travel of sewage effluent between the source and the SPA, i.e. closure of the production area in the event of sewage spill prior to the anticipated impact. At the time of the dye study, the tidal velocity was estimated at 1.6 km/h. The 1,000:1 buffer zone could be extended to the limit of the tidal excursion (maximum distance that the dye-tagged effluent plume is expected to travel on a spring ebb tide) which occurs just past station 4 (Figure 28). This control measure would provide additional buffer to account for higher flows at STW A which may influence NoV loadings arriving at the works and consequently, as observed in Figure 13, NoV removal rates and translate into lower dilution in the estuary. Again, during typical "dry weather" operating conditions, parts of the SPA downstream of the limit of tidal excursion could be classified as "conditionally approved".

Following a STW upgrade, consideration should still be given to the frequency of sewage overflows, or any condition that may reduce the quality of the effluent, to determine the potential frequency of a growing area closure over the course of a year. This assessment

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would determine the feasibility of operating a "conditionally managed" area based on STW performance. Additional sampling could be employed to confirm if coliphage levels in shellfish are below 50 pfu/100 g during periods of normal operation to support an upgrade in classification from "conditionally restricted" to "conditionally approved". The management plan should include provisions that detail the criteria for re-opening the SPA after a closure event due to degraded effluent quality.

Study site 32

During the dye study, STW D was operating within the discharge consent conditions. However, these treatment works operate secondary treatment and do not disinfect the final effluent and, under the NSSP, a STW that either loses or does not utilise disinfection is considered a "malfunctioning" treatment works. In this case, it would be necessary to demonstrate that there is sufficient effluent dilution in the production area to calculate the buffer zone and meet the NSSP microbiological standard for approved areas of 14 FC/100 ml of seawater (as a GM). In the absence of STW performance monitoring data, an assumed bacterial level of 2.8×10^6 faecal coliforms/100 ml for raw sewage discharges (Kay *et al.*, 2008) requiring a 100,000:1 dilution to achieve a concentration of 14 FC/100 ml in the production area (meeting the criteria in the NSSP for "approved" waters[7]) is typically used. However, as the STW provides secondary treatment, and there is final effluent *E. coli* data available from the research (see Section 3.7.1), consideration was given to the 90th percentile *E. coli* level[8] summarised in Table 24. Assuming a concentration of 8.37×10^4 cfu/100 ml discharged from the STW, a dilution of approximately 6,000:1 would be needed to reach the NSSP "approved" classification.

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[7] An 'approved' classification category under the NSSP guidance is broadly equivalent to class A under Regulation (EC) No 854/2004 in the EU. Further detail on equivalence between the US and EU classification systems is given by Lees (2000).

[8] The NSSP prescribes water quality standards as both geometric means and 90% limits.

Table 24 Summary statistics of *E. coli* concentrations in final effluent discharges from sewage treatment works D and dilutions needed to achieve the faecal coliform standard for "approved" waters.

Date of first sample	27/03/2014
Date of last sample	17/06/2015
Number of samples	10
Geometric mean *E. coli* (cfu/100 ml)	1.98×10^4
90th percentile (cfu/100 ml)	8.37×10^4
Dilution needed to achieve 14 faecal coliforms/100 ml	5,979
STW flows/90th percentile flows (factor difference)	1.91
Equivalent dilution necessary for 90th percentile flows	11,419

Considering the STW flows recorded during the dye tracing study and the dye tracking data obtained, parts of the bay encompassing the areas represented by station 5 near the STW D LSO and station 3 had dilutions less than 6,000:1. These areas would be considered impacted by final effluent discharges from STW D as shown in Figure 29.

Figure 29 Rhodamine dye concentrations and sewage effluent dilutions recorded by the towed fluorometer in the bay (site 32) on 16–17 June 2015 with dilutions of less than 6,000:1 highlighted in blue.

However, flows at STW are usually subject to large and rapid variations resulting in long periods of low or no flow followed by full flow conditions in short periods of time (CES, 2002). A flow and load survey undertaken at STW D by Pell Frischmann Consultants (2003) indicates that the diurnal flows at these works varied from 25 l/s to just over 200 l/s. This daily flow variation can affect the level of sewage effluent dilution in the

bay. Under the NSSP, higher flow rates need to be considered when establishing the size of a buffer zone. Consideration of the 90th percentile flows at the works would represent an impact that would include all stations in the bay showing dilutions less than 6,000:1 as shown in Figure 30.

Figure 30 Rhodamine dye concentrations and sewage effluent dilutions recorded by the towed fluorometer in the bay (site 32) on 16–17 June 2015 with dilutions of less than 6,000:1 for the 90th percentile flows through the STW highlighted in blue.

Comparison of the dye tracing data obtained by the fixed fluorometers with data obtained by the towed fluorometer shows that stations 5, 8 and 3 had dilutions < 6,000:1 (based on the maximum dye levels found under the study conditions) and therefore represent areas of the bay that were most impacted by sewage effluent (Table 25). Station 1 had dilutions slightly greater than 6,000:1, and stations 7, and 9 had the greatest dilutions above 6,000:1 based on maximum dye levels detected during the study. However, when the higher 90th percentile flows are considered, all stations show dilutions less than 6,000:1. Based on these results and the fact that STW D does not disinfect the effluent, the entire area within 6,000:1 dilution and encompassing all stations would be classified as "prohibited". However, if STW D were to include a UV disinfection treatment, was monitored to detect malfunctions and changes in effluent quality, and if the shellfish authority has the resources to enforce the conditions of the

management plan, then this production area would meet the requirements for "conditional management" under the NSSP using a 1,000:1 dilution to define the buffer zone. Furthermore, Although STW D does not have a history of frequent overflows, under the NSSP, a CAMP would need to be developed that would describe the protocol for operating the conditional area such as establishing procedure for closing the conditional area in the event of a STW malfunction, including a loss of disinfection.

Table 25 Summary of sewage effluent dilutions obtained in the dye tracing study conducted in the bay (site 32).

Station number	Dilution (peak 1 h)		Dilution (maximum based on stationary fluorometers)		Dilution (maximum based on tracking fluorometers)	
	Study flows	Adjusted (90% flows)	Study flows	Adjusted (90% flows)	Study flows	Adjusted (90% flows)
1	7,757	4,061	6,168	3,229	7,056	3,694
2	2,062	1,080	810	424	11,689	6,120
3	ND	-	ND	-	5,581	2,922
4	23,580	12,346	5,748	3,009	8,658	4,533
5	12,263	6,420	559	293	44	23
6	Err	-	Err	-	7,450	3,900
7	41,328	21,638	7,633	3,996	6,091	3,189
8	12,167	6,370	3,006	1,574	6,702	3,509
9	190,551	99,765	9,021	4,723	9,590	5,021

Err - fluorometer malfunction. ND - not determined.

The change in the size of the prohibited zone as a result of a new disinfection treatment introduced at STW D was estimated using the 1,000:1 dilution ratio. Figure 31 shows that the "prohibited zone" could be as small as a radius around the STW outfall large enough to encompass all of the purple data points which show dilutions less than 1,000:1. These calculations are based on the 90th percentile flows at the STW.
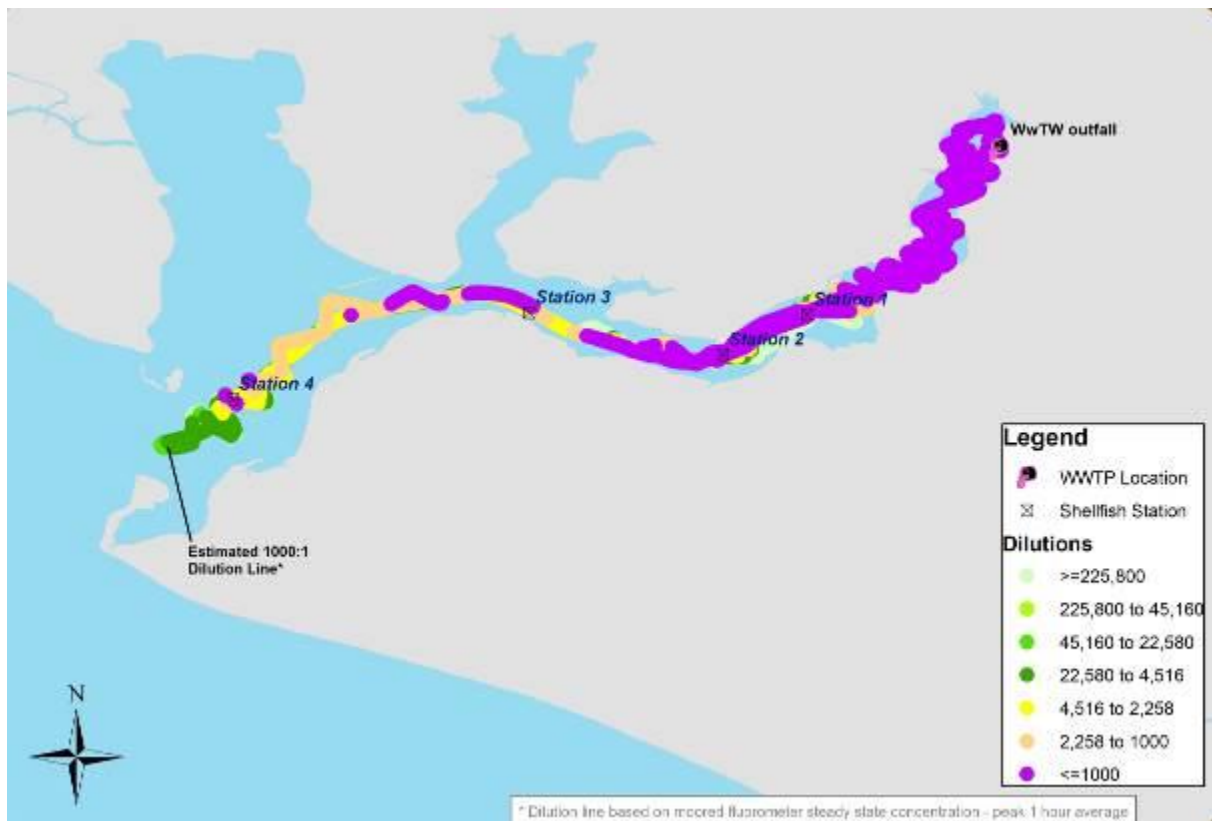
Figure 31 Rhodamine dye concentrations and sewage effluent dilutions recorded by the towed fluorometer in the bay (site 32) on 16–17 June 2015 to illustrate the size of the buffer zone as a result of a new UV disinfection treatment at sewage treatment works D.

Dilutions of less than 1,000:1 for the 90th percentile flows through the STW are highlighted in purple.

Under the NSSP, the time of travel from the STW D LSO to the limit of the SPA would need to be considered so that the competent authority could be notified and respond to a treatment failure (for example a failure of disinfection) before the area was impacted by the contaminated effluent. The time of travel to the edge of the buffer zone may be relatively short and impractical to manage as the time to respond may be much greater than the time for contaminated effluent to travel beyond the borders of the buffer zone. Instead, it may be more practical to consider a prohibited zone that encompasses the area that was impacted by the dye on the first day of tracking showing the geographical extent of dye-tagged effluent that travelled during this timeframe as shown in Figure 32.
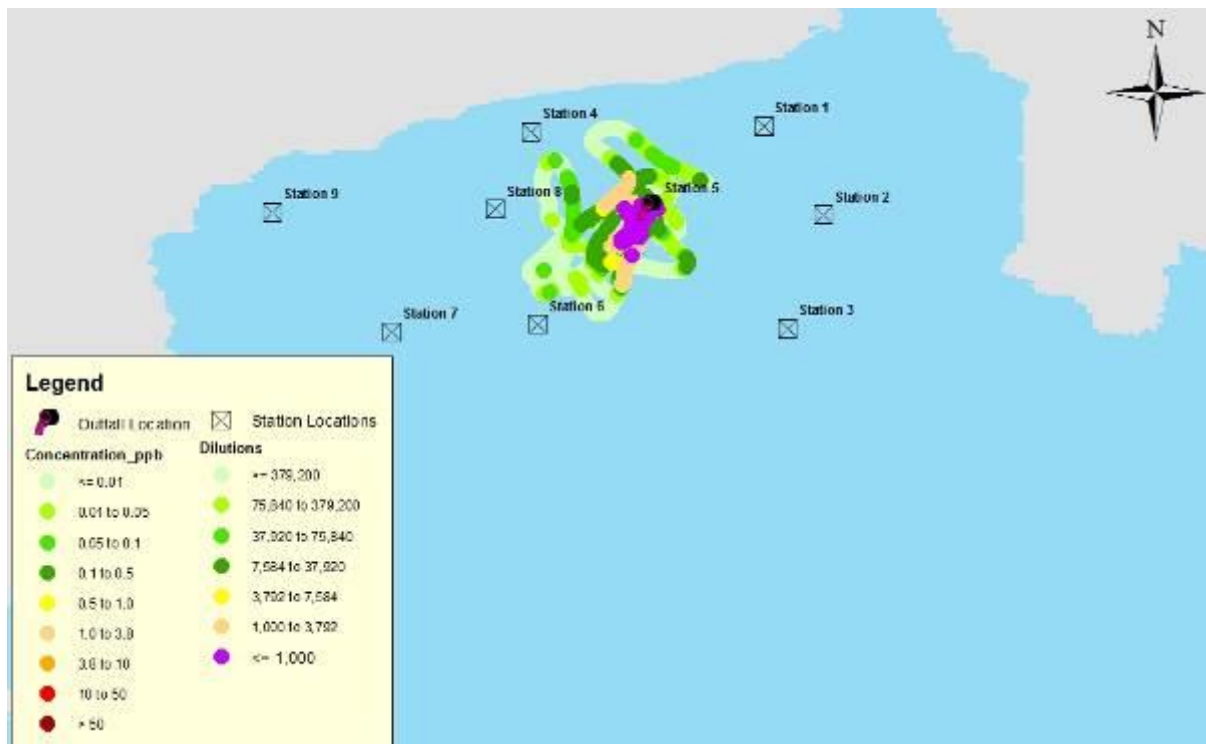
Figure 32 Rhodamine dye concentrations and sewage effluent dilutions recorded by the towed fluorometer in the bay (site 32) on 16–17 June 2015 to illustrate the area impacted by the dye on the first day of tracking. Dilutions of less than 1,000:1 for the 90th percentile flows through the STW are highlighted in purple.

In summary, the conclusions of the assessment on buffer zones in the experimental sites are the following:

Site 13

- At the time of the dye study, the STW A which provides UV disinfection was operating outside of typical "dry weather" operating conditions (frequent sewage overflows) and would therefore be considered malfunctioning.

- In the absence of monitoring data showing water quality improvements in the SPA, the area would be recommended for "conditionally restricted" management classification because of the frequent exposure to NoV from sewage discharges and the relatively low dilution and long residence times in the estuary.

- Monitoring data, post-sewerage improvements indicating a reduction in NoV contamination – reported as, but not exclusively, gene copies – potentially as a result of the new UV disinfection plant at the storm tank or following rectification of the frequent SOs and return of the STW to typical "dry weather" operating

conditions would result in a recommendation for a "conditionally approved" classification regime using a 1,000:1 dilution and a more manageable situation for the production area.

- Under the above classification status, the management plan could consider the time of travel of sewage effluent, i.e. closure of the "conditionally approved" area in the event of sewage spill prior to the anticipated impact on the harvesting area (< 6 h). The buffer zone could be extended to the limit of the tidal excursion (maximum distance that the dye-tagged effluent plume is expected to travel on an ebb tide) which occurs just past the 1,000:1 dilution line.

- Confirmatory sampling could be employed to determine if MSC levels in shellfish are below 50 pfu/100 g during periods of normal operation to support an upgrade in classification from "conditionally restricted" to "conditionally approved" after STW upgrading.

Site 32

- The impacting STW D was operating within the permit conditions but providing only secondary treatment. Consequently, a conditional area management using a 1,000:1 dilution under the NSSP would not apply. As a result of the lack of disinfection at STW D, consideration could be given to the 90th percentile bacterial and STW flow data to identify a buffer zone. The results indicate that the entire area of the bay within 6,000:1 dilution and encompassing all stations would be classified as "prohibited".

- If STW D were to include a UV disinfection treatment, and was appropriately monitored to detect malfunctions and changes in effluent quality, and if the shellfish authority had the resources to enforce the management plan, then this production area would meet the requirements for "conditional management" under the NSSP using a 1,000:1 dilution to size the buffer zone. In these circumstances, the buffer zone would be a relatively small area around the LSO. The time of travel of sewage effluent would need to be considered and the competent authority notified and

respond to a treatment failure before the production area was impacted by the contaminated effluent.

6.2.2 Prediction of Buffer Zone Distances in a Selection of Commercial Production Areas

Further to the assessment above undertaken for the experimental sites, buffer zone distances were identified for a selection of commercial SPAs in England to evaluate the feasibility of implementing this type of risk management approach to commercial shellfisheries in the UK. In this assessment, the concentrations of total NoV (GI + GII) in oysters from the five sites presented in Chapter 2 of this thesis for the period October–March were compared with the concentrations of the virus predicted by the linear model described in Section 5.4.3. Four of the selected production areas are located in estuaries and one production area is located in open coast. Concentrations of *E. coli* for the main sources of sewage contamination impacting the study sites were estimated based on the consented dry weather flow of the discharges and reference faecal coliform concentrations for specific types of sewage effluents published by Kay *et al*. (2008). Dilution levels were estimated using a model of contaminant diffusion based on unidirectional flow developed by Fischer *et al.* (1979). The dispersion model assumes complete vertical mixing in the water column, steady uniform tidal currents, uniform depth and constant source(s) of faecal contaminant input and therefore provides very crude dilution estimates. The predicted NoV concentrations were then compared with actual observed values from previous studies at these sites (Lowther *et al*., 2012).

The established relationship predicted actual NoV concentrations within 0.5 $\log_{10}$ at sites D and F (estuaries) and G (open coast embayment) (Table 26). The relationship overestimated (0.77 $\log_{10}$ difference) actual NoV concentrations for estuary C which was impacted by the lowest microbiological loading in the sewage effluent, and underestimated NoV concentrations in site E (0.81 $\log_{10}$ difference) which was impacted by the highest microbiological loading. However, the differences between the predicted and observed NoV concentrations did not exceed 1 $\log_{10}$ at any study site.

Table 26 Predicted and observed norovirus concentrations and corresponding buffer zone distances between sewage discharges and sampling points in five shellfish production areas.

Characteristics of the shellfish production area			E. coli concentration in effluent (units/s)	Buffer zone distance (m) from STW outfall to the sampling point	Concentrations of norovirus (GI + GII) (copies/g) ($\log_{10}$) in the period of high prevalence (October–March)	
Type	Average water depth (m)[a]	Tidal stream velocity (m/s)[b]			Predicted by the uni-directional flow model developed by Fischer et al. (1979)	Observed in the surveillance data published by Lowther et al. (2012a)
Estuary C	1	0.463	40,333	580	890 (2.95)	152 (2.18)
Estuary D	0.6	0.500	563,500	2,600	1,515 (3.18)	1,298 (3.11)
Estuary E	10	0.257	3,951,041,667	5,433	643 (2.81)	4,201 (3.62)
Estuary F	3.5	0.309	1,220,167	6,744	2,157 (3.33)	7,258 (3.86)
Open coast G	5	1.132	95,750	9,871	133 (2.12)	243 (2.39)

[a] In the tidal area covered by the fishery. [b] On spring tides.

In summary, this study highlights that the geographical extent of buffer zones associated with different possible NoV target concentrations would depend upon:

(a) The hydrodynamic conditions in the receiving waters;

(b) The microbial concentrations in effluent discharges; and

(c) The location of the fisheries in relation to the pollution sources.

It should be noted that the buffer zone analysis is based on predicted NoV concentrations in shellfish flesh for the period of high population disease prevalence. Further analysis could consider the period of low NoV prevalence. In this study, it was not possible to conduct this type of assessment because of the low number of samples collected during the period of low prevalence.

Chapter 7

Conclusions and Recommendations

"Thus, there is considerable immediate potential for better regulation and improved public health with significant additional gains likely in this area and the developing tools become available to underpin a sustainable use of shellfish resources world-wide. It would therefore seem timely, fitting and logical to begin to realise that potential without delay to achieve those health gains in a consistent and collaborative way." In Kay and Rees (2010, p. 327)

This project sought to improve understanding of the fate and behaviour of human NoV, a highly contagious, constantly evolving and environmentally resistant virus which is the cause of most cases of illness associated with the consumption of raw shellfish. The study focused on the main sources of NoV to the marine environment (human sewage discharges and rivers), and the catchment and nearshore conditions driving NoV contamination in SPAs. Improved knowledge on the environmental transmission of NoV is important to regulatory agencies, in developing pollution reduction programmes and improving the microbiological quality of coastal waters; to food safety authorities, in dealing with the public health and legal consequences of NoV outbreaks; to the shellfish industry, in considering risk mitigation measures; and to the water industry, in prioritising future investment in sewerage infrastructure.

A review of the available literature on the environmental transmission of NoV indicated that there is insufficient information on typical NoV concentrations in sewage effluents to inform catchment source apportionment initiatives to mitigate the NoV risk. Although Food Hygiene Regulations contain specific requirements for the examination of the variation of microbiological contaminants in production areas, empirical data on NoV concentrations in shellfish are lacking and decay coefficients are only available for *E. coli* which do not reflect the behaviour of the viruses. Furthermore, hydrodynamic models simulating the fate and transport of NoV in production areas do not currently exist in the UK. This constrains the development of control measures for NoV. The research presented in this thesis contributes to the scientific knowledge in this area and identifies important areas for policy development in the interests of the shellfish industry and

human health. The following paragraphs summarise how the information gaps were addressed, how the objectives of the study were achieved, and what conclusions have been drawn from the findings.

Environmental Drivers of E. coli and NoV Contamination in Shellfish Production Areas

Generic models were developed to predict *E. coli* and NoV concentrations in shellfish. This modelling study used existing data on concentrations of these microbiological contaminants in oysters from 31 commercial harvesting areas on the coast of E&W for the period May 2009–April 2011 and climatic, demographic, hydrometric, pollution source and other characteristics of upstream river catchments. The predictive environmental factor for *E. coli* contamination, as measured by MPN, was rainfall (cumulative over 7 days before sampling) while the predictive factors for total NoV (GI + GII), as measured by PCR, were water temperature, total volume of sewage discharges in the catchment and river flows. The differences observed in the predictive factors reflect the longer persistence of NoV than *E. coli* in the environment and the slow rates of viral elimination from shellfish. It was concluded that reliance on *E. coli* as a regulatory tool is likely to be only partially effective at managing the NoV risk. However, the levels of explanatory variance of the models were low and therefore not suitable for use in a risk management context.

The most operationally useful model found in this study was the model describing the relationship between GM concentrations of total NoV (GI + GII) and the average number of sewage spills to the study sites (R^2 = 74%; *p* = 0.001; n = 10). This model predicted a NoV concentration of 100 copies/g corresponding to an average of 14 sewage spills. This concentration is lower than the average concentration found in oysters associated with cases of NoV gastroenteritis (Lowther *et al.*, 2012). For the first time in the UK, evidence was found that the government policy objective for discharges impacting SWPAs based on an average of 10 spills over 10 years contributes to control the environmental transmission of NoV.

Norovirus in Untreated Sewage, Treated Effluents and Rivers

A programme of field studies was undertaken to obtain data on concentrations of *E. coli* and NoV in untreated sewage and treated effluents and to study their removal efficiencies at four full scale STW characteristic of primary-, secondary- and tertiary treatments and two rivers impacting SPAs. NoV occurrence in untreated raw sewage varied between years and this variation was consistent with the annual variation of the virus in the community as indicated by outbreak laboratory reports published by Public Health England. Significant differences were found between mean NoV concentrations in effluents subject to different levels of treatment. Confirming results from previous research (Da Silva *et al.*, 2008), the study indicated that settlement is not the dominant removal mechanism for NoV. Maximum concentrations of NoV in screened influent were of the same order of magnitude of those in settled stormwater. These results provide evidence that monitoring of NoV in raw sewage can provide early warning of the potential occurrence of NoV in shellfish and contribute to better manage public health risks. Future investment programmes in sewerage infrastructure should consider elimination of stormwater discharges to SWPAs. Large differences were also found in NoV concentrations detected in secondary treated effluents suggest that control measures based on effluent treatment criteria alone may produce very variable levels of environmental protection for NoV. Emergence of new strains and seasonality of NoV and STW performance would have to be considered if end-of-pipe criteria were developed for NoV control. The study also found relatively high removal rates in the modified Ludzack-Ettinger activated sludge treatment studied. Consequently, the most likely area of future research in this area would be to enhance the association between the viruses and the sewage floc to increase virus removal, particularly during periods of high prevalence.

Characteristics of Norovirus in Shellfish Production Areas and Relationships with Sewage Dilution

The microbiological impacts were investigated in the receiving waters of the experimental sites (shallow estuary and deep coastal embayment). The shellfish were placed in cages at different distances from the sewage outfalls and tested for *E. coli* and

NoV. This monitoring programme was complemented by hydrographic drogue tracking and dye tracing studies to evaluate the surface water movements and dispersion and dilution of sewage effluent in the receiving waters. The tracing studies were undertaken using the approach proposed by Goblick *et al.* (2011) which was for the first time tested in UK shellfisheries. High prevalence of NoV was found in the three shellfish species investigated with more than 80% of samples positive for both genogroups at most sampling stations in both sites. This suggest that NoV controls based on absence/presence of genome copies in shellfish would not be viable in the context of UK shellfisheries.

In the shallow estuary, the microbiological monitoring programme showed that the highest *E. coli* and NoV concentrations were detected in shellfish placed in the main meandering channel (stations 1–4), as anticipated. At stations 1 and 4, *E. coli* concentrations exceeding the class B threshold (4,600 MPN/100 g) were detected. The tracer study successfully demonstrated the physical linkage between the identified source of sewage pollution (STW A) and these sampling locations characterised by low dilution where sewage effluent builds up and reaches a steady-state condition approximately 7 days after the discharge event. This is associated with the impact of a high number of sewage spill events observed during the monitoring period.

In the bay, NoV contamination was generally characterised by a high percentage of samples below LoQ with the highest mean concentrations in both oysters and mussels generally detected at the station located near the STW LSO location and stations on the eastern part of the bay. This spatial gradient of NoV contamination in the shellfish was also consistent with the fate and transport suggested by the dye study indicating a clockwise circulation transport regime inside the bay.

The field data were used to evaluate the impact of NoV risk management measures in the experimental sites and in a selection of five other commercial SPAs. The management measures focused on the establishment of NoV limits and buffer zones for SPAs. Concerning NoV limits, the assessment demonstrated that a limit established at LoQ (100 copies/g) would have a high management and compliance impact on both experimental sites. In contrast, a limit established at the upper range of contamination (ex. 1,000 copies/g) would result in substantially different percentages of exceedance at the sites (51% of non-compliance in the estuary and 12% non-compliance in the bay).

For the first time, a linear association was established between total concentrations of NoV (GI + GII) in shellfish and sewage effluent dilution (R^2 = 75%; p < 0.001; n = 11) for the period of high prevalence of the virus in the UK (October–March). This association enabled the development of buffer zone scenarios for the experimental sites based on international guidance. This assessment demonstrated that a requirement for a minimum 1,000:1 dilution of estuarine water to treated effluent for class B areas could help protect consumers from illness arising from NoV contamination. The GM of NoV (GI + GII) associated with this dilution ratio was 600 copies/g. However, the 95% confidence interval associated with this NoV level was large (300–1,260 copies/g) suggesting that the specific conditions for implementing this control measure in the UK would have to consider all the potential environmental drivers, such as the prevalence of NoV in the local human populations, local sewage discharge arrangements and the hydrography in the SPAs.

Significance of the Research

The integrated approach used in this research can be routinely applied by regulatory authorities and/or members of the shellfish industry since reliance on the *E. coli* standards of the legislation does not provide human health protection against NoV. The information reported here can be used to inform pollution reduction programmes under WFD and sanitary survey assessments for SPAs, and help identify sites with low risk of contamination for future development of shellfish farming businesses. The research has therefore successfully addressed several recommendations made by the ACMSF (1998, 2015) and World Health Organization (WHO, 2008) concerning reduction of NoV illness associated with consumption of shellfish.

7.1 Recommendations to the Shellfish Industry

- The harvesting/cultivation of shellfish near sources of human sewage pollution is a high risk practice for NoV. Therefore, commercial production areas should be located away from these sources.

- Members of the industry could consider the NoV epidemiological information available in the public domain to inform their risk assessments. Additional public health protection could be gained through real-time prediction of NoV contamination combined with NoV testing prior to marketing.

- The use of sensors for recording water temperature in SPAs and information on the frequency and duration of sewage discharges could assist the development of models for forecasting NoV contamination in shellfish.

- Dye tracing studies such as those conducted in this research or other modelling studies provide valuable information on the dispersion and dilution of sewage contamination. When combined with NoV monitoring, these methods can help inform the identification of areas that are more vulnerable to contamination. This approach should be considered for both existing and new harvesting/farming sites.

- Members of the shellfish industry should actively participate in the development of the WFD programmes of measures and other plans to reduce pollution in SWPAs.

- Members of the industry could consider the provision of shellfish samples to enable authorities responsible for classifying the production areas to better assess NoV risks, particularly when *E. coli* monitoring could be failing to do so.

- Information on cases of NoV illness associated with shellfish received by members of the industry should be shared with regulators and water companies on a confidential basis.

7.2 Recommendations to Environmental Protection Agencies and Water Industry

- Sewage spills from SOs and other sewage treatment failures should be immediately communicated to the relevant food safety authorities and members of the shellfish industry or their representatives.

- A medium- to long-term plan for control of storm overflows is required. Specific measures should be incorporated in discharge permit conditions and cost-effectiveness built into the WFD river basin management process.

- A science-based policy should be developed for monitoring NoV in effluents from discharges impacting SWPAs.

- For continuous sources of sewage pollution impacting SWPAs, the focus of future sewerage investments should be on biological forms of sewage treatment followed by a disinfection stage.

- Water companies should work collaboratively with regulators in developing a science-based policy for consenting sewage discharges impacting SWPAs that takes into consideration, among others, the seasonality, fate and transport of NoV in receiving waters.

- Water companies could consider working in partnership with scientists on the development of a research agenda focused on the relationships between STW performance and NoV removal from sewage.

7.3 Recommendations to Food Safety Authorities

- Applications for new SPAs in catchments at high risk of NoV contamination should be discouraged. The risk factors identified in Chapter 3 could be considered for this purpose.

- A baseline study of NoV contamination should be conducted as part of the sanitary survey process for new SPAs requiring classification. This information should be included in the annual *E. coli* classification listing.

- Sampling plans for microbiological monitoring of SPAs must consider the differences in the fate and behaviour of *E. coli* and NoV.

- Information on the operation of sewage sources and NoV epidemiology should be considered as part of the microbiological monitoring programme. Reliance on information collated at the time of the sanitary survey may not provide an indication of the potential NoV risk, particularly when new NoV strains emerge in the local population and catchments are subject to sewage infrastructure improvements.

- Shellfish safety authorities should work collaboratively with members of the industry, water companies and other shellfish water users in developing management plans for SPA. These management plans should contain measures to minimise the risk of NoV contamination of shellfish and a communication system for episodes of sewage pollution impacting the production area(s).

7.4 Recommendations for Future Research

- Methods to distinguish between infectious and non-infections NoV in sewage and shellfish should be developed. An important consideration in this area is method reproducibility within the same laboratory and between different laboratories.

- Modelling of the relationships between NoV contamination and sewage effluent dilution could be progressed by analysis of further relevant shellfisheries. These studies could consider different NoV scenarios such as the impact on shellfish risk of inter-annual variation in community levels of virus, the impact in different seasons, the impact of different approaches to standards (e.g. absolute vs mean), the impact of different effectiveness of sewage treatment, etc.

- More information is required on alternative genetic markers and their relationships with the abundance and distribution of pathogens and indicators for UV disinfection treatments to enable the identification of the most suitable molecular targets for detection and quantification of viral pathogens.

- Further studies on the feasibility of implementing buffer zones in SPA and the use of risk flags (such as increasing community levels of NoV or sewer overflows) for flexible mitigation of risk within such zones should be conducted in the UK.

- Further studies are required on whether NoV monitoring of effluents, combined with data on volumes discharged, could be used as a quantitative risk management tool for shellfish production areas. Such studies would need to characterise variables impacting NoV concentrations in STW effluents such as diurnal variation, impact of different flow conditions, etc. Pilot studies to explore this are recommended.

- The physical, chemical and biological processes driving NoV removal during sewage treatment processes remain poorly described. The focus of future research could consider combining PCR and virus cultivation methods to investigate the interactions between NoV removal and STW operational performance parameters (e.g. hydraulic retention times, ecological aspects of treatment processes; UV applied dosing).

- If monitoring programmes for NoV in shellfish production areas are considered in the future, then further information on the variation of NoV concentrations over short spatial scales (<1 km) is required to help inform the degree of resolution of contamination patterns and determine appropriate sampling plans.

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Appendices

Appendix I Additional Publications and Project Dissemination Activities

Abstracts in Conference Proceedings

Campos, C.J.A., Lowther, J., Gustar, N., Avant, J., Stockley, L., Lees, D. 2015. Improving knowledge of human norovirus in the marine environment to reduce the incidence of shellfish-related illness. Proceedings of the EFSA's 2nd Scientific Conference – Shaping the Future of Food Safety, Together, 14–16 October 2015, Milan, Italy, EFSA Journal, Supplement 13(10): 103.

Campos, C.J.A., Avant, J., Gustar, N., Lowther, J., Powell, A., Stockley, L., Lees, D. Fate of human noroviruses in shellfish water catchments in England and Wales. Abstract book of 2014 Water Microbiology Conference: Microbial Contaminants from Watersheds to Human Exposure, Water Institute, University of North California, Chapel Hill, 5–9 May 2014: 3–4.

Posters Presented at Scientific Conferences

Campos, C.J.A., Avant, J., Lowther, J., Till, D., Lees, D. 2015. Effectiveness of sewage treatment processes in removing human norovirus. 8th International Symposium on Health-Related Water Microbiology, 13–19 September 2015, Lisbon, Portugal.

Campos, C.J.A., Avant, J., Gustar, N., Lowther, J., Powell, A., Stockley, L., Lees, D. Characterisation of norovirus and *E. coli* contamination in a commercial oyster fishery. Poster presented at the 17th International Symposium on Health-Related Water Microbiology, 15–20 September 2013, Florianópolis, Brasil.

Campos, C.J.A., Avant, J., Gustar, N., Lowther, J., Powell, A., Stockley, L., Lees, D. Human noroviruses and faecal indicator bacteria in a shellfish water impacted by sewage discharges and freshwater inputs from an ephemeral stream. Poster presented at the workshop Hydrometric Data: The Long Term View, National River Flow Archive and National Groundwater Level Archive. CEH, Wallingford, 22 October 2013.

Campos, C.J.A. Environmental risk factors and management options for norovirus in shellfish waters. Poster presented at the Cefas PhD Student Day, Cefas Lowestoft, 15–16 April 2013.

Articles in Newsletters and Magazines

Campos, C.J.A. 2016. Human norovirus in untreated and treated sewage. Water & Sewerage Journal 93: 46–47.

Campos, C.J.A. 2013. Research helps to characterise the transmission of norovirus from sewage discharges to oyster beds. Shellfish News 36 Autumn/Winter 2013: 17–18.

Campos, C.J.A. 2013. Research by Cefas helps to characterise the transmission of norovirus from sewage discharges to oyster beds. ASSG The Grower, December 2013, 14–15.

Presentations at Conferences and Seminars

Campos, C.J.A. Solving viral pollution...dilution? Presentation at University of East Anglia-Cefas Marine Evidence Conference, 5 July 2016.

Campos, C.J.A. Is dilution the solution to viral pollution? Seminar at Cefas Weymouth Laboratory, 21 March 2016.

Campos, C.J.A. Combined sewer overflows: major sources of water pollution. Seminar at Cefas Weymouth Laboratory, 18 January 2016.

Campos, C.J.A. Norovirus in shellfish waters: a review. Meeting of the UK National Reference Laboratory (NRL) for monitoring bacteriological and viral contamination of bivalve molluscs, Cefas, UK, 29 October 2015.

Campos, C.J.A. Catchment sources of norovirus and their significance for shellfish waters. Seminar at the Department of Biological and Environmental Sciences, University of Stirling, 21 October 2014.

Campos, C.J.A. 2014. Norovirus and oysters: the scale of the problem as indicated by studies in a frequently polluted harvesting area. Seminar at Cefas Weymouth Laboratory, 23 June 2014.

Campos, C.J.A. Fate of norovirus in shellfish waters: new evidence from field studies and implications for shellfish growers and regulators. Seminar at the Annual Conference of the Scottish Association of Shellfish Growers, Oban, Scotland, 3 October 2013.

Campos, C.J.A. Linking marine pathogen incidence to past climate. Presentation at Defra-Strategic Evidence Partnership Funding, Workshop on Human Health, Mary Sumner House, London, 15 March 2013.

Campos, C.J.A. Contamination of shellfish waters with human noroviruses: risk factors and management options. Meeting of the Solent Water Quality Forum, Fareham District Council, 7 November 2012.

Reports Commissioned by Funding Agencies

Campos, C.J.A., Lees, D.N. 2016. Enhancing knowledge of norovirus behaviour in the marine environment to enable better risk management in molluscan shellfisheries. Cefas report to the Food Standards Agency and the Department for Environment, Food and Rural Affairs, FS101088/WT1529.

Campos, C.J.A. 2014. Risk factors for norovirus contamination of oyster producing areas in England and Wales. Cefas report under contract FC003 – Advice and Evidence on Shellfisheries, March 2014.

Campos, C.J.A. 2013. Human noroviruses in a shellfish water on the South coast of England: sources, distribution and relationships with *E. coli*. Report to Defra for Project SEPF/WT0996 – Contamination of shellfish waters with human noroviruses: environmental risk factors and management options (Phase I).

Campos, C.J.A. 2013. Review of knowledge on the fate of human noroviruses in coastal waters. Report to Defra for Project SEPF/WT0996 – Contamination of shellfish waters with human noroviruses: environmental risk factors and management options (Phase I).

Appendix II Detection and Quantification of Norovirus in Sewage and Freshwater

Preparation of sample concentrates

Each sample was shaken by hand to mix; then separate 20 ml volumes were added to each of two polycarbonate centrifuge bottles. A 10 µl volume of Mengo virus strain vMC0 (to act as a process control) was then added to each bottle and the samples were subjected to ultracentrifugation at >150,000 x g and 4 °C for 1 h using a Beckman LE-80K ultracentrifuge. The supernatants were discarded and the two pellets for each sample combined by stepwise resuspension in a single 2 ml volume of glycine buffer (0.25 M, pH = 9.5). The bottle containing the resuspended pellet was incubated on ice for 20 minutes to enable viruses to elute then 2 ml of cold 2 x Phosphate Buffered Saline (PBS) were added. The sample was centrifuged at 12,000 x g and 4 °C for 20 minutes to pellet particulate matter. Then, the supernatant was transferred to a clean bottle and the pellet discarded. A volume of 18 ml 1 x PBS was added to the tube then this was subjected to ultracentrifugation at > 150,000 x g and 4 °C for 1 h to pellet viruses. Finally, the supernatant was discarded then the pellet resuspended in 1 ml 1 x PBS. This was transferred to a clean tube and retained at 4 °C for RNA purification and RT-PCR.

Purification of viral RNA

Viral RNA extraction was carried out using NucliSens magnetic extraction technology (bioMérieux). For each wastewater sample a 500 µl aliquot of sample concentrate was added to 2 ml NucliSens lysis buffer in a 15 ml centrifuge tube. In addition, for each batch of samples tested, a negative extracted control consisting of 500 µl water only was also prepared and tested in parallel. Samples and controls were vortexed briefly then incubated at room temperature for 10 minutes before 50 µl magnetic silica was added to each tube and the samples incubated at room temperature for a further 10 minutes. The tubes were centrifuged at 1,500 x g for 2 minutes and the supernatants removed by aspiration. The pelleted silica beads were resuspended in 400 µl wash buffer 1 then transferred to individually labelled 1.5 ml tubes on the MiniMag extraction station. The magnet of the MiniMag was raised to collect the silica beads on the walls of the tubes; the beads were washed for 30 seconds using the wash function of the MiniMag. The supernatants were removed by aspiration, then the magnet lowered and the silica beads resuspended with 400 µl of wash buffer 1, then washed and the supernatant aspirated

as before. The resuspension/wash/aspiration cycle was then repeated using 500 μl wash buffer 2 then 500 μl wash buffer 3 (wash for 15 seconds). The pelleted silica beads were then resuspended with 100 μl of elution buffer. The tubes were capped and transferred to the thermo-shaker at 60 °C and shaken at 1,400 rpm for 5 minutes to allow elution of nucleic acids from the silica beads. After elution, the tubes were transferred to a portable magnet to collect the silica beads on the walls of the tubes. The supernatant (nucleic acid extract) (NA) was then transferred to a clean 0.5 ml tube and stored at -20 °C until required for reverse transcription.

One-step RT-PCR

For each sample or control and both NoV genogroups I and II, three aliquots of 5 μl NA extract were added to adjacent wells of a 96-well optical reaction plate and made up to 25 μl with (GI or GII) TaqMan reaction mix (final concentration of 1 x each Ultrasense reaction mix, Rox reference dye and RNA Ultrasense enzyme mix (Invitrogen), 500 nM forward primer, 900 nM reverse primer, and 250 nM probe. Positive (dilution series prepared from a known concentration of plasmid carrying a copy of the target sequence) and negative (water only) PCR control materials were also tested. The plate was placed in a Stratagene Mx3005P real-time PCR machine with the following amplification program; 55 °C for 60 minutes, then 95 °C for 5 minutes, followed by 45 cycles of 95 °C for 15 seconds, 60 °C for 1 minutes and 65 °C for 1 minute. For analysis, threshold values were set at 0.20 fluorescence units, then threshold cycle (Ct) values were determined using the Mx3005P system software. Unexpected results in any positive or negative extraction or RT-PCR control triggered retesting of any affected samples.

Calculation of extraction efficiency

Two aliquots of 5 μl NA extract from each sample were added to adjacent wells of a 96-well optical reaction plate and made up to 25 μl with Mengo virus-specific TaqMan reaction mix (final concentrations as described above). A dilution series prepared from the Mengo virus process control material was also tested. The plate was placed in a Stratagene Mx3005P real-time PCR machine and amplified using the program described above. The percentage extraction efficiency for each sample was determined by

comparing the Ct values for the sample NA extract with those for the Mengo virus dilution series.

Quantification of NoV from dsDNA standard curve analysis

On each TaqMan run, a log dilution series of dsDNA control corresponding to a range of approximately 1 to 10,000 template copies/μl (quantified using spectrophotometry at 260 nm) was included. The Ct values from this dilution series were then used to produce a standard curve. For each TaqMan replicate for the samples under test, a quantity in copies/μl was determined using the corresponding standard curve. Not detected replicates were ascribed a quantity of zero. The average quantities from the three replicates in each NoV genogroup-specific TaqMan assay were calculated to give an overall quantity in detectable copies/μl NA extract for that sample and genogroup. This quantity was corrected using the percentage extraction efficiency and converted into a concentration in copies/ml wastewater considering the various concentration factors involved in the testing.

Appendix III Frequency Distributions and Probability Density Functions for Log_{10}-transformed Concentrations of Norovirus in Sewage (A, B) and Shellfish (C, D) During the Study Period.

A
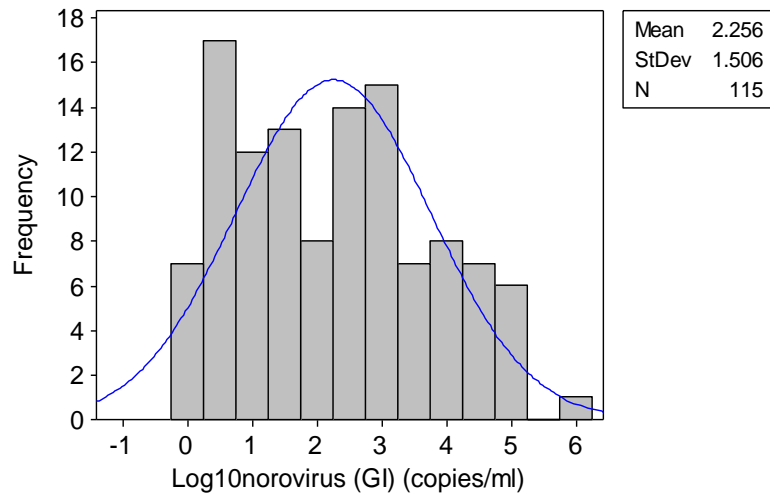
B
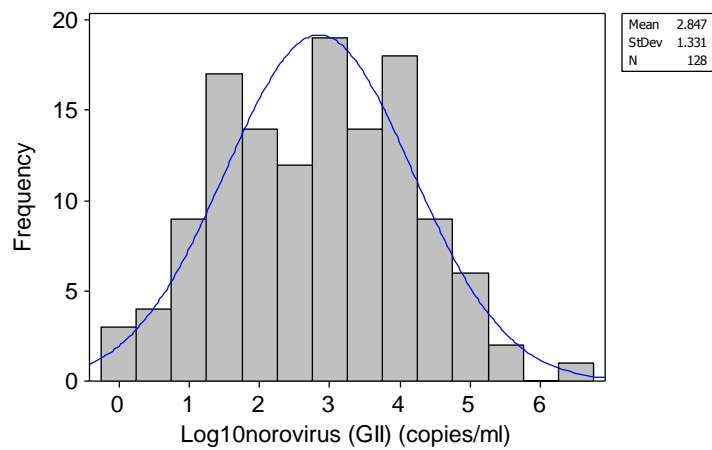
C
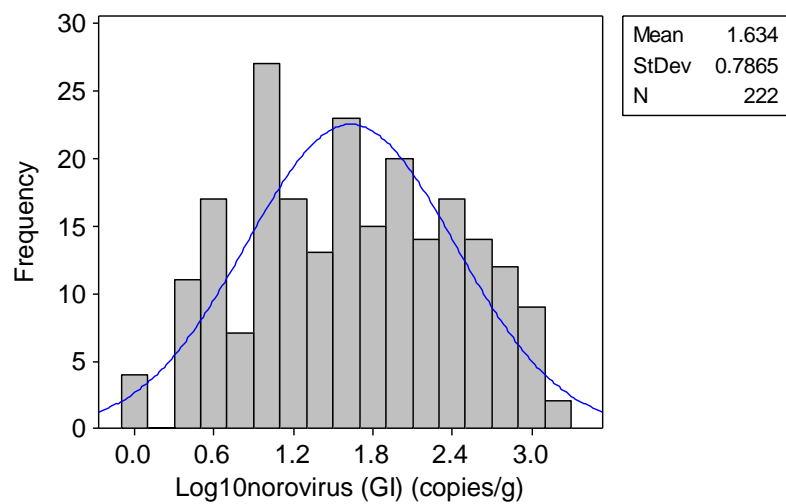
D
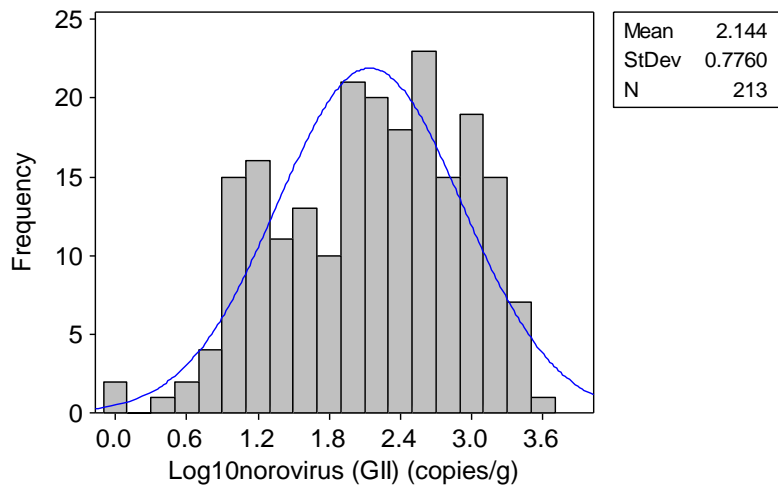
Sewage:

Variable	N	N*	Skewness
$\log_{10}$NoV GI result	115	55	0.27
$\log_{10}$NoV GII result	128	42	0.01

Shellfish:

Variable	N	N*	Skewness
$\log_{10}$NoV GI	222	16	0.00
$\log_{10}$NoVGII	213	25	-0.33

Appendix IV Determination of Quantities of Dye for Injection.

The calculation spreadsheet uses the conservation of mass equation to determine dye quantities as follows:

$C_{jug} \times Q_{jug} = C_{out} \times Q_{out} = C_{est} \times Q_{est}$ where:

C_{jug} is the concentration of dye in the dosing container; Q_{jug} is the flow rate of the dye injection pump; C_{out} is the concentration of dye in the STW outfall; Q_{out} is the flow rate in the STW outfall; C_{est} is the concentration of dye in the receiving water; and Q_{est} is the flow rate in the receiving water.

Records of flows to full treatment (FFT) and disinfected storm flow (15-minute interval measurements) for STW A and FFT for STW D for the periods of the dye injections were supplied by the water companies. The flow rate data were used to determine the concentration of dye in the effluents according to the equation:

$C_{out} = (C_{jug}*Q_{jug})/Q_{out}$ where:

C_{out} is the concentration of dye in the STW outfall; C_{jug} is the concentration of dye in the dosing container; Q_{jug} is the flow rate of the dye injection pump; and Q_{out} is the flow rate in the STW outfall.

STW flows, calculated dye concentrations in the final effluent and predicted dye concentrations for four dilution ratios in the estuary (site 13)

Date time	Flow full treatment (l/s)	Disinfected storm flow (l/s)	Total flow (l/s)	Q_{out} (gal/day)	C_{out} (ppb)	35:1 (ppb)	100:1 (ppb)	1,000:1 (ppb)	10,000:1 (ppb)
20-Jan-15 04:00:00	291.8	219.4	511.2	11655075	2314	66	23	2.31	0.23
20-Jan-15 04:15:00	308.0	156.1	464.1	10580625	2549	73	25	2.55	0.25
20-Jan-15 04:30:00	278.0	216.5	494.5	11275311	2392	68	24	2.39	0.24
20-Jan-15 04:45:00	303.1	197.3	500.4	11408550	2364	68	24	2.36	0.24
20-Jan-15 05:00:00	283.9	168.0	451.8	10302039	2618	75	26	2.62	0.26
20-Jan-15 05:15:00	292.6	208.8	501.4	11432775	2359	67	24	2.36	0.24
20-Jan-15 05:30:00	303.8	158.4	462.1	10536450	2560	73	26	2.56	0.26
20-Jan-15 05:45:00	265.5	235.0	500.5	11410689	2363	68	24	2.36	0.24
20-Jan-15 06:00:00	322.0	140.6	462.6	10547139	2557	73	26	2.56	0.26
20-Jan-15 06:15:00	278.9	213.5	492.4	11226861	2402	69	24	2.40	0.24
20-Jan-15 06:30:00	300.8	212.3	513.0	11696400	2306	66	23	2.31	0.23
20-Jan-15 06:45:00	300.4	148.9	449.3	10242900	2633	75	26	2.63	0.26
20-Jan-15 07:00:00	276.1	222.8	498.9	11374350	2371	68	24	2.37	0.24
20-Jan-15 07:15:00	313.0	203.9	516.9	11785461	2288	65	23	2.29	0.23
20-Jan-15 07:30:00	310.0	204.1	514.1	11721339	2301	66	23	2.30	0.23
20-Jan-15 07:45:00	291.4	208.6	499.9	11398575	2366	68	24	2.37	0.24
20-Jan-15 08:00:00	307.0	228.0	535.0	12198000	2211	63	22	2.21	0.22
20-Jan-15 08:15:00	298.6	234.3	532.9	12150975	2219	63	22	2.22	0.22
20-Jan-15 08:30:00	295.8	245.0	540.8	12329100	2187	62	22	2.19	0.22
20-Jan-15 08:45:00	298.6	273.1	571.7	13034475	2069	59	21	2.07	0.21
20-Jan-15 09:00:00	313.9	274.3	588.2	13411389	2011	57	20	2.01	0.20
20-Jan-15 09:15:00	304.8	275.1	579.8	13219725	2040	58	20	2.04	0.20
20-Jan-15 09:30:00	307.6	274.2	581.8	13266039	2033	58	20	2.03	0.20
20-Jan-15 09:45:00	301.0	270.9	571.9	13040175	2068	59	21	2.07	0.21

20-Jan-15 10:00:00	307.1	267.4	574.5	13099311	2059	59	21	2.06	0.21
20-Jan-15 10:15:00	308.6	265.9	574.5	13099311	2059	59	21	2.06	0.21
20-Jan-15 10:30:00	308.3	268.7	576.9	13154175	2050	59	21	2.05	0.21
20-Jan-15 10:45:00	301.8	272.5	574.2	13092189	2060	59	21	2.06	0.21
20-Jan-15 11:00:00	298.1	253.2	551.3	12569925	2145	61	21	2.15	0.21
20-Jan-15 11:15:00	294.1	238.0	532.2	12133161	2223	64	22	2.22	0.22
20-Jan-15 11:30:00	295.0	222.0	517.0	11786889	2288	65	23	2.29	0.23
20-Jan-15 11:45:00	307.6	219.8	527.4	12024861	2243	64	22	2.24	0.22
20-Jan-15 12:00:00	303.8	254.4	558.2	12726675	2119	61	21	2.12	0.21
20-Jan-15 12:15:00	286.4	258.0	544.4	12411750	2173	62	22	2.17	0.22
20-Jan-15 12:30:00	298.8	260.8	559.6	12758739	2114	60	21	2.11	0.21
20-Jan-15 12:45:00	303.5	249.2	552.7	12600561	2140	61	21	2.14	0.21
20-Jan-15 13:00:00	302.1	210.8	512.9	11694975	2306	66	23	2.31	0.23
20-Jan-15 13:15:00	306.6	193.4	500.0	11400000	2366	68	24	2.37	0.24
20-Jan-15 13:30:00	300.6	234.4	535.0	12198711	2211	63	22	2.21	0.22
20-Jan-15 13:45:00	301.8	248.4	550.2	12543561	2150	61	21	2.15	0.21
20-Jan-15 14:00:00	290.1	245.9	536.1	12222225	2206	63	22	2.21	0.22
20-Jan-15 14:15:00	315.4	221.3	536.7	12236475	2204	63	22	2.20	0.22
20-Jan-15 14:30:00	304.1	205.3	509.4	11614461	2322	66	23	2.32	0.23
20-Jan-15 14:45:00	302.5	224.9	527.4	12024150	2243	64	22	2.24	0.22
20-Jan-15 15:00:00	297.3	225.9	523.1	11927250	2261	65	23	2.26	0.23
20-Jan-15 15:15:00	295.3	222.3	517.6	11800425	2285	65	23	2.29	0.23
20-Jan-15 15:30:00	291.9	218.2	510.1	11630139	2319	66	23	2.32	0.23
20-Jan-15 15:45:00	303.0	210.9	513.9	11717061	2302	66	23	2.30	0.23
20-Jan-15 16:00:00	307.3	199.4	506.7	11551761	2335	67	23	2.33	0.23
20-Jan-15 16:15:00	305.1	208.8	513.9	11717061	2302	66	23	2.30	0.23
20-Jan-15 16:30:00	292.6	199.6	492.2	11222589	2403	69	24	2.40	0.24
20-Jan-15 16:45:00	300.3	196.8	497.1	11333025	2380	68	24	2.38	0.24
20-Jan-15 17:00:00	293.3	215.0	508.3	11588811	2327	66	23	2.33	0.23

STW flows, calculated dye concentrations in the final effluent and predicted dye concentrations for four dilution ratios in the bay (site 32)

Date time	Flow to full treatment (l/s)	Qout (gal/day)	Cout (ppb)	35:1 (ppb)	100:1 (ppb)	1,000:1 (ppb)	10,000:1 (ppb)
16/06/2015 04:44:40	52.89	1205980	5785	165	58	5.78	0.58
16/06/2015 04:45:00	52.89	1205980	5785	165	58	5.78	0.58
16/06/2015 04:53:40	42.47	968291	7205	206	72	7.20	0.72
16/06/2015 04:56:50	32.21	734499	9498	271	95	9.50	0.95
16/06/2015 05:00:00	39.31	896205	7784	222	78	7.78	0.78
16/06/2015 05:00:40	51.95	1184549	5889	168	59	5.89	0.59
16/06/2015 05:03:40	62.46	1424187	4898	140	49	4.90	0.49
16/06/2015 05:07:50	44.18	1007257	6926	198	69	6.93	0.69
16/06/2015 05:15:00	44.26	1009205	6913	198	69	6.91	0.69
16/06/2015 05:15:50	54.35	1239101	5630	161	56	5.63	0.56
16/06/2015 05:18:00	66.57	1517704	4597	131	46	4.60	0.46
16/06/2015 05:23:50	56.48	1287808	5417	155	54	5.42	0.54
16/06/2015 05:26:50	42.38	966343	7219	206	72	7.22	0.72
16/06/2015 05:30:00	43.24	985826	7077	202	71	7.08	0.71
16/06/2015 05:31:00	56.48	1287808	5417	155	54	5.42	0.54
16/06/2015 05:34:50	71.61	1632652	4273	122	43	4.27	0.43
16/06/2015 05:38:00	53.15	1211825	5757	164	58	5.76	0.58
16/06/2015 05:45:00	43.32	987774	7063	202	71	7.06	0.71
16/06/2015 05:47:10	75.45	1720324	4055	116	41	4.06	0.41
16/06/2015 05:48:10	85.79	1956065	3566	102	36	3.57	0.36
16/06/2015 05:49:10	72.63	1656031	4213	120	42	4.21	0.42
16/06/2015 05:53:10	55.54	1266377	5509	157	55	5.51	0.55
16/06/2015 05:56:20	44.86	1022843	6820	195	68	6.82	0.68
16/06/2015 05:59:20	56.48	1287808	5417	155	54	5.42	0.54
16/06/2015 06:00:00	56.48	1287808	5417	155	54	5.42	0.54
16/06/2015 06:01:20	69.98	1595635	4372	125	44	4.37	0.44
16/06/2015 06:06:20	50.76	1157274	6028	172	60	6.03	0.60
16/06/2015 06:10:20	39.82	907895	7684	220	77	7.68	0.77
16/06/2015 06:11:20	52.38	1194291	5841	167	58	5.84	0.58
16/06/2015 06:14:30	63.06	1437825	4852	139	49	4.85	0.49

16/06/2015 06:15:00	63.06	1437825	4852	139	49	4.85	0.49
16/06/2015 06:19:30	50.42	1149480	6069	173	61	6.07	0.61
16/06/2015 06:23:30	66.82	1523549	4579	131	46	4.58	0.46
16/06/2015 06:25:30	81.09	1848910	3773	108	38	3.77	0.38
16/06/2015 06:28:40	65.80	1500169	4650	133	47	4.65	0.47
16/06/2015 06:30:00	64.43	1468997	4749	136	47	4.75	0.47
16/06/2015 06:32:50	80.41	1833324	3805	109	38	3.81	0.38
16/06/2015 06:33:50	63.58	1449514	4813	138	48	4.81	0.48
16/06/2015 06:34:50	76.39	1741755	4005	114	40	4.01	0.40
16/06/2015 06:36:50	93.06	2121668	3288	94	33	3.29	0.33
16/06/2015 06:39:50	78.19	1782669	3913	112	39	3.91	0.39
16/06/2015 06:44:50	95.19	2170375	3214	92	32	3.21	0.32
16/06/2015 06:45:00	95.19	2170375	3214	92	32	3.21	0.32
16/06/2015 06:48:50	82.46	1880082	3711	106	37	3.71	0.37
16/06/2015 06:49:50	108.44	2472357	2822	81	28	2.82	0.28
16/06/2015 06:50:50	86.31	1967755	3545	101	35	3.55	0.35
16/06/2015 06:55:00	105.87	2413909	2890	83	29	2.89	0.29
16/06/2015 07:00:00	89.12	2032048	3433	98	34	3.43	0.34
16/06/2015 07:02:00	75.97	1732014	4028	115	40	4.03	0.40
16/06/2015 07:05:10	87.76	2000875	3487	100	35	3.49	0.35
16/06/2015 07:06:10	102.03	2326237	2999	86	30	3.00	0.30
16/06/2015 07:10:10	84.68	1930738	3613	103	36	3.61	0.36
16/06/2015 07:13:20	98.95	2256099	3092	88	31	3.09	0.31
16/06/2015 07:14:20	114.67	2614581	2668	76	27	2.67	0.27
16/06/2015 07:15:00	114.67	2614581	2668	76	27	2.67	0.27
16/06/2015 07:15:20	132.19	3013977	2315	66	23	2.31	0.23
16/06/2015 07:24:30	146.03	3329597	2095	60	21	2.10	0.21
16/06/2015 07:26:30	132.19	3013977	2315	66	23	2.31	0.23
16/06/2015 07:29:30	108.35	2470409	2824	81	28	2.82	0.28
16/06/2015 07:30:00	108.35	2470409	2824	81	28	2.82	0.28
16/06/2015 07:31:30	123.82	2823046	2471	71	25	2.47	0.25
16/06/2015 07:37:30	108.01	2462616	2833	81	28	2.83	0.28
16/06/2015 07:38:30	122.88	2801615	2490	71	25	2.49	0.25
16/06/2015 07:40:30	111.68	2546391	2740	78	27	2.74	0.27

16/06/2015 07:42:30	130.57	2976960	2343	67	23	2.34	0.23
16/06/2015 07:45:00	136.21	3105545	2246	64	22	2.25	0.22
16/06/2015 07:46:40	117.49	2678874	2604	74	26	2.60	0.26
16/06/2015 07:48:40	130.06	2965270	2353	67	24	2.35	0.24
16/06/2015 07:56:50	107.84	2458719	2837	81	28	2.84	0.28
16/06/2015 07:57:50	118.01	2690564	2593	74	26	2.59	0.26
16/06/2015 08:00:00	115.02	2622374	2660	76	27	2.66	0.27
16/06/2015 08:11:10	140.31	3199062	2181	62	22	2.18	0.22
16/06/2015 08:15:00	138.60	3160097	2208	63	22	2.21	0.22
16/06/2015 08:15:10	124.50	2838632	2458	70	25	2.46	0.25
16/06/2015 08:18:10	113.39	2585357	2698	77	27	2.70	0.27
16/06/2015 08:19:10	133.90	3052942	2285	65	23	2.29	0.23
16/06/2015 08:23:20	116.73	2661340	2621	75	26	2.62	0.26
16/06/2015 08:24:20	128.86	2937994	2375	68	24	2.37	0.24
16/06/2015 08:26:30	117.24	2673029	2610	75	26	2.61	0.26
16/06/2015 08:30:00	121.85	2778236	2511	72	25	2.51	0.25
16/06/2015 08:35:30	106.90	2437288	2862	82	29	2.86	0.29
16/06/2015 08:38:30	124.33	2834736	2461	70	25	2.46	0.25
16/06/2015 08:41:30	104.76	2388581	2921	83	29	2.92	0.29
16/06/2015 08:42:40	118.61	2704202	2580	74	26	2.58	0.26
16/06/2015 08:43:40	93.91	2141151	3258	93	33	3.26	0.33
16/06/2015 08:45:00	95.45	2176220	3206	92	32	3.21	0.32
16/06/2015 08:47:50	107.24	2445081	2853	82	29	2.85	0.29
16/06/2015 08:52:50	93.40	2129461	3276	94	33	3.28	0.33
16/06/2015 08:53:50	114.33	2606788	2676	76	27	2.68	0.27
16/06/2015 08:57:00	101.94	2324288	3001	86	30	3.00	0.30
16/06/2015 08:59:00	126.12	2875649	2426	69	24	2.43	0.24
16/06/2015 09:00:00	119.80	2731477	2554	73	26	2.55	0.26
16/06/2015 09:00:50	109.12	2487943	2804	80	28	2.80	0.28
16/06/2015 09:05:00	95.62	2180116	3200	91	32	3.20	0.32
16/06/2015 09:06:10	117.92	2688615	2595	74	26	2.59	0.26
16/06/2015 09:07:10	105.36	2402219	2904	83	29	2.90	0.29
16/06/2015 09:09:10	116.30	2651598	2631	75	26	2.63	0.26
16/06/2015 09:11:10	101.43	2312599	3017	86	30	3.02	0.30

16/06/2015 09:12:10	116.98	2667184	2616	75	26	2.62	0.26
16/06/2015 09:13:10	106.64	2431443	2869	82	29	2.87	0.29
16/06/2015 09:14:10	93.31	2127513	3279	94	33	3.28	0.33
16/06/2015 09:15:00	93.31	2127513	3279	94	33	3.28	0.33
16/06/2015 09:15:10	113.22	2581460	2702	77	27	2.70	0.27
16/06/2015 09:22:20	96.90	2209340	3158	90	32	3.16	0.32
16/06/2015 09:24:20	107.75	2456771	2840	81	28	2.84	0.28
16/06/2015 09:25:20	92.03	2098289	3325	95	33	3.32	0.33
16/06/2015 09:27:20	112.71	2569771	2715	78	27	2.71	0.27
16/06/2015 09:30:00	108.09	2464564	2831	81	28	2.83	0.28
16/06/2015 09:31:30	92.71	2113875	3300	94	33	3.30	0.33
16/06/2015 09:36:30	109.55	2497685	2793	80	28	2.79	0.28
16/06/2015 09:42:40	88.36	2014513	3463	99	35	3.46	0.35
16/06/2015 09:45:00	91.60	2088547	3340	95	33	3.34	0.33
16/06/2015 09:46:50	110.91	2528857	2759	79	28	2.76	0.28
16/06/2015 09:50:40	90.32	2059323	3388	97	34	3.39	0.34
16/06/2015 09:57:00	124.25	2832787	2463	70	25	2.46	0.25
16/06/2015 09:57:50	108.86	2482098	2811	80	28	2.81	0.28
16/06/2015 10:00:00	111.68	2546391	2740	78	27	2.74	0.27
16/06/2015 10:01:00	94.25	2148944	3246	93	32	3.25	0.32
16/06/2015 10:02:00	81.09	1848910	3773	108	38	3.77	0.38
16/06/2015 10:06:00	99.21	2261944	3084	88	31	3.08	0.31
16/06/2015 10:12:10	88.36	2014513	3463	99	35	3.46	0.35
16/06/2015 10:14:10	76.31	1739807	4010	115	40	4.01	0.40
16/06/2015 10:15:00	76.31	1739807	4010	115	40	4.01	0.40
16/06/2015 10:15:10	96.73	2205444	3163	90	32	3.16	0.32
16/06/2015 10:16:10	111.00	2530805	2757	79	28	2.76	0.28
16/06/2015 10:21:10	95.79	2184013	3194	91	32	3.19	0.32
16/06/2015 10:24:20	80.15	1827479	3817	109	38	3.82	0.38
16/06/2015 10:26:30	95.70	2182064	3197	91	32	3.20	0.32
16/06/2015 10:28:30	107.07	2441185	2858	82	29	2.86	0.29
16/06/2015 10:30:00	103.91	2369099	2945	84	29	2.94	0.29
16/06/2015 10:32:30	91.26	2080754	3353	96	34	3.35	0.34
16/06/2015 10:38:40	115.44	2632115	2650	76	27	2.65	0.27

16/06/2015 10:39:40	102.80	2343771	2977	85	30	2.98	0.30
16/06/2015 10:41:40	88.70	2022306	3450	99	34	3.45	0.34
16/06/2015 10:42:40	72.46	1652135	4223	121	42	4.22	0.42
16/06/2015 10:44:40	57.51	1311187	5321	152	53	5.32	0.53
16/06/2015 10:45:00	57.51	1311187	5321	152	53	5.32	0.53
16/06/2015 10:48:40	67.85	1546928	4510	129	45	4.51	0.45
16/06/2015 10:50:40	19.74	450051	15501	443	155	15.50	1.55
16/06/2015 11:30:10	79.38	1809945	3854	110	39	3.85	0.39
16/06/2015 11:31:10	114.08	2600943	2682	77	27	2.68	0.27
16/06/2015 11:37:30	99.89	2277530	3063	88	31	3.06	0.31
16/06/2015 11:42:30	76.65	1747600	3992	114	40	3.99	0.40
16/06/2015 11:43:30	93.40	2129461	3276	94	33	3.28	0.33
16/06/2015 11:45:00	100.15	2283375	3055	87	31	3.06	0.31
16/06/2015 11:47:30	103.91	2369099	2945	84	29	2.94	0.29
16/06/2015 11:48:30	90.41	2061272	3384	97	34	3.38	0.34
16/06/2015 11:50:30	78.87	1798255	3879	111	39	3.88	0.39
16/06/2015 11:53:30	89.47	2039841	3420	98	34	3.42	0.34
16/06/2015 11:54:30	104.34	2378840	2933	84	29	2.93	0.29
16/06/2015 11:58:40	91.26	2080754	3353	96	34	3.35	0.34
16/06/2015 12:00:00	85.71	1954117	3570	102	36	3.57	0.36
16/06/2015 12:02:40	103.14	2351564	2967	85	30	2.97	0.30
16/06/2015 12:04:40	113.91	2597046	2686	77	27	2.69	0.27
16/06/2015 12:09:40	100.66	2295064	3040	87	30	3.04	0.30
16/06/2015 12:12:40	89.55	2041789	3417	98	34	3.42	0.34
16/06/2015 12:14:40	100.15	2283375	3055	87	31	3.06	0.31
16/06/2015 12:15:00	100.15	2283375	3055	87	31	3.06	0.31
16/06/2015 12:16:40	112.71	2569771	2715	78	27	2.71	0.27
16/06/2015 12:19:40	100.23	2285323	3053	87	31	3.05	0.31
16/06/2015 12:24:40	84.25	1920996	3632	104	36	3.63	0.36
16/06/2015 12:26:40	95.45	2176220	3206	92	32	3.21	0.32
16/06/2015 12:30:00	105.36	2402219	2904	83	29	2.90	0.29
16/06/2015 12:34:40	76.31	1739807	4010	115	40	4.01	0.40
16/06/2015 12:38:50	88.10	2008668	3473	99	35	3.47	0.35
16/06/2015 12:39:40	99.38	2265840	3079	88	31	3.08	0.31

16/06/2015 12:42:50	88.36	2014513	3463	99	35	3.46	0.35
16/06/2015 12:45:00	80.67	1839169	3793	108	38	3.79	0.38
16/06/2015 12:46:50	74.68	1702790	4097	117	41	4.10	0.41
16/06/2015 12:49:50	92.03	2098289	3325	95	33	3.32	0.33
16/06/2015 12:52:00	105.10	2396375	2911	83	29	2.91	0.29
16/06/2015 12:54:00	89.47	2039841	3420	98	34	3.42	0.34
16/06/2015 12:57:10	78.53	1790462	3896	111	39	3.90	0.39
16/06/2015 13:00:00	76.91	1753445	3979	114	40	3.98	0.40
16/06/2015 13:02:10	96.64	2203495	3166	90	32	3.17	0.32
16/06/2015 13:06:10	83.57	1905410	3661	105	37	3.66	0.37
16/06/2015 13:15:00	91.86	2094392	3331	95	33	3.33	0.33
16/06/2015 13:15:10	97.67	2226875	3133	90	31	3.13	0.31
16/06/2015 13:17:10	85.02	1938531	3599	103	36	3.60	0.36
16/06/2015 13:25:20	104.68	2386633	2923	84	29	2.92	0.29
16/06/2015 13:29:20	87.67	1998927	3490	100	35	3.49	0.35
16/06/2015 13:30:00	87.67	1998927	3490	100	35	3.49	0.35
16/06/2015 13:33:20	76.82	1751497	3983	114	40	3.98	0.40
16/06/2015 13:38:30	87.76	2000875	3487	100	35	3.49	0.35
16/06/2015 13:44:40	77.59	1769031	3944	113	39	3.94	0.39
16/06/2015 13:45:00	77.59	1769031	3944	113	39	3.94	0.39
16/06/2015 13:49:40	92.71	2113875	3300	94	33	3.30	0.33
16/06/2015 13:55:50	78.36	1786565	3905	112	39	3.90	0.39
16/06/2015 13:57:50	89.04	2030099	3436	98	34	3.44	0.34
16/06/2015 13:58:50	78.27	1784617	3909	112	39	3.91	0.39
16/06/2015 13:59:50	88.36	2014513	3463	99	35	3.46	0.35
16/06/2015 14:00:00	88.36	2014513	3463	99	35	3.46	0.35
16/06/2015 14:08:50	76.91	1753445	3979	114	40	3.98	0.40
16/06/2015 14:15:00	86.39	1969703	3542	101	35	3.54	0.35
16/06/2015 14:15:10	88.18	2010617	3470	99	35	3.47	0.35
16/06/2015 14:17:10	73.49	1675514	4164	119	42	4.16	0.42
16/06/2015 14:18:10	52.64	1200136	5813	166	58	5.81	0.58
16/06/2015 14:21:20	35.03	798791	8734	250	87	8.73	0.87
16/06/2015 14:24:20	48.02	1094929	6371	182	64	6.37	0.64
16/06/2015 15:02:50	42.47	968291	7205	206	72	7.20	0.72

16/06/2015 15:03:50	81.61	1860600	3749	107	37	3.75	0.37
16/06/2015 15:08:00	68.87	1570307	4443	127	44	4.44	0.44
16/06/2015 15:13:00	81.43	1856703	3757	107	38	3.76	0.38
16/06/2015 15:13:50	93.57	2133358	3270	93	33	3.27	0.33
16/06/2015 15:15:00	89.21	2033996	3430	98	34	3.43	0.34
16/06/2015 15:19:00	82.29	1876186	3718	106	37	3.72	0.37
16/06/2015 15:23:10	67.42	1537187	4538	130	45	4.54	0.45
16/06/2015 15:25:10	80.75	1841117	3789	108	38	3.79	0.38
16/06/2015 15:26:10	97.76	2228823	3130	89	31	3.13	0.31
16/06/2015 15:27:10	85.71	1954117	3570	102	36	3.57	0.36
16/06/2015 15:30:00	90.49	2063220	3381	97	34	3.38	0.34
16/06/2015 15:31:10	74.51	1698893	4106	117	41	4.11	0.41
16/06/2015 15:32:20	85.54	1950220	3577	102	36	3.58	0.36
16/06/2015 15:33:10	71.95	1640445	4253	122	43	4.25	0.43
16/06/2015 15:41:20	87.33	1991134	3504	100	35	3.50	0.35
16/06/2015 15:44:20	76.91	1753445	3979	114	40	3.98	0.40
16/06/2015 15:45:00	76.91	1753445	3979	114	40	3.98	0.40
16/06/2015 15:51:20	89.38	2037892	3423	98	34	3.42	0.34
16/06/2015 15:54:30	77.93	1776824	3926	112	39	3.93	0.39
16/06/2015 15:58:30	64.34	1467049	4755	136	48	4.76	0.48
16/06/2015 16:00:00	64.43	1468997	4749	136	47	4.75	0.47
16/06/2015 16:01:30	75.20	1714479	4069	116	41	4.07	0.41
16/06/2015 16:15:00	81.43	1856703	3757	107	38	3.76	0.38
16/06/2015 16:21:50	63.92	1457307	4787	137	48	4.79	0.48
16/06/2015 16:22:50	77.33	1763186	3957	113	40	3.96	0.40
16/06/2015 16:30:00	83.91	1913203	3646	104	36	3.65	0.36
16/06/2015 16:34:00	87.42	1993082	3500	100	35	3.50	0.35
16/06/2015 16:42:00	68.96	1572255	4437	127	44	4.44	0.44
16/06/2015 16:45:00	78.19	1782669	3913	112	39	3.91	0.39
16/06/2015 16:47:00	85.79	1956065	3566	102	36	3.57	0.36
16/06/2015 17:00:00	91.35	2082703	3350	96	33	3.35	0.33
16/06/2015 17:05:30	73.49	1675514	4164	119	42	4.16	0.42

Appendix V Time Series of Water Current Speeds Measured by the Acoustic Doppler Current Profiler.
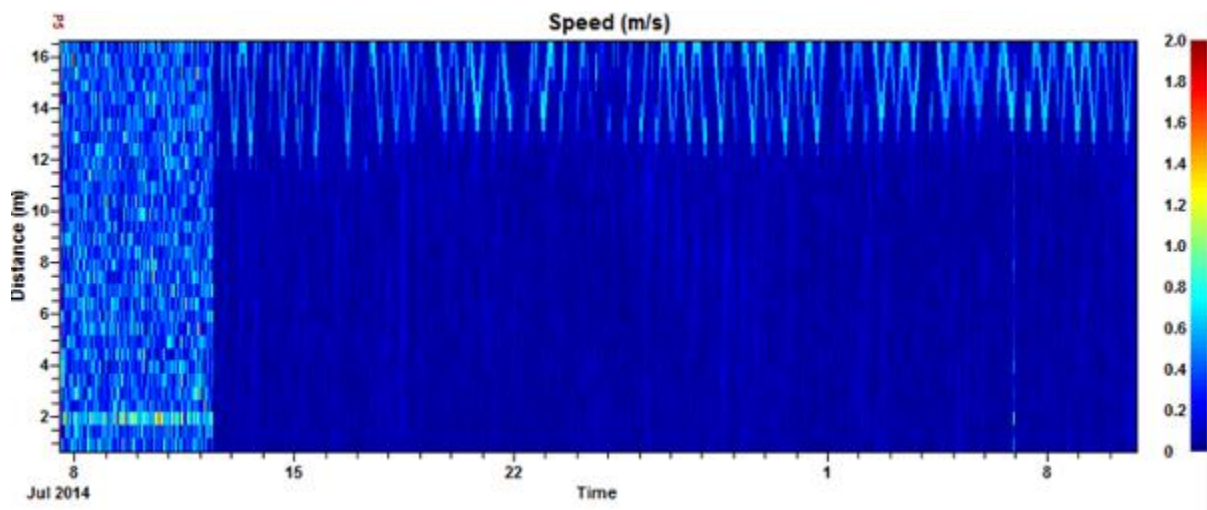
Appendix VI Temperature Profiles Obtained in the Bay (Site 32).

Station 1

Station 2

Station 3

Station 4

Station 5

Station 6

Station 7
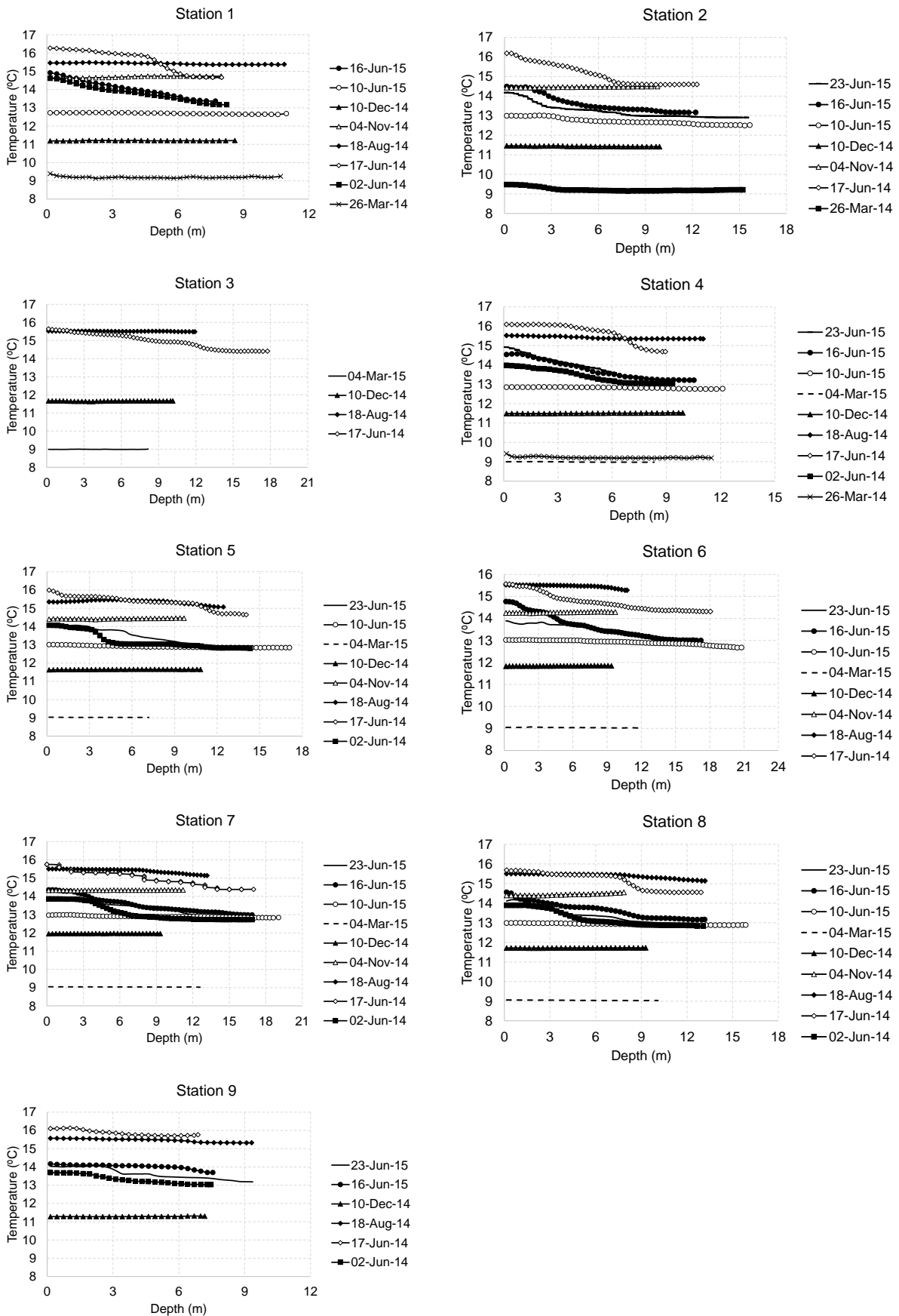
Station 8

Station 9

Appendix VII Background Fluorescence Concentrations Recorded in the Estuary (site 13) and Bay (site 32).

Study site	Station number								
	1	2	3	4	5	6	7	8	9
13	66	73	39	54	-	-	-	-	-
32	49	48	-[a]	53	49	-[b]	41	44	-[b]

All fluorescence concentrations in ppb. [a] This sampling station was lost in the week prior to the dye study during stormy weather. [b] The fluorescence data recorded at this station were considered erroneous and were excluded from the analyses.