DOCTOR OF PHILOSOPHY

Norovirus contamination of Mytilus edulis shellfisheries : assessing the threat to human health

Winterbourn, Ben

Award date:
2014

Awarding institution:
Bangor University

Norovirus Contamination of *Mytilus edulis* Shellfisheries; Assessing the Threat to Human Health

J. Ben Winterbourn

June 2014

PRIFYSGOL
BANGOR
UNIVERSITY

Thesis submitted to Bangor University for the degree of Doctor of Philosophy

School of the Environment, Natural Resources and Geography

Bangor University, Bangor, Gwynedd, LL57 2UW

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This Doctorate thesis is dedicated to Mum and Dad

Summary

Contamination of bivalve molluscan shellfish (BMS) with enteric viruses from human faecal sources is recognised as an important human health risk. Norovirus (NoV) is the principal agent of acute non-bacterial gastroenteritis worldwide and infection with NoV is the most common health risk associated with the consumption of faecally contaminated BMS. Advances in molecular methods provide new opportunities for assessment and management of this risk. Previous research has clearly demonstrated that oysters are a vector in NoV disease outbreaks. However, mussels (e.g. *Mytilus edulis*) have also been implicated in transmission of viral illness. There is a need for more information on the practical application of molecular methods to establish robust sampling strategies *for M. edulis* end-product testing and production area surveillance. The aim of my PhD was to reduce knowledge gaps associated with NoV accumulation and elimination in *Mytilus edulis*, and to provide mitigation recommendations to the industrial partner. My research determined that *Mytilus edulis* are efficient accumulators of NoV but variability in bioaccumulation can be detected between individual animals. However, composite samples (pool of 10 animals - as prescribed by ISO TS 15216) will effectively reduce the effect of animal-to-animal variation and provide a good sampling model for surveillance and monitoring purposes. NoV concentrations in *M. edulis* show high spatial variability within production areas. However, the spatial distribution of contamination can be effectively predicted using advection-dispersion modelling of effluent plumes, with implications for future risk assessment and management. Offshore cultivation or relaying represent options for avoidance or elimination of viral contamination of BMS but production efficiency may be compromised. It was shown that traditional commercial BMS depuration systems, optimised for elimination of pathogenic bacteria, are ineffective for elimination of NoV from *M. edulis*. NoV is currently regarded as non-culturable, with complications for the direct determination of infectivity. However, The T90 value for infectious FRNA bacteriophage, a morphologically similar, non-enveloped RNA virus, in mussel digestive tissue at 90°C was determined to be 42 s. Experiments showed that domestic cooking of whole animals at temperatures >90°C reduced infectious concentrations of FRNA Bacteriophage to undetectable levels within 3 minutes. The results of this thesis support the argument that control of harvesting area pollution is likely to represent a more effective strategy for controlling the risk of viral illness associated with consumption of BMS than currently available post-harvest processing strategies. However, the health risks associated with consumption of *Mytilus edulis* when thoroughly cooked are far less than for any BMS consumed raw.

Contents

Acknowledgements

I would like to thank Prof. Davey Jones and Dr. Shelagh Malham (Bangor University) and Mr. James Wilson (Bangor Mussel Producers) for offering me the KESS studentship and for continual support and supervision over the last three years. I am also indebted to Dr. James McDonald of Bangor University for welcoming me into his molecular laboratory, supporting and advising me, and to Dr. James Lowther of CEFAS Weymouth for the training and ongoing support provided by him and his team; Nicole Gustar and Justin Avant most especially.

Thanks are due to all at Bangor Mussel Producers for funding this project, introducing me to all aspects of the industry and making samples available. James Wilson, Kim Mould, all skippers and crew. And especially Trevor Jones for making his own boat and time available for the offshore experiments.

Thanks are also due to Gwynne Jones, Peter Hughes and Ben Powell, of the School of Ocean Sciences, for advice on the construction of offshore moorings and for time aboard the Maconna, also conducting experiments in rough seas up to 12 km off the North Wales Coast. Gwynnes's skilful skippering made "surfing" the Cheetah catamaran off of the Rhyl Flats an exhiting experience.

I would also like to thank my peers and colleagues in the department. Those with whom I have collaborated, Tracy Perkins and the now Dr. Katie Clements, and those who I have had the good fortune of working alongside in the Environment Centre Wales. Especially Dr. Helen Glanville for support, advice and a ready supply of laughter.

Thanks to DigiCal, Felix Iyah, Papa Si and all of the Freedom Soundsystem family for support and for keeping the Freedom Fyahphone blazing. Forward ever!

This PhD work was made possible through a studentship from Knowledge Economy Skills Scholarships.

Finally, a very big thank you is owed to my parents for providing so much support and ultimately, for making this work possible.

CHAPTER 1

Introduction to the thesis

1. Introduction

This chapter will introduce the literature informing the aims and objectives of the thesis and will conclude with a description of its structure. The thesis focusses upon *Mytilus edulis* mussels as a vehicle for human infection with norovirus (NoV). The research and its conclusions must be placed within a wider context of public health, foodborne illness, epidemiology, official controls and especially of human viral illness occurring after consumption of faecally contaminated bivalve molluscan shellfish (hereafter 'shellfish'). Shellfish are an ancient human food source evidenced by archaeological findings. However, viruses, bacteria and toxic chemicals may accumulate during their natural filter feeding. Consequently, shellfish are potential carriers of several food-borne illnesses (Lees 2000). The potential for shellfish to vector human pathogens was recognised over 150 years ago (Maalouf et al. 2010b). Shellfish consumption is still frequently implicated in disease outbreak investigations (e.g. Doyle et al. 2004; Le Guyader et al. 2006; 2008; Nenonen et al. 2009; Westrell et al. 2010). Of forty internationally reported outbreaks of food/waterborne NoV 2000-2007, 17.5% were accounted for by bivalve shellfish (Baert et al. 2009). Random sampling at farms and markets has found NoV in up to 55% of oysters from Europe and the US (cited by Westrell et al. 2010).

Bacterial pathogens remain responsible for most serious illnesses / fatalities (Oliveira et al. 2011). However, most contemporary outbreaks involve self-limiting gastro-enteric illness and the most common health risk associated with consumption of faecally contaminated shellfish is infection with NoV (Lees et al. 2010). This constitutes a significant risk to human health and sometimes causes economic losses within the shellfish industry. Shellfish viral safety concerns are large because consumption is often of raw or lightly cooked whole animals, including digestive parts which are the focus of contamination (Maalouf et al. 2010b; Lees et al. 2010). The following sections will review important aspects of shellfish contamination with human viral pathogens.

2. Shellfish

2.1 Bivalve molluscs

Bivalve molluscs tend to be static animals, attached to the seabed or submerged surfaces (exceptions include scallops). A bentho-pelagic life cycle with pelagic larval stage and sessile benthic adult stage is typical for most bivalve molluscs (Ackerman et al. 1994). Larvae tend to settle close to their con-specifics (Grünbaum 2011). Therefore they can form dense beds in productive areas which may be natural, or artificially created for commercial species (Quayle & Newkirk 1989). Sheltered areas with high nutrient levels are favourable conditions for settlement (Potasman et al. 2002). Bivalve molluscs may be harvested from natural populations or cultured in open sea without escape prevention. Harvesting of "wild" shellfish may be at elevated risk of human illness due to the absence of water quality monitoring programmes which are in place in commercial production areas. Those bivalve

species which are adapted to drying conditions (oysters, mussels and clams) close their shells to maintain internal conditions so can be sold live (Table 1).

Table 1. Commonly Commercially Exploited Bivalves

Common Name	Latin Name
Native/Flat Oyster	*Ostrea edulis*
Pacific Oyster	*Crassostrea gigas*
Common Blue Mussel	*Mytilus edulis*
Mediterranean Blue Mussel	*Mytilus galloprovincialis*
Cockle	*Cerastoderma edule*
King Scallop	*Pecten maximus*
Queen Scallop	*Chlamys opercularis*
Native Clam	*Tapes decussates*
Hard Shell Clam	*Mercenaria mercenaria*
Manilla Clam	*Tapes philippinarum*
Razor shells	*Ensis spp*

2.2 Shellfish Consumption and Production

Shellfish production and consumption are growing worldwide (Potasman et al. 2002; Soto 2009). Growing public awareness of potential health benefits of shellfish consumption has been suggested as an explanation for this increase (Woolmer 2010; Oliveira et al. 2011; Glude 1983). Shellfish contain low levels of saturated fat compared with chicken, beef or salmon, but contain high levels of polyunsaturated fats and nutritionally important vitamins and minerals (Woolmer 2010). Significant levels of long chain n-3 fatty acids (omega-3) are also present in shellfish (Arts et al. 2001). Molluscan shellfish represented 71% of 19.9 million tonnes of wild caught shellfish landed globally in 2010 and aquaculture is the fastest growing food supply sector in the world (Anon 2012b; Anon 2012a). Techniques for shellfish aquaculture include rope culture, long lines and shellfish racks. Mussels are the main shellfish group cultivated in Europe, followed by pacific oysters, with 477,000 and 105,000 tonnes, respectively, produced in 2010 (Anon 2012a). Mussels were also the most cultivated shellfish in the UK in 2010, representing 95% (by weight) of an industry worth £25.5m . Of 30,212 tonnes of mussels produced in the UK in 2010, approximately 30% was produced in Wales (Anon 2012a). Production volumes and value for 2011 are shown in table 2.

Table 2. Quantity and value of shellfish aquaculture in UK in 2011 (Anon 2013)

Tonnes	Scotland	England	Wales	Northern Ireland	UK Total (tonnes)	UK Total (£ value)
Pacific Oyster	251	447	6	50	754	949,400
Native Oyster	28	86	-	-	114	125,400
King Scallops	9	-	-	1	10	31,000
Queen Scallops	1	-	-	-	1	3,100
Mussels	6,996	3,127	8,370	7,665	26,158	17,966,590
Manila Clams	-	5	-	-	5	16,120
Hard Clams	-	10	-	-	10	31,000
Cockles	-	6	-	-	6	7,000
Total Volume	7,285	3,681	8,376	7,716	27,058	19,130,000

An increasing demand for marine-derived food products certified as being sustainable has also been observed in consumer trends in recent years and sustainable methods are available for commercial production of bivalve molluscs e.g. the Marine Stewardship Council (MSC) recognises the North Menai Strait Mussel Fishery as the first MSC certified enhanced fishery worldwide. The fishery is considered to have a negligible or potentially positive impact on mussel stocks (Welsh Assembly Government 2010). Seed mussels are relayed in the Menai Strait, grown for 18-24 months and produce a sustainable harvest of 8-10,000 metric tonnes annually (Shellfish Association of Great Britain 2010).

2.2 Contamination during natural feeding

Bivalve shellfish consume a mixed diet including phytoplankton and detritus (Lesser et al. 2010). They do so by filtering large amounts of water over their gills. Oysters may pump 10-20 litres per hour at peak activity (Jørgensen 1952; Riisgard 1988) and mussels 3 litres per hour (Famme et al. 1986; Riisgard et al. 2003). Large quantities of virus are shed in human faeces when enteric viruses replicate in the human alimentary tract (Atmar 2008; Haramoto et al. 2008; Mcleod et al. 2009a). Such enteric viruses may persist within the environment and within shellfish tissues (Costantini et al. 2006). Faecal pollution of production areas allows human enteric viruses to be accumulated during the filter feeding process (Lees et al. 2010; Potasman et al. 2002). In this manner the viruses may be concentrated. *Mytilus chilensis* concentrated Hepatitis A virus (HAV) 100-fold from surrounding water and *Crassostrea virginica* concentrated F+ coliphage by up to 99-fold (Enriquez et al. 1992; Burkhardt & Calci 2000). During filter feeding, shellfish may rapidly accumulate NoV in their gills, digestive glands and other tissues but when placed in clean sea water, the virus is eliminated at a slower rate (Wang et al. 2008; Ueki et al. 2007; Campos & Lees 2014). Studies have shown NoV and HAV accumulate primarily within the gut but may be internalized within non-digestive tissues to a

lesser extent (Romalde et al. 1994; Schwab et al. 1998; Le Guyader et al. 2006b; Mcleod et al. 2009b). Human epidemiology, persistence through sewage treatment and within the environment and shellfish uptake characteristics are all factors affecting bivalve mollusc contamination. According to Maalouf et al. (2010b), viral accumulation within shellfish depends on factors including water temperature, glycogen content of connective tissues, mucus production, ligand expression and gonadal development. Because shellfish may be exposed to the sewage of a large population they may be simultaneously and multiply contaminated with enteric viruses. This can explain the occurrence of mixed infections with multiple genogroups e.g. with NoV GI and GII (Ando et al. 1995; Sugieda et al. 1996; Schmid et al. 2005); with multiple viruses causing gastroenteritis (Caul 1996); or with multiple viruses causing discrete symptoms. e.g. Viral gastroenteritis with viral hepatitis (Halliday et al. 1991).

The bivalve molluscan shellfish are considered to feed according to similar principles but some authors disagree on the precise mechanisms, especially with regard to whether the feeding mechanism is autonomous or can be selective and regulated according to environmental conditions (Bayne 1998; Dolmer 2000; Jørgensen 1996). Filtration and clearance rates are difficult to measure but appear to vary between individual bivalves of the same species under similar conditions and fluctuations are reported in experiments with single animals. This may introduce variability in the uptake of virus and 100 fold differences among individuals within a batch have been observed (Seraichekas et al. 1968). A classic sieve model does not explain the retention mechanism of smaller particles unless they are aggregated (Bayne et al. 1976). It would also not adequately explain the retention of viral particles (e.g. NoV virions, ~30 nm). Retention of viral particles has been explained by a number of mechanisms including adhesion to latero-frontal cilia aided by mucus, which may play a role in feeding but not fully account for high retention efficiencies. NoV may also associate with organic and inorganic matter before being accumulated by shellfish (Campos & Lees 2014).

NoV persistence in shellfish may be aided by the binding of histoblood group antigens (HBGA). Virus like particles (VLPs), which resemble viruses but lack viral genetic material, have been synthesised and used to study accumulation and persistence in shellfish. GI NoV VLPs and native GI NoV showed no difference in tissue distribution after accumulation (Maalouf et al. 2010a). NoV was shown to bind in oysters to ligands similar to human histo-blood group A used for attachment to carbohydrate on human epithelial cells i.e. an "A-like carbohydrate structure" (Tan & Jiang 2007). It was shown that NoV-like particles (VLP) can specifically bind to oysters; *Crassostrea virginica, Crassostrea sikamea,* clams; *Venerupis virginica* and mussels; *Mytilus edulis* (Tian et al. 2007). This may contribute toward difficulty in eliminating the pathogen during depuration in clean seawater and the expression of these ligands may vary seasonally (Maalouf et al. 2010a). Specificities for HBGA ligands vary between NoV strains (Maalouf et al. 2010a). Some viral particles have been detected in

phagocytes within the epithelium or connective tissues of oysters and this may affect retention (Le Guyader et al. 2006b). McLeod et al. (2009b) showed NoV GII.4 bound in gills. NoV GI.1 appeared to be mostly restricted to digestive tissues, whilst NoV GII.4 bound to various organs using two different ligands (Maalouf et al. 2010a). Strong evidence for differential depuration is, however, lacking at this time. Exhibition of various binding patterns according to strain and genogroup suggests that this group of viruses, their host and carrier vector may possibly have coevolved (Le Pendu et al. 2006; Tan et al. 2009).

2.3 A History of the transmission of human viral illness via shellfish

Shellfish have been recognised as transmitting viral gastroenteritis for many decades (Rippey 1994). The first officially documented shellfish-borne transmission of viral gastroenteritis in the UK was via cockles. Electron microscopy revealed small-round-virus-like (SRVL) particles in human faeces after no bacterial pathogens were isolated (Appleton & Pereira 1977). The agent was epidemiologically linked to 33 incidents affecting 800 people. Similar SRVL particles were then observed in 90% of samples associated with shellfish vectored outbreaks of gastroenteritis (Appleton & Pereira 1981). NoV (then 'norwalk virus'), specifically, was associated with shellfish consumption in 1978 after an outbreak affecting 2000 people in Australia (Murphy et al. 1979) and in 1980, in Florida, USA (Gunn et al. 1982). 103 NoV outbreaks associated with clams or oysters affected >1000 persons in New York state during a wider US epidemic in 1982 then imported depurated English clams resulted in a further 2000 cases in New York and Jersey (Lees 2000). Lees (2000) reports attack rates 79 – 92% associated with shellfish consumption in outbreaks across multiple continents 1983-1991. These high attack rates observed in specifically investigated outbreaks e.g. within catered settings, suggest a much greater extent of unreported illness. An attack rate of 62% amongst a group who consumed oysters originating from Louisiana suggests that the total harvest of four million oysters may potentially have caused illness in up to 186,000 people (Dowell et al. 1995). The bed from which these were harvested was considered sufficiently remote to be free of sewage pollution. But harvesters "routinely" disposed of faeces overboard (Lees 2000). One harvester had gastroenteritis. Sequencing showed the outbreak to be caused by a single strain (Ando et al. 1995). According to Kohn et al. (1995) the epidemiological evidence suggested that disposal of diarrhoeal stool from one sick individual caused this potentially very large outbreak. The high concentration of NoV shed in stool, low infectious dose and potential for shellfish bioaccumulation make this plausible (Lees 2000; Dowell et al. 1995). Further outbreaks have also been linked to overboard dumping of faeces (Farley et al. 1998).

However, according to documented outbreaks, contamination of shellfish beds most commonly occurs after heavy rain (Grodzki et al. 2012). The capacity of wastewater treatment facilities may be exceeded and untreated sewage, heavily contaminated with enteric viruses, may be released to the

aquatic environment (Maalouf et al. 2010b). Murphy et al. (1979) noted this link: Shellfish grown in an urbanised coastal area and harvested after heavy rain resulted in a 2000 person outbreak of viral gastroenteritis. Similar occurred in New York, 1982 (Morse et al. 1986). Failure of one sewage treatment plant after heavy rain has twice caused large outbreaks through contamination of a lagoon where oysters are grown (Le Guyader et al. 2006; Le Guyader et al. 2008). The "first flush" of an overloaded Wastewater Treatment Plant (WTP) may release waste which has been accumulated over time and Le Guyader et al. (2008) described a broad range of virus strains isolated from shellfish and infected patients' stool.

3. Viruses of Concern

Although many human viruses which are transmitted by the faecal oral route have been isolated in bivalves, relatively few are associated with outbreaks of shellfish-borne illness. This may be due to factors including pre-existing immunity, under-reporting or higher infectious doses for some viral pathogens. Viruses typically associated with infection after shellfish consumption cause gastroenteritis or hepatitis and the most common pathogenic agents worldwide are NoV and HAV (Woods & Burkhardt 2010; Richards et al. 2010). Characteristics of the main shellfish-vectored viruses are summarised by Maalouf et al. (2010b) and shown in Table 2.

Table 2 - Characteristics of the main enteric viruses (Maalouf et al. 2010b)

Name	Size	Capsid	Genome[a]	Incubation	Illness	Season
Adenovirus	70 nm	Complex	DsDNA-35,900 bp	3-10 d	Gastroenteritis	All year
Aichi virus	27-32 nm	Icosahedral	ssRNA -8,251 bases	1-2 d	Gastroenteritis	All year
Astrovirus	27-32 nm	Icosahedral	ssRNA-6,797 bases	3-5 d	Gastroenteritis	Winter
Calicivirus	27-32 nm	Icosahedral	ssRNA-7,642 bases	2-3 d	Gastroenteritis	Winter
Enterovirus	20-30 nm	Icosahedral	ssRNA-7,200 bases	7-30 d	Diverse	Summer
Rotavirus	70 nm	Triple layer icosahedral	dsRNA, 11 genes (667 - 3302 bp	3 d	Gastroenteritis	Winter
Hepatitis A virus	27-32 nm	Icosahedrol	ssRNA-7,487 bases	Up to 6 weeks	Hepatitis	All year

a *ds* double strand, *bp* base pairs, *ss* single strand

Together these viral contaminants represent significant concerns regarding shellfish safety and threaten the economic viability of the shellfish industry and associated trades (Richards et al. 2010).

3.1 Caliciviridae

Those caliciviridae which are known to infect humans are transmitted by the faecal-oral route and classified under the generas NoV and sapovirus. Caliciviruses are positive sense, single stranded non-segmented RNA viruses. Their morphology is generally round, they are small (27-40 nm) and non-enveloped (Clarke & Lambden 1997; Dimmock et al. 2007; Buckow et al. 2008). Sapoviruses have not been associated with shellfish vectored illness, perhaps due to a high seropositivity rate in adults.

3.1.2 Norovirus

NoVs cause approximately 90% of epidemic non-bacterial gastroenteritis outbreaks worldwide (Lindesmith et al. 2003; Koopmans et al. 2002) and are the most significant cause of infectious intestinal disease (Lees 2000). However, biological knowledge of Human NoVs is partly limited by inability to culture NoV *in vitro.* They are icosahedral, ~ 38 nm in diameter, with a 7.5–kb positive-sense single stranded RNA genome, containing three open reading frames coding structural and non-structural proteins (Donaldson et al. 2010). NoV can be divided into 5 genogroups comprising >31 genetic clusters and >164 strains (Zheng et al. 2006). Some strains of NoV are uniquely associated with animals. Genogroup 1 (GI) and Genogroup 2 (GII) cause almost all human infections (Lees et al. 2010; Zheng et al. 2006). The most prominent strain has been GII.4 for many years (Donaldson et al. 2008; Siebenga et al. 2009; Lopman et al. 2009; Campos & Lees 2014). Whilst GI is a rarer causative agent in outbreaks, it is most commonly transmitted by food and often associated with shellfish-borne outbreaks (Noda et al. 2008; Siebenga et al. 2009; Maalouf et al. 2010a). GIV NoVs also occasionally infect humans but GIII and GV infect cows and mice respectively (Di Martino et al. 2010; Eden et al. 2012). Clinical infection caused by NoV located on GII has been detected in pigs but these belong to different clusters within GII (GII.11, GII.18 & GII.19) to human NoVs (the remaining 16 x GII clusters) (Wang et al. 2005; Sugieda et al. 1998). Because known human strains have been shown to replicate and induce immune response in gnotobiotic pigs, and porcine strains are quite genetically similar, there is a possibility of co-infection and emergence of porcine-human recombinants (Cheetham et al. 2006; Mattison et al. 2007). NoVs infecting cattle appear to be less closely related and form a distinct genogroup (Oliver et al. 2003). However detection in a bovine sample of NoV RNA showing a sequence similarity to the human GII.4 "Farmington Hills" cluster contributes to concerns of potential co-infection and subsequent recombination (Mattison et al. 2007). Bovine NoVs have also been detected in shellfish but do not appear to bind (Zakhour et al. 2010). The possibility of zoonotic/reverse-zoonotic transmission and recombination requires further study, especially because new NoV variants infecting humans, with mutations in the polymerase gene and increased virulence, have been added to the GII.4 cluster recently (Lopman et al. 2004; Bull et al. 2006; Oliver et al. 2003). Different characteristics for viral faecal loads, asymptomatic shedding, and behaviour during wastewater treatment may help explain differences in epidemiological patterns for NoV GI and GII (Chan et al. 2007; Atmar 2008; Da Silva et al. 2007)

3.2 Hepatitis Viruses

Hepatitis viruses cause liver inflammation. Enterically transmitted Hepatitis viruses, A and E, show a worldwide distribution but are variably endemic. Transmission is often linked to sanitary conditions within the population and especially associated with contaminated water. No clear seasonal pattern is

observed in general infection rates but shellfish-vectored hepatic illness does show some seasonal pattern (Pintó & Saiz 2007).

3.2.1 Hepatitis A Virus

HAV is an icosahedral picornavirus of the hepatovirus family with a positive sense single stranded RNA genome and no envelope (Fauquet et al. 2005; Dimmock et al. 2007). There is an efficient vaccine. Improvements observed in public hygiene have reduced prevalence in many regions. However, HAV is the most common hepatitis virus worldwide causing large economic losses including for global food trade (Pintó et al. 2010). The distribution pattern for HAV is closely related to that of socioeconomic development (Previsani et al. 2004; Hollinger & Emerson 2007). Due to a long incubation (2-6 weeks), the source of infection is unidentifiable in ~40% of cases and consumption of contaminated product can continue for a considerable period before outbreak recognition. Thus, seafood and HAV association is probably underreported (Rippey 1994). Salamina & D'argenio (1998) suggest that 70% of HAV cases in Italy may be shellfish-borne. Confirmed shellfish-borne outbreaks may represent a small part of a larger burden including sporadic cases also acquired after consumption of shellfish (Lees 2000). HAV replicates in the human liver, reaches the intestine via the bile duct and large quantities of up to $5x10^{11}$ genome copies/g are shed in faeces prior to, during and post symptoms and also in asymptomatic individuals (Costafreda et al. 2006; Pintó et al. 2010). The clinical infection is relatively severe and the most serious viral illness directly associated with shellfish. It causes serious debilitating disease although it is self-limiting and rarely fatal; Non-specific illness (fever, malaise, headache, nausea) progresses to anorexia, vomiting, diarrhoea and jaundice (Lees 2000). However, young children <5 years, tend to be asymptomatic or develop mild symptoms and seropositivity by age 6 is common in endemic countries. Declining prevalence is observed in many countries particularly in Southern Europe (Germinario et al. 2000; Van Damme & Van Herck 2005; Domínguez et al. 2008) Eastern Europe (Cianciara 2000; Tallo et al. 2003) and several Asian (Barzaga 2000), South American (Tanaka 2000) and Middle Eastern countries (Gdalevich et al. 1998). Improving sanitary conditions result in declining prevalence but a growing population susceptible to adult infection. This has implications for average age of exposure and hence severity of illness (Pebody et al. 1998). In adults suffering overt hepatitis, clinical illness usually lasts <2months with 10-15% suffering relapses or prolonged symptoms for up to six months (Sjogren et al. 1987; Glikson et al. 1992). Chronicity is rare except in the immuno-compromised or those with existing liver damage who may develop fulminant hepatitis (Previsani et al. 2004). Recovery is usually complete and confers long term immunity (Hollinger & Emerson 2007). Genotypes I, II and III (of six) are of human origin but there is one serotype, conferring long lasting immunity to GI, II & III. Low antigenic variability is associated with low variability of the HAV capsid (Sanchez et al. 2003; Pintó et al. 2010).

HAV presence in sewage effluent, receiving waters and bivalve shellfish has been demonstrated frequently. Shellfish vectored outbreaks can be dramatic and geographically wide ranging (Conaty et al. 2000; Sanchez et al. 2002; Shieh et al. 2007). The first documented occurred in Sweden in 1955, on consumption of oysters, causing 629 cases (Roos 1956). The largest documented shellfish-borne viral outbreak was of HAV in China, 1988, where consumption of clams was implicated in 300,000 cases (Tang et al. 1991; Halliday et al. 1991). However, HAV is a rare contaminant of shellfish in non-endemic areas (Lees et al. 2010).

HAV is stable in the acid pH of the stomach, in biliary and other salts, resistant to detergents and able to survive long extracorporeal periods and persist on contaminated fomites (Abad et al. 1994b; Abad et al. 1994a). This explains frequent transmission by food and water (Dentinger et al. 2001; Sanchez et al. 2002; Pintó et al. 2009). If HAV is circulating in the population, shellfish-borne outbreaks are hard to prevent because excretion precedes symptoms, which take time to appear, and inactivation of virus by sewage treatment is difficult (Blatchley et al. 2007; Bosch 2007).

3.2.2 Hepatitis E

Hepatitis E virus (HEV), a single-stranded RNA virus, has been classified into the genus hepevirus. It shares some morphological features with the caliciviridae. HEV infects all ages and is hyperendemic primarily in Asia. It causes a similar acute, self-limiting liver disease to HAV although HEV is associated with a higher death rate in pregnancy. Four distinct genotypes have been reported but there is one serotype. HEV1 and HEV2 appear to be restricted to humans, whilst HEV3 and HEV4 infect humans, pigs and other animal species. In the UK, HEV infection was previously mostly associated with travel to endemic countries. However, in the last decade an increase in non-travel associated human cases has been observed. Occurrence of HEV in patients who did not travel outside of England and Wales but habitually ate shellfish suggested that shellfish consumption is a risk factor (Ijaz et al. 2005). During investigation of an HEVIII outbreak aboard a cruiseship, consumption of shellfish was strongly associated with infection (Said et al. 2009). Genotype 3 is the main type of HEV found in the United Kingdom and a recent study has demonstrated presence of HEV3 in mussels produced on both the East and West coast of Scotland (Crossan et al. 2012). The sequences isolated from the mussels corresponded with sequences isolated from a human source.

3.3 Other viruses warranting further study

Astroviruses, positive sense, single stranded RNA viruses, are a common agent of childhood diarrhoea and frequently present in both pre- and post-treatment sewage (Le Cann et al. 2004; Myrmel et al. 2006). Astrovirus distribution is worldwide and infection often occurs in winter in temperate regions, causing vomiting, diarrhoea, fever and abdominal pain after 2-4 days incubation. Recovery is normally complete, without complications and results in immunity to one of seven

recognised serotypes. Astroviruses are most often seen in sporadic individual cases. Whilst epidemiological links are not well documented, nor are outbreaks as frequent as for NoV, astroviruses have been implicated in shellfish-borne outbreaks (Le Guyader et al. 2008).

Rotaviruses, of the reoviridae, are double stranded RNA viruses causing near-ubiquitous infection of children under five. Immunity appears to develop after early exposure but many children acquire immunity after several infections, subsequent infections being less severe. Initial immunity may be serotype specific but repeat infections increase cross-reactive antibodies (Anderson & Weber 2004). Adults are therefore, less frequently infected (Velázquez et al. 1996). Due to large viral loads $>10^{12}$ particles/g in the faeces of infected patients, rotavirus is also frequently detected in sewage (Gajardo et al. 1995; Dubois et al. 1997; Kamel et al. 2010). Presence in shellfish has been detected (Le Guyader et al. 2008) but not epidemiologically linked with shellfish-borne disease. This may be due to age-related resistance and adults being primary consumers of seafood (Maalouf et al. 2010b). However, the non-group A rotaviruses are less common, with lower antibody prevalence in the community and may cause severe gastroenteritis in all ages. Therefore they are likely to represent the greatest risk for shellfish vectored illness.

Adenoviruses are double stranded DNA viruses causing respiratory, ocular and gastroenteric infection in animals and humans. Fifty five serotypes in six subgenera affect humans. Serotypes 40 and 41 are those predominantly associated with gastroenteritis (Verma et al. 2009). However, non-enteric adenoviruses may also be shed in faeces (Lees 2000). Adenoviruses have been detected in sewage effluent, seawater and but no seafood related outbreaks are recorded and age-related immunity may be a factor (Girones et al. 1995; Vantarakis & Papapetropoulou 1998; Pina et al. 1998; Myrmel et al. 2006; Katayama et al. 2008; Wyn-Jones et al. 2010).

Enteroviruses form a genus within the picornaviridae. They are single stranded RNA viruses and include the poliovirus, Group A & B Coxsackieviruses and echoviruses (Muir et al. 1998). More than 66 immunologically distinct serotypes causing human infection exist. All age groups can be infected. Infection by enteroviruses may be mild or clinically inapparent but in some cases they can develop into fatal disease. An example is poliomyelitis, causing neurological disease, which is still common in some countries despite vaccination campaigns (Maalouf et al. 2010b). Symptoms may present in different areas of the body, but many enteroviruses replicate within the intestinal tract after faecal-oral transmission. The virions are acid resistant and so can survive the gut but they do not commonly cause gastroenteric symptoms. As, some enteroviruses are prevalent and may be cultured, they are sometimes used as an indicator of human viral contamination. Despite being isolated in sewage effluent, seawater and bivalves (Jaykus et al. 1994), shellfish have not been linked to transmission. This is interesting because consumers have probably been exposed. It is possible that long incubation

periods complicate the identification of a food vehicle and that enterovirus transmission via shellfish impacts public health (Lipp and Rose 1997).

4. NoV and Human Health

4.1 Characteristics of human NoV infection

In the UK, reporting of gastroenteritis by GPs to public health authorities is not mandatory and patients suffering relatively mild symptoms associated with NoV may not present to their doctor (Lees 2000). Reported foodborne incidents tend to be those occurring in restaurants, and principally, large functions. Therefore reported NoV illness probably underestimates the true numbers affected (Sugieda et al. 1996; Tam et al. 2003; Potasman et al. 2002; Wheeler et al. 1999). The rate of under-reporting is probably greater at the global scale (Potasman et al. 2002). The UK age-adjusted community incidence of NoV associated infectious intestinal disease is estimated to be 4.7/100 person-years, representing 3 million disease episodes and 130,000 consultations per year (Tam et al. 2012; Phillips et al. 2010). There appears to be a dose response relationship between NoV titre ingested and disease outcome. Whilst 18 NoV GI genomes represents the estimated ID_{50}, the probability of displaying NoV symptoms is 1% at 10^3 and rises to 70% at 10^8 genome copies (Teunis et al. 2008). This may explain asymptomatic excretion in up to 16% of healthy individuals, and also why shellfish concentrations in investigated outbreaks are typically in excess of typical background levels (Campos & Lees 2014; Phillips et al. 2011; Lowther et al. 2012a). Woods & Burkhardt (2010) compared NoV GII virus titres in retail shellfish and those implicated in outbreaks. Retail oysters returned 20-100 RT-PCR units/100 g and outbreak implicated oysters 300-1500/100g. Therefore typical levels in retail shellfish may represent a risk of asymptomatic infection and transmission.

NoV potential to cause large outbreaks and secondary infections may be explained by resilience to treatments inactivating other viruses (Patel et al. 2009), potential for aerosolisation (Barker & Jones 2005) and low infectious dose rendering low levels of food contamination a risk (Donaldson et al. 2008; Lees et al. 2010; Le Guyader et al. 2003; Le Guyader et al. 2006). Incubation is 24-48 hrs, clinical symptoms last 12-72 hrs but Rockx et al. (2002) observed shedding >3 weeks after symptoms subsided. According to Atmar (2008) those infected shed NoV at 5×10^8 – 1.6×10^{12} RNA copies/g of stool and post symptomatic shedding continues with a median of 28 days despite most being non-symptomatic after 4 days. Clinical infection causes nausea, diarrhoea, abdominal pain and sometimes vomiting, myalgia and fever. Death and lasting ill health is very rare but infection presents increased risk in the immunocompromised, the elderly, those with underlying health concerns or those without access to plentiful clean water (Donaldson et al. 2008; Patel et al. 2009; Harris et al. 2008). Unlike rota-, astro- and adenoviruses, which tend to infect children, NoV affects all age groups and repeat infection is common. Immunity appears to be incomplete, temporary and strain specific (Lindesmith

et al. 2003). Therefore epidemic spread is facilitated by the susceptibility of most of the population. NoV susceptibility in humans is dependent upon blood group (Tan & Jiang 2007). Studies show that expression of histoblood group antigens (HBGA) is linked to susceptibility (Shirato 2011; Lindesmith et al. 2008; Huang et al. 2005). Individuals of blood type O appear to be infected disproportionately (Lindesmith et al. 2003). NoV may be transmitted by person to person contact, or by faecally contaminated food and water with large outbreaks sometimes traced to one infected food handler (Goodgame 2006). Attack rates as high as 100% have been detailed and this is especially apparent in closed communities such as hospitals (Lees 2000).

4.2 Health impact of shellfish-borne transmission of NoV

Illness associated with seafood contaminated with NoV is estimated to cost the US $184m annually (Batz et al. 2011). In January and February 2010 the European Centre for Disease Control was informed of 334 cases in 65 clusters of NoV infection associated with consumption of raw oysters, mostly in catered settings (Westrell et al. 2010). Stool samples from infected patients often tested positive for both NoV genogroups I and II (Table 3). This may reflect environmental contamination by sewage and is common in shellfish related outbreaks (Le Guyader et al. 2006b; Bon et al. 2005; Gallimore et al. 2005; Symes et al. 2007). Single strains often characterise person-to-person transmission but shellfish-borne outbreaks have featured up to seven detectable strains (Bon et al. 2005). Within the UK, the Health Protection Agency was informed of 22 outbreaks (120 persons affected) of gastroenteritis associated with consumption of oysters in restaurants in January 2010 (Westrell et al. 2010). The aforementioned studies implicate oysters, whilst studies which confirm mussels as the vehicle NoV transmission are relatively few. This may reflect that oysters are more frequently consumed raw. Nothwithstanding this, several outbreak investigations have found cases of norovirus illness to be strongly associated with raw mussel consumption (Prato et al. 2004, Rizzo et al. 2007). These studies are based in the Puglia region, Italy, where traditional consumption of raw mussels is reported.

Table 3. NoV clusters linked to consumption of oysters, UK, Norway, France, Sweden and Denmark. Jan-Mar 2010 (adapted from Westrell 2010)

Country	Clusters	Verified	Total n of cases	NoV detection (genogroup)		Origin of Oysters
				Cases	Oysters	
United Kingdom (England and Wales)	22	3	120	+ (I,II)	+ (I,II)	England, Scotland and Ireland
Norway	8	8	39	NA	+ (I,II)	Brittany, France
France (1)	6	6	22	NA	+	Brittany, France
France (2)	4	4	45	+ (I,II)	+	Brittancy, France
Sweden	16	0	50	NA	NA	The Netherlands and France
Denmark	9	6	58	+ (I,II)	+ (I,II)	Different locations in France

NA; Not available

5. Seasonality & Weather Effects

NoV has been colloquially referred to as "Winter vomiting bug/disease" due to its marked seasonal pattern of infection. The timing of annual outbreaks of shellfish-vectored NoV is also highly consistent. The epidemiology of NoV infection in the human population shows a clear, winter-biased, seasonal trend but important differences exist between years eg. the number of laboratory reports during the winter of 2009 / 2010 was greater than in subsequent years (Anon 2014). A clear, Winter-biased trend is also evident in norovirus prevalence and levels in shellfish, being most frequently detected and at highest levels between October and February (Burkhardt & Calci 2000; Lowther et al. 2008). Some studies have demonstrated no substantial seasonality in NoV levels in shellfish, but are outnumbered by studies demonstrating generally higher levels of NoV in shellfish during Winter (Lowther et al. 2012b; Rajko-Nenow et al. 2012; Campos et al. 2013). Average norovirus levels in *Crassostrea gigas* may be 17 times higher in the period Oct-Mar than the remainder of the year (Lowther et al. 2008). This is consistent with the winter biased seasonality in shellfish related outbreaks in temperate climates. Between March 2006 and March 2010 the Rapid Alert System for Food and Feed recorded 19 cases of NoV detected in EU oysters, 17 of which occurred between the months of January and April (Westrell et al. 2010). In the US, the consumption of oysters harvested between November and January was implicated in 78% of NoV illnesses associated with gulf coast shellfish in the 1990s (Burkhardt & Calci 2000). According to Lopman et al. (2008) and Dowell (2001), viral gastroenteritis (including NoV) prevalence is greatest in colder months but does not disappear in summer. The prevalence and quantity of NoV in commercial oysters from UK harvesting areas reflects this seasonal pattern: NoV GI and GII are often present in shellfish throughout the year, but with peaks of prevalence in Winter being consistent with the incidence of NoV infections in the UK (Nordgren et al. 2009; Campos & Lees 2014; Lees 2000). Between September 2008 and August 2011 a 90% NoV positivity rate was observed in UK commercial oysters

in the months Oct - Mar and 62.4% Aril – September, with highest levels detected December – March (Lowther et al. 2012b). That contamination and illness by NoV are more prevalent in cooler months is true for all temperate continents. Oysters and mussels are considered to be of the best organoleptic quality / meat content outside the breeding season i.e. also in cooler months when they are consumed more. A winter-biased seasonality is evident in the pattern of all viral outbreaks associated with shellfish consumption in the UK (Lees 2000). Sampling live US market oysters, Woods and Burkhardt (2010) showed that detection rates for both NoV and also HAV were highest during Winter months.

However, many enteric viruses circulate endemically throughout the year and enteric viruses are detected in untreated effluent all year (Da Silva et al. 2007; Patel et al. 2008; Lopman et al. 2008). Therefore, shellfish-borne viral illness outbreak seasonality is likely to be influenced by conditions affecting the environmental persistence of viruses. HAV and NoV remain active longer in colder environments and exposure to sunlight is detrimental to the persistence of viruses (Burkhardt et al. 2000; Flannery et al. 2009). Doré et al. (1998) suggested that depuration inefficiencies might also influence the seasonal pattern of illness, where shellfish may purge pathogens less efficiently under depuration during Winter months when shellfish metabolic activity may be lower. Seasonal expression of ligands to which NoV may bind may also form a factor affecting prevalence and levels of virus retained in shellfish (Maalouf et al. 2010a). Rainfall, which may be seasonally influenced, has been shown to be an important factor in the contamination of shellfish, sometimes influenced by the release of untreated sewage as discussed later. Both Miossec et al. (2000a) and Riou et al. (2007) were able to define risk periods, according to rainfall, for commercial shellfisheries.

6. Wastewater treatment and environmental persistence

Stools from individuals infected with NoV or other enteric viruses contain high virus titres and enter the environment via both untreated and treated sewage at lower levels. Causative links between sewage effluents, freshwater, shellfish and outbreaks of human gastroenteritis have been demonstrated by detection of clinical strains in environmental samples (Ueki et al. 2005; Nenonen et al. 2008; Campos & Lees 2014). Routes of introduction into the marine environment include the discharges of water treatment works, septic tanks and overflows of such systems (Campos & Lees 2014). Currents, tides and other estuarine / coastal processes subsequently affect the distribution of discharged viruses (Pommepuy et al. 2005). Factors controlling the abundance and distribution of NoV in shellfish waters are site specific and include level and mode of sewage treatment, discharge proximity, water temperature and salinity and rainfall (Campos & Lees 2014).

6.1 Modes of sewage treatment

Conventional biological sewage treatment processes achieve smaller reductions of NoV than of faecal indicator bacteria (FIB). But significant differences are observed in the performance of different treatment technologies (Da Silva 2007). Factors affecting the concentration of infective viruses in effluent after sewage treatment are likely to include initial raw sewage concentrations, predation by bacteria and protozoa, residence time, solar radiation, temperature, adsorption, enzymatic destruction and process / level of treatment (Le Cann et al. 2004; Myrmel et al. 2006; Katayama et al. 2008; Maalouf et al. 2010b). During processing, NoV may be physically removed from the effluent e.g. NoV associated with solids may be physically removed during clarification with the proportion being associated or adsorbed influencing removal rates (Hejkal et al. 1981; Da Silva et al. 2008). However, it is difficult to characterise processes causing the inactivation of viruses for which culture based techniques are not available e.g. NoV (Da Silva et al. 2008). For example the polymerase chain reaction method may not properly reflect reductions in virus viability during e.g. Ultraviolet (UV) tertiary treatment (Campos et al. 2013; Flannery et al. 2013). Treatments based upon membrane bioreactor technology (biological activated sludge + membrane filtration) appear to remove NoV most efficiently, with reported log reductions (GI+GII) of 3.3 to 6.8 $\log_{10}$ units (Campos & Lees 2014; Sima et al. 2011; Simmons et al. 2011).

Contrasting evidence exists regarding the prevalence of different NoV strains in effluent. This may reflect different patterns of strain prevalence in respective communities but also strain-dependent resistance to different forms of treatment (Campos & Lees 2014). As an estimate, typical treated wastewater may contain up to 10^3-10^4 NoV genome copies/litre during non-epidemic periods and 100-1000-fold higher during the annual (winter) epidemic (Maalouf et al. 2010b).

6.1.1 Non-municipal sewerage

Properties not served by municipal sewerage may be connected to septic tanks. Poorly maintained septic tanks may result in groundwater contamination and, as NoV (GI) may remain infective for more than 61 d, could subsequently contaminate surface waters (Campos & Lees 2014; Seitz et al. 2011; Borchardt et al. 2011). Soil chemistry may affect the movement of NoV through groundwater, with pH, mineral content, soil organic matter and particle size, being recognised as factors (Campos & Lees 2014).

6.2 Overload of sewage treatment capacity

Wastewater treatment systems have a designed capacity. If this capacity is exceeded wastewater arriving may be redirected to storage or discharge with little or no treatment. This may occur after heavy rain resulting in faecal contamination of shellfish growing waters and exposure to enterically transmitted viruses (Miossec et al. 2000b; Le Guyader et al. 2006; Le Guyader et al. 2008). Such

discharges represents a significant risk for shellfish contamination because shellfish may accumulate many infective doses (ID_{50} 18 virus particles) from mean NoV concentrations (10^2 – 10^4 GI + GII gc/ml) present in crude sewage (Campos & Lees 2014). Therefore, it is necessary to consider the adequacy of storm-water storage and treatment (Lees 2000). Overflows which are designed to discharge a combination of rain water and diluted sewage (e.g. Combined Sewer Overflows) when treatment capacity is exceeded may differ in character to accidental overflows resulting from malfunction but both are likely to present a high risk for shellfish contamination (Campos & Lees 2014; Hata et al. 2014; Rodríguez et al. 2012; Flannery et al. 2013). The association between heavy rain and release of untreated sewage is likely to be a factor influencing seasonality in many climates (Mounts et al. 2000).

6.3 Disposal of faeces by boats

Large outbreaks in areas far from point-source discharges have been explained by overboard dumping of faeces (Berg et al. 2000; Kohn et al. 1995). Large ships represent closed settings in which NoV outbreaks are common. They are expected to have sewage treatment systems but these may not be effective in reducing NoV. However, due to the high concentrations of virus shed in the faeces of infected individuals, and the low infectious doses, overboard dumping of faeces from a single case poses a significant risk of shellfish contamination (Campos & Lees 2014).

6.4 Post-discharge environmental behaviour

Enteric virions may persist in an infective state for weeks to months whilst suspended in the water column, attached to matter or accumulating in sediments (Lees 2000; Maalouf et al. 2010b). HAV has been shown to survive more than one month in seawater (Callahan et al. 1995; Gantzer et al. 1998). Conditions in receiving water affect survival but human NoVs appear to be highly resistant to degradation in aquatic environments (Seitz et al. 2011). Gentry et al. (2009) examined the distribution of NoV across samples of oysters, water and plankton in an estuarine environment and found the most frequently contaminated was oysters (55%), followed by 63-200 μm plankton (11.1%), water (8.3%) and <200 μm plankton (2.8%), reflecting the ability of oysters to bioaccumulate virus. But the highest concentrations of NoV observed in the study were found in samples of <200μm plankton. Gentry et al. (2009) speculate that electrostatic interactions may cause adsorption to plankton. Resuspension of bacterial pathogens from sediments has been shown to be important and may also be important for viral pathogens (Wilkinson et al. 2006). A number of currently unpublished studies demonstrate that the extent of NoV contamination about a discharge point can be large (Campos & Lees 2014). The spatial pattern of this contamination is principally determined by plume hydrodynamics resulting in less impact upon discharge-proximate animals outside of the concentrated plume than those within and at greater distance (Goblick et al. 2011). The key post-discharge factors upon which enteric virus concentrations in marine water depend are either physical (e.g. dilution,

dispersion, sedimentation), or physico-chemical (e.g. sunlight exposure, salinity, temperature, pH) (Maalouf et al. 2010b).

7. Detection and Quantification of Norovirus

Recently standardardised (ISO TS 15216) methods for the detection of norovirus in foods and the environment employ a quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Anon 2013b). These assays quantify copies of a NoV GI or GII-specific RNA sequence, and allow the sensitive detection and quantification of virus genome copies in water, sewage, food, air and on fomites. In October 2011 the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) shared bench protocols and trained the author in the quantification of NoV in BMS. Protocols were based on the draft European Committee for Standardisation (CEN) method (Lees et al. 2010). The following chapters apply this method, which is now internationally standardised under technical specification ISO TS 15216 and suitable for use in a legislative context (EFSA Panel on Biological Hazards 2012; Anon 2013b; CEFAS 2013). The fluorogenic probe-based TaqMan technology relies upon the amplification of a necessarily short region of reverse-transcribed genome. The ISO specification allows scope for published primers but those used in its development, and adopted for the experiments described in this thesis, amplify 86 bp and 89 bp products (of ca. 7600 nt) for NoV GI and GII respectively. Therefore this widely-applied method cannot differentiate between infective virus particles and residual RNA from degraded particles. This limitation raises problems when assessing the risk to human health posed by food samples determined positive by PCR assays (Knight et al. 2012). An infectivity assay for human norovirus would help answer key research questions.

Straub et al. (2007) published details of an *in vitro* cell culture infectivity assay for NoV but the results were not proven reproducible and NoV remained generally regarded as unculturable. Therefore detection has relied upon molecular techniques, particularly qRT-PCR (as above), and currently there is no validated assay to directly measure infectivity (Campos & Lees 2014). However, Jones et al. (2014) very recently published three important findings: Firstly, human B cells were identified as a cellular target of NoV GII.4-Sydney (the current globally dominant strain), where the cellular tropism has previously remained elusive. Secondly, free HBGA or presence of HBGA-expressing bacteria enhance infection in human norovirus cell culture (and murine norovirus *in vivo*), suggesting that HBGAs have a role as stimulatory carbohydrate molecules. This may help explain correlation between a human individual's HBGA profile human and their susceptibility to NoV infection, and may explain failures to culture human NoV in their absence (Jones et al. 2014). Thirdly, the findings allowed the development of an *in vitro* infection model for a human norovirus, which could prove a large step towards a reliable human NoV infectivity assay.

In the mean time the standardised qRT-PCR method for determining NoV GI and GII genome copies is well characterised. Despite potential to detect non-infective particles, superior sensitivity (potentially 1 genome copy in a reaction) make qRT-PCR the leading choice for detection of viruses with low infectious doses in food. Alternative RNA based methods include Nucleic Acid Sequence Based Amplification (NASBA) and Reverse Transcribed Loop-Mediated Isothermal Amplification (RT-LAMP) but appear to offer no advantages in discriminating between infectious and non-infectious NoV (Knight et al. 2012). Enzyme-Linked-Immunosorbent-Assay (ELISA) technology is useful in clinical detection of NoV in stool, but is insufficiently sensitive to detect food/environmental contamination at levels representing infectious doses, cannot detect all genotypes so may result in false-negatives, and does not distinguish infectious particles from empty capsids and free antigen (Knight et al. 2012). Electron microscopy is not useful in detecting food/environmental contamination due to its insensitivity to low infectious doses.

However, potential adjuncts to the qRT-PCR method could help predict infectivity by distinguishing signal derived from intact infective virus particles, intact but defective particles, ribonucleoprotein complexes arising from degraded particles, or naked RNA (Knight et al. 2012). Infectivity relies upon intact viral capsid and genome. These methods attempt to differentiate PCR signals according to determination of capsid integrity or genome integrity, providing more informed data for use in risk assessment.

7.1 Genome Integrity

A virus particle could be rendered non-infectious by a single strand break in the genome but this will not be discovered by qRT-PCR if the target region remains intact. A longer region of genomic RNA may be targeted for amplification in qRT-PCR, but amplification efficiency decreases with increasing fragment size. Therefore there is a trade-off for sensitivity, which is crucial when studying viruses with low infective doses. However, by priming the reverse transcription at the 3' end, then amplifying only a small and distant region of the cDNA, intact viral genomes may be detected with greater efficiency. This approach has been used to demonstrate the sensitivity of NoV to e.g. UV irradiation (Wolf et al. 2009).

7.2 Capsid Integrity

The capsid is important for infection and therefore its integrity may be a marker for infectivity (Knight et al. 2012). Knight et al. (2012) identified in the literature three main approaches to measurement of capsid integrity using subsequent detection of RNA by qRT-PCR. These are summarised below.

7.2.1 Enzyme Pretreatment qRT-PCR approaches

These approaches use RNase treatment to measure RNA exposure resulting from capsid damage. However, the approach may not completely abolish RT-PCR signals from inactivated virus due to protection of RNA by the partially degraded capsid i.e. formation of RNase resitiant RNA-protein complexes described as ribonucleoprotein complexes (RNPs). The additional use of Proteinase K may help degrade the capsid of damaged virus particles allowing RNase activity without destroying intact particles. However the use of Proteinase K and RNase proved difficult to control and report (Knight et al. 2012). Use of RNase ONE alone shows promising results but there is a potential for matrix protection resulting in a remaining discrepancy between qRT-PCR results and loss of infectvity.

7.2.2 "Integrated" qRT-PCR approaches

These approaches assume that damaged capsids will be unable to bind to appropriate receptors and therefore measure the affinity of virus particles for ligands including antibodies, carbohydrates, negatively charged magnetic particles or receptors involved in cellular attachment. For example, the selective properties of antibodies may be used in immunomagnetic-capture PCR, promoting detection of viral genome recovered from intact viral particles (Jothikumar et al. 1998). Synthetic HBGAs have also been used as capture ligands (Knight et al. 2012). However, the development of broadly reactive assays is complicated by occurrence of non-specific binding, different antigenic epitopes amongst human NoVs and limited information regarding which receptors and binding conditions are appropriate.

7.2.3 qRT-PCR with intercalators

Nucleic acid intercalating agents can cross-link RNA forming stable monoadducts which cannot be amplified by PCR. However, these agents cannot penetrate intact viral capsids. Therefore, pretreatment with intercalating agents, including ethidium monoazide and propidium monoazide, should prevent qRT-PCR signal generation from damaged capsids. However, a region of the genome with extensive secondary structure is required for interaction with intercalating agents and finding suitable regions of the NoV genome which are also highly conserved has been challenging (Knight et al. 2012). The approach has not proven reliable in differentiating infective / non-infective bacteriophage T4 (Fittipaldi et al. 2010).

7.3 Conclusion to Section

The development of a suitable cell culture system for human noroviruses is aided by recent findings that NoV GII.4-*sydney* targets B-cells and infection is enhanced by presence of HBGA-expressing enteric bacteria. However, a broadly reactive infectivity assay is not yet available. The widely applied ISO method cannot distinguish between infective and non-infective virus. Though adjuncts to this

qRT-PCR approach may help in determining signal origin, all currently have limitations, caveats and no method represents a direct measure of infectivity. Although the ISO TS 15216 method does not discriminate infectivity, RNA fragments do not appear to accumulate in oysters (Dancer et al. 2010) and levels of norovirus RNA present in bivalve shellfish appear to be related to infectious risk (Lowther et al. 2010). Therefore the method is valuable in determining shellfish hygiene and predicting risk to human health. Currently the ISO TS 15216 method represents the most rigorously tested and data in this thesis should be comparable to that of the national and European reference laboratories for monitoring viral and bacterial contamination of bivalves molluscs.

8. Use of urrogate and indicator viruses in norovirus research

Research on the susceptibility of NoV to inactivation during wasterwater treatment / shellfish processing is complicated by the inability to propagate Human NoV *in vitro* or to differentiate between infective / non-infective virus. This, combined with a need to understand persistence, inactivation and transmission, has led to the use of surrogates including Murine NoV (MNV) and Feline Calicivirus (FCV) (Cannon et al. 2006; Bae & Schwab 2008). However, these surrogates may not accurately mimic the inactivation characteristics, or the behaviour in shellfish, of NoV. FCV is transmitted via the respiratory route and is likely to be less acid tolerant than human NoV which must survive low pH conditions in the human stomach (Buckow et al. 2008). Human NoV appears to persist in shellfish longer than FCV during depuration (Ueki et al. 2007). MNV-1 is possibly a more useful surrogate due to greater genetic similarity to Human NoV (Bae & Schwab 2008) and ability to survive under gastric pH (Cox et al. 2009). It replicates in small animals and its infectivity may be determined by cell culture therefore providing a very useful model. However, despite being shed in mouse faeces similar to Human NoV, MNV demonstrates different clinical presentations in mice including hepatitis, pneumonia and inflammation of the nervous system (Wobus et al. 2006). Although caliciviruses share many characteristics including particle and genome size and structure, there are considerable differences in capsid sequence (de Roda Husman et al. 2004). Information derived from studies using surrogates for Human NOV should therefore be considered presumptive (Richards et al. 2010).

The above surrogate candidates share the disadvantage of being absent in typical environmental / food samples. Therefore experiments concerning shellfish rely upon seeding practices, where the surrogate virus must be artificially incorporated. This may not be representative of the behaviour of naturally accumulated viruses and may not be possible during e.g. investigation of a commercial process. In this context, viral indicators (e.g. male-specific (F+) bacteriophages) offer advantages. F+ bacteriophages are bacterial viruses originating from the faeces of humans and warm blooded animals and are present in high numbers in sewage and (Wolf et al. 2007). F+ bacteriophages have similar

genomic and physical structures to NoV, being single stranded RNA viruses with a capsid size ~27nm (Doré & Lees 1995). They have been proposed (and in some cases adopted) as indicators of viral pollution in the marine environment and of contamination of BMS, where they appear to more accurately indicate the likely presence of human enteric viruses than *E. coli* (Doré & Lees 1995, Doré et al. 1999, Formiga-Cruz et al. 2002). The winter-biased seasonality in levels appears to coincide with that of NoV and with with increased viral risk associated with shellfish harvested in Winter (Doré et al. 2003). Because the genotypes are associated with different hosts, genotyping allows human and animal sources to be distinguished providing useful information for microbial source tracking (Wolf et al. 2007). F+ RNA bacteriophage is used in chapter 2 to compare uptake characterisitics in *Mytilus edulis* with those of NoV GI and GII. In chapter 6 F+ RNA bacteriophage is used as an indicator of viral infectivity after cooking *Mytilus edulis.*

9. Shellfish processing options

There are post-harvest options to reduce shellfish-vectored virus risk but these may be less effective than potential control measures applied during primary production, pre-harvest (Campos & Lees 2014). Shellfish safety can be improved using one, or a combination, of the processing techniques. No currently available method guarantees total virus inactivation/elimination without impacting organoleptic qualities or failing to meet demand for raw / live product. Multiple methods may however have additive advantage (Richards et al. 2010).

9.1 Depuration

Depuration is the practice of extending the filter feeding process of bivalves, in clean seawater in order to purge microbiological contaminants. The practice originated over a century ago (Herdman & Scott 1896). Depurated shellfish can be sold live and organoleptic characteristics remain unaffected (Lees et al. 2010). The process is subject to legal control in the E.U. under Directive 853/2004 (Anon 2004b) (See section 4.1). Disinfection of recirculated water by UV irradiation, ozonation, chlorination or addition of iodophores serves to inactivate any pathogens purged from the animals to prevent re-uptake or cross-contamination (Doré 2003). The process is optimised for the removal of *E. coli* but improved virus detection methods allow the optimisation for specific pathogens to be investigated (Lees 2000). *E. coli* and most bacteria can be effectively removed from various shellfish species by depuration but the removal of viruses is problematic (Ueki et al. 2007; Nappier et al. 2008; Power & Collins 1989; Muniain-Mujika et al. 2002). Variation exists in the findings of different authors and this may reflect numerous parameters which should be controlled (Richards et al. 2010). Shellfish take considerably longer to purge NOV than FIB when transferred to uncontaminated waters (Campos & Lees 2014). Strong epidemiological evidence links outbreaks of viral illness with consumption of depurated shellfish (Grohmann et al. 1981; Chalmers & McMillan 1995; Perrett & Kudesia 1995; Ang 1998; Conaty et al. 2000).

Rates of viral elimination may vary between species e.g. The T_{90} value for elimination of male specific F+ bacteriophage in mussels and oysters naturally contaminated over a short period was 47.3 and 54.6 hr respectively. In animals exposed over a longer period the difference in T_{90} for mussels and oysters was larger (41.3 and 60.8 hr) (Doré & Lees 1995). The T_{90} for *E. coli* was 6.5 hr or less in all cases. Responses to factors affecting depuration may also be species-specific. Salinity of depuration water affected the retention of NoV in *C. virginica* but not *C. ariakensis* (Nappier et al. 2008). Early studies of depuration efficacy for virus risk reduction attended to the concurrent pathogens of concern. Moderate levels of Poliovirus (PV) could be purged from *C. virginica, C. gigas, M. edulis, M. mercenaria* and *M. arenaria* in about three days (Mitchell et al. 1966; Hamblet et al. 1969; Power & Collins 1989; Hoff & Becker 1969; Liu et al. 1967b; Liu et al. 1967a). *Venerupis philippinarum*, *Tapes japonica*, *Tapes philippinarum* and *Ostrea lurida* appeared to require longer periods (Hoff & Becker 1969). However, some factors may not have been controlled between those experiments.

Enteric viruses are eliminated from shellfish more slowly than most bacteria but may differ in rate (Doré & Lees 1995; Richards et al. 2010; Bosch et al. 1995). Poliovirus was eliminated more rapidly than NoV or HAV in Pacific oysters (Mcleod et al. 2009a) and more rapidly than HAV in mussels (Enriquez et al. 1992). Typical depuration times to achieve *E. coli* standards are two days (Richards et al. 2010). Reductions in infectious HAV and rotaviruses of 98.7% and 97.0% can be achieved in mussels after 96 hr but the viruses were not completely eliminated (Abad et al. 1997). Enriquez et al. (1992) and De Medici et al. (2001) showed residual infective HAV remaining in *Mytilus edulis* after 7 d and 5 d depuration respectively. Kingsley & Richards (2003) demonstrated persistence of infectious HAV in oysters during depuration for 3 weeks and detection of PCR signal for 6 w. Residual contamination is important because enteric viruses may be infectious at low doses.

Specific binding of virus to shellfish ligands may help explain difficulties in eliminating NoV (Le Guyader et al. 2012; Maalouf et al. 2010a; Tian et al. 2007). Depuration for 48 hr resulted in <7% reduction in NoV concentrations in oyster and clam (Schwab et al. 1998). Grossly contaminated shellfish may be rapidly purged of high coliform concentrations (35,000 / 100 g – 330 / 100 g, 72 hr) but after 15 d extended depuration with UV disinfection, be implicated in outbreaks of presumptive NoV (Heller et al. 1986). Heavy rain and flooding resulted in elevated faecal contamination in a shellfish production area during a period of high gastroenteritis prevalence. Prolonged depuration, to achieve European regulatory *E. coli* standards, did not prevent >205 shellfish-vectored human enteric viral illnesses including mixed infections (Le Guyader et al. 2008). Therefore shellfish exposed to unusually high levels of faecal contamination should not be considered for depuration.

Extending the depuration time period is recommended for enhancing removal of viruses in typically contaminated shellfish (Doré & Lees 1995; Muniain-Mujika et al. 2002). Metabolic / physiological

condition of shellfish may also be a factor affecting depuration of virus and process temperature appears to be important (Doré et al. 2010). A temperature of 18-20°C was found to be optimal for removal of F+ bacteriophage from *C. gigas* (Doré et al. 1998). Depurators in the UK have been recommended to increase process time and temperature by the Food Standards Agency and Shellfish Association of Great Britain but seawater in commercial depuration tanks is often not heated. However, there is contrasting evidence regarding improved NoV elimination at elevated temperature (Neish 2013). No significant reduction in NoV concentration was observed during depuration of *C. gigas* for 14 d at 8 °C and at 16 °C 59% of the initial concentration remained after 14 d. The effect of elevated temperature was considered to be smaller than expected and is inadequate (Neish 2013). NoV persisted in *C. ariakensis* oysters under depuration for 29 d at 20 °C across a range of salinities (Nappier et al. 2008). Possibly the effect of increased temperature upon elimination rates is smaller for some public health significant viruses than those in which the effect was observed. Poliovirus was depleted more rapidly than NoV or HAV during depuration at 20 °C (Mcleod et al. 2009a). Heating of the large volumes of water used in depuration may not be economically viable. The use of ozone during depuration is sometimes practiced but Neish (2013) determined that removal of NoV was not improved by ozone disinfection of water, nor direct contact with oysters compared to UV alone. The UV dose required to inactivate some viruses may be high but the major problem for viral depuration appears to be that some viruses (e.g. NoV) may be very hard to release from the shellfish tissues therefore they are not exposed to disinfection (Mcleod et al. 2009a; Ko et al. 2005; Souza et al. 2013).

Improper or poorly controlled depuration presents opportunity for post-harvest contamination e.g. where oysters were linked to illness but environmental contamination was not evident, depuration water supply was determined vulnerable to storm water contamination (Guillois-Becel et al. 2009). The effect of process temperature on depuration efficacy requires further study as do other physiologically important parameters including salinity, dissolved oxygen, food availability and condition of shellfish.

9.2 Relaying

Relaying of shellfish represents an option for improving microbial quality prior to harvesting (EFSA Panel on Biological Hazards 2012). Shellfish may be moved to cleaner areas for self-purification in a natural environment. In Europe, shellfish grown in Class C classified waters must be relayed for a period of 2 months according to directives 853/2004 and 854/2004 (Anon 2004a,b) (See section 5.2). Shellfish can also be relocated to very clean areas as an alternative to depuration. This typically takes longer than commercial depuration to achieve similar standards (Richards et al. 2010) In Europe, animals which have been relaid for this purpose must be demonstrated to meet Class A criteria before they are marketed (Anon 1991a) (See section 5.2).

Information regarding efficacy of relaying for virus elimination is limited and relaying is not widely practiced due to high economic cost associated with moving shellfish (Diagne et al. 2004). NoV was detectable in Pacific oysters 8 w after relaying in an area considered uncontaminated (Greening et al. 2003). But Doré et al. (2010) showed that a combination of relaying followed by subsequent depuration could be used to restore the virological quality of heavily contaminated oysters to typical levels and illness was not associated with these treated oysters (Doré et al. 2010). It can be assumed that information derived from studies of viral depuration of shellfish also have similar implications for relaying of shellfish, i.e. that virus is less rapidly eliminated than FIB when shellfish are transferred to clean water. Similarly, clearance of virus is dependent on water temperature and differences may be observed between species (Doré et al. 1998). Barriers to the widespread use of relaying include the availability of sufficiently clean waters which, in addition to predictable sources of contamination, may be impacted by boat waste discharge (Richards et al. 2010). Therefore potential recontamination cannot easily be ruled out both in studies and in practice.

9.3 Hydrostatic Pressure Processing

High Hydrostatic Pressure (HHP) processing, aka hydrostatic pressure processing (HPP) or pascalisation, has been used in food processing to inactivate microorganisms and enzymes causing food spoilage. It may also be used to inactivate pathogenic microorganisms. HPP treated shellfish appear slightly cooked due to partial protein denaturation but changes in texture and taste are usually minimal after the non-thermal treatment (Richards et al. 2010; Jay et al. 2005). However, increased lipid oxidation has the potential to impair flavour and nutritional value (Ohshima et al. 1993; Henry & Chapman 2002) HPP treated shellfish are accepted by some consumers but cannot be sold live (Kingsley et al. 2007). HPP denatures proteins, fortifies hydrogen bonds and disrupts non-covalent bonds (He et al. 2002; Brown 2007). Determination of HPP efficacy for unculturable viruses e.g. NoV is complicated and surrogates have been used.

HAV inactivation is dependent upon pressure and time. Kingsley et al. (2002) showed that 450 MPa maintained for 5 minutes could reduce a 7-$\log_{10}$ PFU / ml HAV stock to undetectable levels (Kingsley et al. 2002). In the same study FCV as a surrogate for human NoV could be inactivated by lower pressures but it is not clear how accurately FCV represents human NoV. Results of an RNase protection assay suggested FCV and HAV viral capsids remained intact and inactivation was associated with denaturation of capsid proteins without lysis. However, PV titre was unaffected by 5-min at 600 MPa (Kingsley et al. 2002). This suggests that while PV appears easier to purge from shellfish during depuration than NoV or HAV, it may be more resistant to inactivation by HPP. PV may be conferred resistance by an unusually large capsid wall thickness (Oliveira et al. 1999).

The shellfish matrix may introduce important factors not reflected by those experiments using virus stock. High salt content has been shown to decrease the effectiveness of HPP on inactivation of HAV and FCV (Kingsley et al. 2002; Kingsley & Chen 2008; Grove et al. 2009). Food matrix pH may also affect inactivation rates (Kingsley & Chen 2009). Virus may accumulate in different anatomical structures within shellfish possibly affecting HPP efficacy (Kingsley et al. 2007) It was shown that HAV bioaccumulated in oysters could be reduced by 1- 2- and 3-$\log_{10}$ PFU by 1-minute treatments at 350, 375 and 400 MPa (Calci et al. 2005).

Temperature also appears to affect HPP efficiency but in a virus-specific manner. HAV appears to be more susceptible to HPP at elevated temperatures whilst inactivation of FCV and MNV-1 appears to be more efficient at refrigerated temperatures (Kingsley et al. 2006; Kingsley et al. 2007; Buckow et al. 2008). Low temperature optimised inactivation is also observed in some picornaviruses (e.g. foot-and-mouth disease virus) but HAV appears to be an exception (Oliveira et al. 1999; Kingsley et al. 2006).

Although the ability of HPP to inactivate surrogates for NoV has been described, pressure inactivation thresholds have been shown to vary widely between closely related viruses (Kingsley et al. 2002; Kingsley et al. 2004). Once available, Kingsley et al. (2007) applied an infectivity assay for MNV-1, considered a more genomically similar calicivirus to Human NoV than FCV and found MNV-1 to be less sensitive than FCV to HPP. Leon et al. (2011) applied a human challenge study to investigate inactivation human NoV by HPP in oyster matrix. Oysters were not bioaccumulated but injected with an inoculum of 1000 genomic copies NoV GI.1 per participant portion and subjected to a range of HPP treatments or no treatment. RT-PCR analysis of participant stools demonstrated no infection in those who consumed oysters treated with 600 MPa for 5-minutes (6 °C). Infections occurred in those who consumed oysters treated at 400 MPa for 5-minutes (6°C or 25°C) at a rate which was not significantly different to those who consumed untreated oysters. This would suggest Human NoV is more resistant to HPP than suggested according to MNV-1 or FCV as surrogates. However, diverse human NoV strains may demonstrate differential sensitivity to HPP, as shown in HAV strains (Kingsley et al. 2004; Grove et al. 2008; Shimasaki et al. 2009).

In contrast with heat treatments, pressure is transmitted instantaneously and uniformly throughout a product (Knorr 1999). HAV inactivation did not differ between whole in-shell oysters and pre-shucked oysters at any pressure (Kingsley et al. 2009). The process separates the adductor muscle from the shell causing shells to open. Therefore HPP may be used as a combined food safety / shucking process. Although not suitable for shellfish intended for live sale, HPP is a viable method for reducing the risk of enteric virus infection after consumption of uncooked shellfish.

9.4 Irradiation

Irradiation techniques for inactivation of virus associated with shellfish have not been demonstrated to be reliable or commercially viable. Variability in reported results probably reflects factors including shellfish species or virus type studied, radiation used and exposure method, or variable characteristics of the matrix (Richards et al. 2010). Surrogate culturable caliciviruses for human NoV in suspensions containing high or low protein content can be inactivated by UV or gamma irradiation (de Roda Husman et al. 2004). MNV-1 is sensitive to UV and results of long range reverse transcription PCR suggest that UV causes damage to the human NoV viral genome which would render it inactive (Wolf et al. 2009). However, UV is insufficiently powerful to penetrate and inactivate viruses in shellfish tissue and is useful only for surface sanitation.

Jung et al. (2009) demonstrated the importance of matrix effects in showing that 90% of PV in PBS buffer, culture broth, and within oysters could be inactivated by 0.46, 2.84 and 2.94 kGy respectively (Jung et al. 2009). To inactivate 90% of HAV in *C. virginica* and *M. mercenaria* required doses of 2.0 and 2.4 kGy, respectively which resulted in minimal change to organoleptic characteristics (Mallett et al. 1991). However, the dose of gamma radition required to inactivate 90% of PV in in-shell *Crasostrea gigas* and *Ostrea lurida* caused the products to become 'unpalatable' (Girolamo et al. 1972). Harewood et al. (1994) reported that doses of 2.71 and 13.5 kGy were required for the inactivation of 90% of the bacterial pathogen *C. perfrigens* and of F-coliphage, respectively, in *M. mercenaria,* where >0.5 kGy resulted in significant shellfish mortality. In summary, UV irradiation is not suitable for improving the microbial quality of shellfish flesh and required doses of gamma irradiation impair organoleptic qualities and cause shellfish mortality, which subsequently cannot be sold live.

9.5 Cooking and Heat Treatment

Thorough cooking or heat treatments represent effective methods for reduction of viral load in food (Richards et al. 2010). However, several shellfish species are frequently consumed raw and others are often consumed only lightly cooked. Shellfish cooking methods adopted by many consumers are considered unlikely to inactivate human NoV (Hewitt & Greening 2006). Variable results are reported for thermal resistance of viruses in shellfish. Important parameters are likely to include the manner and duration of cooking and the maximum internal temperature achieved. Other factors are likely to include virus strain, food composition and shellfish species, where smaller shellfish may be easier render safe (Lees 2000). High protein and fat content matrices appeared to protect PV and HAV from thermal inactivation (Bidawid et al. 2000). But milk did not appear to protect HAV and MNV from inactivation (Hewitt et al. 2009).

An early experiment suggested that 7% of PV in *C. gigas* and *O. lurida* oysters could remain viable after 30-min steaming (DiGirolamo et al. 1970). But cooking at >85°C for 3-min was shown to have inactivated PV in cockles and raising the internal temperature of cockle to 85 - 90°C for 1-minute could inactivate HAV by >4$\log_{10}$ infectious units (Millard et al. 1987). In 2001, the Ministry of agriculture, Fisheries and Food recommended that an internal temperature >90°C should be maintained for > 90s when cooking bivalve molluscs (Waterman 2001). These parameters are adopted under legislation covering commercial heat treatments of BMS (See section 5.4). These parameters may not be achieved by traditional cooking processes in which it is common to use shell opening as a time reference when boiling or steaming mussels. Inactivation of HAV and rotavirus was incomplete when *Mytilus edulis* were steamed for 5-min after shell opening (Abad et al. 1997). Hewitt and Greening (2006) showed that steaming for 300 s only achieved 83°C mean internal temperature despite all *Perna canaliculus* (50) having opened. However, during boiling, *Perna canaliculus* reached an internal temperature of 90 °C after 170 s. All mussels (50) were open 40s later suggesting boiling should continue for approximately 50 s after shell opening to achieve 90 °C for 90 s. Directly comparing the processes showed that steaming for 180 s resulted in a mean internal temperature of 63 °C accompanied by 1.5-log decreased in infective HAV, boiling for the same duration achieved 92 °C internal temperature and total inactivation of HAV (Hewitt & Greening 2006). The authors subsequently recommended shellfish be immersed in boiling water for 3 min, and not steamed.

Regulatory cooking parameters were based on inactivation data for HAV. As NoV is unculturable and responsible for the majority of shellfish-vectored illness, surrogates have been used. FCV was shown to be more readily inactivated than HAV (Slomka and Appleton 1998). However, according to qRT-PCR data, HuNoV appears less susceptible to heat than either HAV or MNV (Hewitt et al. 2009). Although this cannot be confirmed by culture and may not fully reflect infectivity status, differences observed in the reduction in qRT-PCR titre suggests different degrees of RNA destruction and could be explained by variable susceptibility to heat.

In a multistate outbreak of NoV in the US, cooking of oysters did not appear to significantly reduce attack rate (McDonnell et al. 1997). But outbreaks of viral gastroenteritis associated with cooked mussels are rare in the UK and comparison with species eaten raw suggests that cooking reduces risk (Lees 2000). In an outbreak of HAV associated with clam consumption, the attack rate was 18% after consumption of raw clams, 7% after consumption of cooked clams, suggesting that cooking of clams reduced risk but did not eliminate it (Wang et al. 1990). There are limited studies applied to *Mytilus edulis* and these have investigated culturable HAV or rotavirus (Croci et al. 2005; Abad et al. 1997). Studies using surrogates for NoV have investigated MNV in clams (Toffan et al. 2014) or FCV in

Mytilus galloprovincialis (Croci et al. 2012). Further information is required for other shellfish species and may be derived using other methods to estimate reductions in NoV during cooking.

Other heat treatments include canning, which should provide sufficient heat to effectively sterilise food products and therefore canned shellfish should not present a risk of viral illness (Richards et al. 2010). Some consumers accept pasteurised and refrigerated oysters. However, RNase pre-treatment, to ensure RT-PCR detection of intact viral capsids, suggested that pasteurisation is insufficient to inactivate NoV in a food matrix (Mormann et al. 2010). Furthermore, many consumers prefer to purchase live shellfish.

9.6 Other Techniques

Live shellfish may be refrigerated and presented on ice, and non-live shellfish may be frozen, reducing replication of many bacteria and especially spoilage bacteria. However, neither assist the control of virus risk because viruses do not replicate outside of host. Furthermore, freezing is a laboratory practiced method to preserve virus (Richards et al. 2004). Freeze-thaw cycles may reduce virus infectivity but would provide inadequate protection to the consumer and damage shellfish quality (DiGirolamo et al. 1970; Richards et al. 2010).

The methods for smoking seafood are non-standardised. The heat applied is likely to vary between facilities and batches. Outbreaks of *Listeria monocytogenes* have been associated with smoked mussels (Brett et al. 1998; Baek et al. 2000). It seems likely that enteric viruses would survive typical smoking processes.

Acidification is used in the preservation and microbial risk abatement of some food products. However, Mormann et al. (2010) suggest that acidification would be insufficient to inactivate enteric viruses including NoV within a food matrix. Enteric viruses must tolerate the acidic environment of the gut to reach their site of replication. They may be acid resistant to <3.0 pH (Richards et al. 2010). Hewitt & Greening (2004) subjected mussels to a marinating process at pH 3.75 and, although FCV (a calicivirus transmitted by the respiratory route) was inactivated, NoV remained detectable and infectious HAV was recovered. MNV-1 appears stable across a wide range of pH (pH 2-10) and Cannon et al. (2006) report outbreaks of human NoV associated with highly acidic products including frozen raspberries and orange juice. There is some evidence of reduced viral infectivity after desiccation but this is unlikely to be important outside of the preparation of dietary supplements which may contain freeze-dried shellfish (Richards et al. 2010). Furthermore, many viruses are known to persist on dry surfaces.

9.7 Handling

Although the production and marketing of shellfish are regulated (See section 5), improper handling can result in contamination via hands, gloves, knives and surfaces. Contaminated ice and water used in storage and rinsing of shellfish have been epidemiologically linked to outbreaks of NoV and HAV (Richards et al. 2010). Re-immersing shellfish to revive before sale is not approved in the US or EU but has been practiced and linked to outbreaks of viral illness in both (Richards et al. 2010; Nenonen et al. 2009). This thesis focusses upon the contamination of shellfish during production and subsequent methods to control viral risk. Nevertheless, food products may also become contaminated during retail food preparation. Poor personal hygiene of infected food handlers can result in viral contamination of food products. Contact with contaminated hands appears to be the primary transmission mode and gloving and handwashing are most effective when practiced simultaneously (Mokhtari & Jaykus 2009).

10. Official Control Measures

The potential for shellfish to vector human pathogens is well recognised. In response, official control measures aim to reduce this risk. This section will review the directives and regulatory devices associated with controlling the risk of infectious human illness transmission via faecally contaminated shellfish. Other substances harmful to human health may be accumulated by shellfish but their respective control measures are outside the scope of this thesis. This section will cover the current control measures which attempt to prevent contamination during primary production, through the regulations applying to post-harvest processes and sale. Unfortunately, regulatory efforts can be compromised by illegal activities which may have an important role in outbreaks. Nenonen et al. (2009) detail an outbreak in which shellfish were harvested from an approved area but re-immersed in a harbour prior to consumption. This practice is not permitted (Anon 2004a). Other breaches of regulation associated with outbreaks include document fraud and harvest from unapproved areas. Oysters associated with outbreaks of viral illness have carried documents for an approved area, and fishermen have subsequently admitted harvesting from a forbidden area (Le Guyader et al. 2010). Because post-harvest processes may be ineffective or not well accepted the most effective strategies for reduction of viral risk can be developed in the control of harvesting area pollution (Campos & Lees 2014; EFSA Panel on Biological Hazards 2012; Maalouf et al. 2010b). The section will conclude with a discussion of potential future control options.

10.1 Initial Control of Harvesting Area Pollution

The European Community Shellfish Waters Directive 2006/113/EC superseded 79/923/EEC and sought to improve the quality of directly edible shellfish by maintaining and improving designated shellfish growing waters. The directive prescribed guideline values and minimum standards of water quality according to chemical, physical and microbiological criteria. The legislation was subsequently

incorporated within the Water Framework Directive, the aim of which is to combine the various aspects of water quality legislation for all water sources up to one nautical mile offshore. The Water Framework Directive applies a catchment-based approach rather than traditional administrative boundaries. This enables a holistic approach to ensuring that each catchment achieves a "good ecological standard" according to chemical, biological, and physical parameters by 2015 (Anon 2000; Kay et al. 2008; Stapleton et al. 2008). However, periodic monitoring programs provide less protection against intermittent spills, including storm water discharges, than they do against continuous discharges. Sewage infrastructure improvements may be more easily justified in areas where the shellfish industry is concentrated than those in which it is dispersed (Maalouf et al. 2010b). However, EU environmental quality legislation should support the balance of economic arguments in areas without concentrated industry. EU bathing water standards have also positively influenced expenditure on sewage infrastructure (Lees 2000). Sewerage infrastructure has improved in Europe and many parts of the world by investment but storm overflows remain a significant problem (Burian et al. 2000; Sato et al. 2013; Campos & Lees 2014).

10.1.1 Faeces from boats

As described in section 2.3, disposal of both untreated and treated human waste can cause significant contamination of shellfish. International Convention MARPOL 73/78 prevents discharge by ships of untreated sewage into inshore waters. In some circumstances treated sewage may be discharged but on-board treatment may not effectively remove enteric viruses. Since 2003 in Europe, newly built recreational craft must have provision to fit (or have fitted) holding tanks for waste (European Communities 2003). But European regulations do not ban overboard discharge and only some countries have introduced regulation against overboard discharge into coastal waters. In the US, the Clean Water Act does prohibit discharge of untreated waste into recreational waters and vessels with a toilet must be equipped with a Marine Sanitation Device. Nevertheless MSDs may not be effective in removing NoV (Campos & Lees 2014). The risk would be most effectively controlled by banning the overboard dumping of any effluent in the vicinity of shellfish waters (Campos & Lees 2014).

10.2 Harvesting area classification and mandatory processing

Bacteriological criteria are often employed to assess the impact of sewage on BMS and their growing waters. *Escherichia coli* (*E. coli*) is used as an indicator of faecal contamination within hygiene frameworks implemented both in Europe and the US (Anon 2004a; US/FDA 2011). In Europe, regulations 854/2004 (Anon 2004a) require that harvesting areas are classified according to levels of *E. coli* in shellfish tissue. Regulations 853/2004 state that gatherers may only harvest from these fixed and bounded areas (Anon 2004b) In England and Wales this is undertaken by the Centre for the Environment, Fisheries and Aquaculture Science (CEFAS) on behalf of the Food Standards Agency (FSA) (CEFAS 2009). These classifications specify acceptable levels of the indicator bacteria and

dictate the level of processing required before sale of animals from a particular production area (Table 4). New areas gain a temporary classification until they meet the criteria for long term classification. Monitoring is by monthly samples taken from a representative sampling point informed by the sanitary survey.

Table 4. Microbial Classification of Shellfish Harvesting Waters

Classification	Microbial standard	Information
A	≤230 *E. coli* per 100 g flesh and intervalvular liquid. Determined by 5 tube, 3 dilution MPN.	Live shellfish may be collected and sold for direct human consumption.
B	≤4,600 *E. coli* per 100 g flesh and intervalvular liquid in 90% samples. Determined by 5 tube, 3 dilution MPN.	Live shellfish may be collected but placed on the market for human consumption only after treatment in an approved purification centre or after relaying so as to meet the standards for class A.
C	≤46,000 *E. coli* per 100 g flesh and intervalvular liquid. Determined by 5 tube, 3 dilution MPN.	Live shellfish may be collected but placed on the market for human consumption only after relaying over a long period to meet the standards for class A.
	>46,000 *E. coli* per 100 g flesh and intervalvular liquid. Determined by 5 tube, 3 dilution MPN.	Harvesting not permitted

Adapted from EC 854/2004

The U.S. Sanitation Programme bases classification on *E. coli* in shellfish growing waters rather than shellfish tissue (WHO 2010). Either method is relatively cheap, standardised and widely available.

10.3 The depuration process

The depuration process is subject to legal control in the E.U. under Directive 853/2004 (Anon 2004a). The legislation stipulates that depurated shellfish comply with an end-product standard equivalent to the class A standard for shellfish sold for direct human consumption i.e. bivalve shellfish harvested from class 'B' areas (230 ≤ 4,600 *E. coli* per 100 g flesh in 90% of samples) must be purified in an approved depuration system and the end products must comply with the EC 854/2004 microbial standards of ≤230 *E. coli* per 100 g flesh (Anon 2004a; Anon 2004b). In England and Wales commercial depuration systems must meet the conditions of approval determined by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS). There are several generally approved systems which all employ UV irradiation for disinfection of recirculated water within a closed system (Lee et al. 2008). The CEFAS protocol for approval of depuration systems specifies minimum temperatures, salinities and dissolved oxygen levels according to species and in all cases depuration must be for a minimum of 42 hr (CEFAS 2010).

Importantly, compliance with bacterial standards after depuration may not reflect virological safety (Doré & Lees 1995; Schwab et al. 1998; EFSA Panel on Biological Hazards 2012). It is quite widely accepted that BMS can be successfully purged of *E. coli* and marketed in compliance with legislation whilst continuing to contain harmful levels of other potential pathogens (Power and Collins 1989, Muniain-Mujika et al. 2002, Nappier et al. 2008, Barile et al. 2009, Nappier et al. 2010). For example, depurated and compliant mussels from Italy and Greece have tested positive for infectious Hepatitis A (Chironna et al. 2002) while an oyster-vectored outbreak in Scandinavia caused NoV gastroenteritis in at least 356 patients despite oysters complying with all European faecal coliform standards (Christensen et al. 1998). The minimum approved depuration temperatures may be ineffective in reducing norovirus levels even over a prolonged period (Neish 2013).

10.4 Cooking / Marketing Regulation

The cooking parameters advised by the Ministry of Agriculture (90 °C, 90 s) are adopted under legislation: According to EU directive 853/2004, commercial heat treatments of category B or C shellfish must be approved (Anon 2004a). The conditions apply to shellfish sold as processed. Shellfish sold live are subject to different regulation and may be cooked or eaten raw at home or in a restaurant. Contrasting evidence exists regarding inactivation of virus under heat treatment (section 4.5) but significantly fewer illnesses associated with shellfish commercially cooked shellfish suggests the treatment and associated legislation are effective (Lees 2000; Richards et al. 2010).

11. Potential Future Control Options

The processing dictated by bacterial analysis has successfully reduced the occurrence of seafood vectored bacterial illness by protecting consumers from infection with sewage-derived bacterial pathogens (Lees 2000). However, viral contamination is not readily identified using bacterial indicators due to differences in behaviour during wastewater treatment, in the environment and within bivalve molluscs (Lees 2000; Lees et al. 2010; Schwab et al. 1998). A correlation has been observed between harvesting area classification (according to *E. coli*) and NoV levels in UK production areas. Furthermore, within sites, *E. coli* levels were shown to correlate temporally with NoV levels in shellfish and including temperature as a factor could predict NoV risk (Lowther 2011). However, the correlation between viral pathogens and FIB has often been demonstrated to be weak (Mena & Gerba 2009; Fong & Lipp 2005; De Donno et al. 2012). Therefore, the risk of NoV illness may not be contained by legislative standards based on FIB (Doré et al. 2010; EFSA Panel on Biological Hazards 2012). Contamination may occur after an accidental contamination event, rather than exposure to a continuous sewage discharge because beds near to major outfalls are unlikely to gain certification (Maalouf et al. 2010b). Faecal coliforms including *E. coli* may provide a good indicator of recent contamination events but if such events are missed e.g. by undersampling, the longer persistence demonstrated by some viral pathogens may not be reflected. This disparity means the use

of *E. coli* as an indicator of production area quality and end-product hygiene compliance may allow shellfish containing harmful levels of enteric virus to reach the market.

NoV is often detected in oysters harvested from class A and B waters (Doré et al. 2010; EFSA Panel on Biological Hazards 2012; Lowther et al. 2008). Those from class A waters may be placed directly onto the market and those from class B waters must first be depurated; but as currently practiced, this does not appear to effectively reduce NoV concentrations. Shellfish demonstrating compliance with the regulatory *E. coli* standard, following depuration in approved plants and after production in officially classified waters have been linked to outbreaks of viral illness (Lees 2000; Lowther et al. 2010).

Virological analysis is relatively expensive and difficult compared with bacteriological analysis but it is widely noted that current regulation does not assure protection of the consumer or producer (Pintó & Bosch 2008). Whilst NoV is the most common health risk associated with consumption of faecally contaminated shellfish, it is not always prevalent in the population when other viruses may present a risk. Therefore a number of viruses have been proposed as having potential for use as indicators of faecal viral contamination. Bacteriophages have been identified as candidates due to sharing some physical and genomic characteristics with human enteric viruses (Tartera & Jofre 1987; Tartera et al. 1989). They are abundant in sewage and relatively easy to detect (IAWPRC 1991). The distribution of male specific (F+) RNA bacteriophage is not restricted to human effluents but can be speciated to determine human or animal source (Hsu et al. 1995; Beekwilder et al. 1996). Levels of F+ bacteriophage have been shown to predict the risk of viral contamination more effectively than *E. coli* (Doré et al. 1998; Chung et al. 1998; Doré et al. 2000; Lees 2000; Doré 2003). In addition, FRNA Bacteriophage has the advantage that its infectivity can be determined using standard culture-based methods (Anon. 1995). However, its elimination kinetics under depuration appear to be different to those for NoV (Neish 2013).

The alternative to the use of viral indicators is the direct detection of human pathogenic viruses. Screening for all potential pathogens is not feasible and selection must be on the grounds of either being sufficiently prevalent in contaminating effluents in order to routinely reflect the presence of enteric viruses in general, or being of sufficient concern in isolation for monitoring to represent a direct benefit to public health.

The significant public health impact of NoV, and that NoV contamination can be quite readily detected and monitored has been recognised for some time (Henshilwood et al. 1998; Le Guyader et al. 1998). Development of an ISO standard method which is considered suitable for use in a legislative context has led to the proposed introduction of virological monitoring to the official

classification and control of shellfish harvesting areas (EFSA Panel on Biological Hazards 2012; CEFAS 2013). For this purpose, a requirement for information regarding the distribution of NoV contamination within BMS including the potential for differences between individuals, within batches and temporal/geographical variations acrossa harvesting areas was identified (Lees et al. 2010).

12. Conclusion

BMS including *Mytilus edulis* represent a vehicle for transmission of human enteric viruses, including NoV, when their growing waters are contaminated with human faecal material. NoV is the most common risk associated with shellfish consumption. The illness caused by HAV is more severe but its transmission via shellfish is rare outside of endemic areas. Illness associated with shellfish viral contamination is estimated to be at a large cost to society. Generally, post-harvest processing methods are ineffective or poorly accepted by the consumer, although commercial cooking regimes appear highly effective. Control of harvesting area pollution is complicated by the numerous routes for introduction of NoV into the marine environment and by site specific factors. A growing coastal population and climate change induced changes to rainfall patterns represent threats to risk control. However, recently standardised methods for quantification of NoV in BMS represent a valuable tool available to risk managers. Further information is required prior to the implementation of virological monitoring, which should enable the continued production of a high quality product of considerable nutritional value.

13. Key objectives of this thesis:

The thesis is presented as a series of manuscripts intended for publication and one published paper.

Objective One

- Determine the appropriate sample size for *Mytilus edulis* when applying the ISO method to with regard to the variability between individuals.

Inter-mussel variability in accumulation of NoV GI and GII and FRNA bacteriophage assessed during laboratory controlled bioaccumulation. Results presented in chapter two.

Objective Two

- Evaluate the spatial pattern of NoV contamination around a coastal sewage discharge.

Caged mussels experimentally deployed around a sewage outfall to investigate spatial patterns of contamination and the correlation with hydrodynamic model predictions. Differences between NoV and *E. coli* patterns investigated and results presented in chapter three.

Objective Three

- Assess the potential for offshore cultivation / relaying of *Mytilus edulis* to mitigate against viral and bacterial contamination

Caged mussels relocated from the Menai Strait to experimental moorings at 1 -12 km North of Great Orme for a total of 124 days. Time-series sampling for faecal indicator bacteria and NoV concentrations of shellfish tissues. Results presented in chapter four.

Objective Four

- Investigate the ability of a commercial depuration process applied to exported *Mytilus edulis* to improve their virological quality post-harvest

Commercially harvested mussels exported prior to depuration were intercepted and concentrations of NoV determined before and after depuration. A laboratory-simulated depuration allowed for *E. coli* analysis and extended depuration under similar conditions. Results reported in chapter five.

Objective Five

- Investigate the effectiveness of domestic cooking practices to reduce concentrations of infective virus in *Mytilus edulis.*

Cooking experiments conducted upon mussels contaminated with NoV and FRNA Bacteriophage to determine reductions in the concentrations of infective FRNA Bacteriophage according to plaque assay compared with NoV and FRNA Bacteriophage concentrations determined by RT-qPCR. Results presented in chapter 6.

Norovirus Contamination of *Mytilus edulis* Shellfisheries; Assessing the Threat to Human Health		
Chapter One	Introduction	
Chapter Two	Assessing ISO 15216 sample size when applied to *Mytilus edulis*	Manuscript
Chapter Three	Investigating spatial patterns of contamination around an outfall	Manuscript
Chapter Four	Assessing potential for offshore cultivation to reduce contamination	Component of desk study for LCRI
Chapter Five	Investigating effectiveness of a commercial depuration	Manuscript
Chapter Six	Investigating effectiveness of cooking	Journal of Applied Microbiology
Chapter Seven	General Discussion, Conclusions and Further Work	
Appendix 1	Inter-mussel norovirus variability in environmentally contaminated mussels	
Appendix 2	Site-Specific and Industry directed research	Reports
Appendix 3	Appendices for chapter three	Conference manuscript
Appendix 4	Training Undertaken	
Appendix 5	Participation and Industrial Engagement during this PhD	

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CHAPTER 2

Assessing the suitability of the ISO 15216 prescribed minimum sample size for norovirus analysis when applied to *Mytilus edulis*

Authors: J. Ben Winterbourn[†], John T. Flannery[‡], James Lowther[§], Shelagh K. Malham[·] and David L. Jones[†]

[†]School of the Environment, Natural Resources & Geography, Bangor University, Bangor, Gwynedd, LL57 2UW, UK

[‡]The Pirbright Institute, Ash Road, Pirbright, Surrey, GU24 0NF, UK

[§]CEFAS, The Nothe, Barrack Road, Weymouth, Dorset, DT4 8UB, UK

[·]School of Ocean Sciences, Bangor University, Bangor, Gwynedd, LL59 5AB, UK

Corresponding Author: James B. Winterbourn, Bangor University, Bangor, Gwynedd, LL57 2UW, United Kingdom

Tel. +44 1248 38 3052 Email: afpc58@bangor.ac.uk

Author Contributions: JBW conducted the experiment using FRNA bacteriophage propagated by JTF. JL invented the analysis of theoretical averages having applied a similar methodology investigating *Crassostrea Gigas.* JL shared spreadsheets which assisted the analysis and provided standards and control materials for the norovirus assay. JBW prepared the manuscript reviewed by JTF DLJ and SKM.

Keywords: Norovirus, mussels, intraspecies variability, RT-qPCR, FRNA bacteriophage, sampling protocol

Abstract

Contamination of shellfish by pathogenic viruses represents a major threat to human health; however, factors that control bioaccumulation by individual shellfish animals remain poorly understood. This is currently limiting our potential to formulate robust risk assessment guidelines for shellfish harvesting and end-product testing. The aim of this study was therefore to evaluate the inter-mussel variability in the accumulation of Norovirus (NoV) when exposed to marine water contaminated with Norovirus (NoV) genogroups I and II and FRNA Bacteriophage GA under laboratory conditions. Our results showed a log-normal distribution in viral accumulation. Some individuals demonstrated low levels of accumulation for all three viruses, suggesting that they were at a sub-optimal physiological state. There was no evidence of upward distortion of measured virus in pooled samples occurring due to the inclusion of mussels accumulating exceptionally high levels. The importance of pooling animals for analysis was, however, demonstrated due to the observation of a small proportion of animals accumulating low levels. Overall, the results are in general agreement with those obtained for viral accumulation in *Crassostrea gigas*. Based on our data, it appears that a sample of ten animals is sufficient to contain the effects of animal-to-animal variation in viral contamination for use in surveillance and monitoring. However, in some situations a larger sample may be appropriate to yield 2 ± 0.2 g of pooled digestive gland (DG) as recommended by the ISO method.

1. Introduction

Infection with norovirus (NoV) is the most common health risk associated with the consumption of faecally contaminated bivalve molluscan shellfish (BMS). *E. coli* is commonly used in risk management to indicate the presence of sewage contamination, however, this approach is unsuitable to reflect the risk from human enteric viruses (Lees 2000). Advances in molecular methods, which enable quantification of NoV genome copies in shellfish, provide new opportunities for assessment and management of shellfish-borne NoV risk. A molecular method has recently been adopted as an international standard (Anon 2013) and there is scope for official control of NoV concentrations in BMS sold for human consumption.

However, sampling protocols for risk assessment must be fit for purpose and demonstrated not to introduce bias if they are to be adopted in official monitoring programmes. Whilst official controls could be applied to all live BMS sold for human consumption, a greater amount of work has focussed upon *Crassostrea gigas,* a commercially exploited BMS which is often consumed raw. To establish robust sampling strategies for end-product testing or production area surveillance, further information regarding the method's practical application in *Mytilus edulis* is required. An important part of this requirement is to establish any significant between animal variability in NoV concentrations (Lees & CEN WG6 TAG4 2010) that may occur due to metabolic/physiological differences between individuals when simultaneously bioaccumulated under controlled laboratory conditions. Several studies have investigated between animal variability in NoV concentration for the commercially important filter-feeding BMS *Crassostrea gigas* (Lowther 2008; Rangdale 2007). It had been posited that the inclusion of one or more "hot oysters" (animals which for metabolic, physiological or spatial reasons may accumulate higher concentrations of virus particles) might skew PCR results. Generally, the authors found that although between between-animal variability could be detected, it was generally manifested in a small proportion of animals which accumulated significantly lower levels, and use of composite samples which pool together 10 oysters should provide a suitable model of a batch. Correspondingly, the ISO method stipulates that a minimum of ten BMS be pooled together prior to analysis (Anon 2013). The aim of this study was to determine whether this conclusion is also applicable to *Mytilus edulis* when the ISO method is applied and, in this context, whether between animal variability can adversely affect composite *Mytilus edulis* sample results. In the studies conducted by Rangdale (2007) and Lowther (2008), *C. gigas* provided sufficient material for comparison between individual half-glands and composite samples comprised of the remaining half-glands. However, *M. edulis* possess smaller digestive glands and this approach is not practical. The approach used here was to determine 5000 theoretical average concentrations for ten individually assayed mussels originating from a pool of thirty, and to consider the range of results. These were processed individually after simultaneous exposure to waters with homogenous

contamination. Additionally, a pool of ten digestive glands originating from the same bioaccumulation was processed according to the ISO method, for direct comparison with the averages and individual results.

This investigation simulated exposure of *Mytilus edulis* to NoV GI and NoV GII when grown using broadcast / bottom-culture methods. Additionally, animals were simultaneously exposed to high concentrations of F-specific RNA (FRNA) bacteriophage. FRNA bacteriophage demonstrates similarities in morphology and environmental persistence to non-enveloped RNA human enteric viruses including NoV (Grabow 2001) and has been used previously in the monitoring of shellfish beds to indicate the presence of enteric viruses (Doré et al. 2000; Flannery et al. 2009). In the present study they were used to provide an alternative measure of virus accumulation by *Mytilus edulis,* for comparison with data representing the two human NoV genogroups most frequently associated with shellfish-borne human illness, NoV GI and GII. The principle aim was to determine any significant mussel-to-mussel variability in viral concentrations after bioaccumulation. This allows investigation of whether the finding that ten oysters is suitable for routine viral contamination testing, is also applicable to mussels, or whether a different minimum number of animals for analysis as per ISO15216 is appropriate.

2. Methodology

2.1 Preparation of virus stocks

Human stool samples positive for either NoV GI.4 or NoV GII.4 were diluted 20% w/v with PBS. Genotype was determined according to sequencing of the capsid NS domain. *S. Enterica* serovar Typhimurium WG49 was used as host for the propagation of FRNA bacteriophage GA by broth enrichment (Anonymous 1995). Concentrations of FRNA bacteriophage GA, NoV GI and NoV GII determined by RT-qPCR in the resulting aliquots were 7.56 $\log_{10}$, 3.59 $\log_{10}$ and 6.76 $\log_{10}$ genome copies ml^{-1}, respectively.

2.2 Bioaccumulation experimental setup

Blue mussels (*Mytilus edulis*) (n=55) were collected from within a 1 m^2 quadrat on a commercial, intertidal, broadcast harvesting area at low tide before being washed and labelled with correction fluid. Animals were acclimatised in an aerated tank containing 10 l of UV disinfected seawater at 10 ±1 °C supplemented with 1.2 g yeast extract (Oxoid, UK) as a food source and recirculated at 450 l h^{-1} (total volume turnover approximately 80 s) using a Micro-Jet MC450 (Aquarium Systems, Sarrebourg, France). A sample of 10 mussels was analysed to determine viral concentrations after 24 h acclimatisation.

To simulate broadcast, bottom-culture methods employed locally, the remaining mussels (n=45) were distributed in a mono-layer on the raised floor of a second recirculating tank containing 10 l fresh seawater and 1.2 g yeast extract. This permitted equal access to surrounding water, which was also kept well circulated at 450 l h^{-1}. A volume of 3.33 ml of each previously prepared NoV GI and NoV GII faecal material suspension and FRNA bacteriophage GA culture were then added to the tank. Mussels were allowed to feed for 24 h at 10 ±1 °C. The location within the tank of each labelled animal was recorded before and after the accumulation.

Acclimatisation and bioaccumulation tanks were filled from the same seawater supply (salinity 36 ppt, dissolved oxygen, 8.1 mg l^{-1}).

2.3 Digestive gland preparation

Following bioaccumulation, 40 extant mussels were randomly selected and placed in individual plastic bags. Animals were considered to be in suitable condition if any exposed flesh reacted to touch; percussion on the shell caused shell closing or movement; shellfish closed of their own accord or remained tightly closed. These animals were then stored alive out of water (4°C, 2 d) before freezing (-20 °C, 7 d) prior to analysis. This was adopted after Neish (2013) observed that NoV levels in oysters could increase during a period of time following bioaccumulation and attributed this to the movement of NoV through the oyster digestive tract prior to removing the digestive gland for analysis. After thawing on ice, the bagged animals were randomly split into two groups comprising 30 and 10 mussels. For each individual, shell length and width were measured with Vernier calipers (Mitutoyo, Japan). For the group of 30 mussels, individual digestive glands were finely chopped using a sterile razor blade and weighed into sterile 1.5 ml tubes. For the group of ten mussels, digestive glands were pooled, finely chopped and a 2 g portion weighed into a sterile 50 ml tube.

2.4 Virus extraction from Mussel DG for RT-qPCR analysis

Each sample (individual or pooled digestive gland) was spiked with 10 µl of Mengo virus strain MC_0 to control for extraction efficiency. Then Proteinase K solution (100 µg ml^{-1}; 30 U mg^{-1}; Sigma-Aldrich) was added to the 2 g portion of pooled digestive (2 ml) gland and to each individual gland (500 µl). All samples were then incubated at 37 °C with shaking at 300 rpm for 1 h followed by incubation at 60 °C for 15 min. Following centrifugation at 3,000 ×*g* for 5 min, the supernatant was retained, the volume recorded, and stored at 4°C until RNA extraction was carried out (within 24 h).

2.5 Viral RNA extraction

Viral RNA was extracted from 500 µl of each individual mussel DG proteinase K extract and from 500 µl of each NoV positive faecal material suspension and FRNA bacteriophage GA culture using the NucliSENS® miniMAG® and magnetic extraction reagents (bioMérieux, France) according to the manufacturer's instructions. Each run included a negative extraction control (molecular biology

grade water) and RNA was eluted into 100 µl of elution buffer. RNA was stored at -80 °C until RT-qPCR analysis was undertaken (<7 d).

2.6 RT-qPCR assay for NoV and FRNA bacteriophage GA

A Life Technologies Quantstudio 6 instrument was used for RT-qPCR analysis. Twenty microlitres of the NoV GI, NoV GII, FRNA bacteriophage GA, or Mengo Virus reaction mix was prepared using RNA Ultrasense one-step qRT-PCR system (Invitrogen). The reaction mix consisted of 1 × reaction mix, 500 nM forward primer, 900 nM reverse primer, 250 nM probe, 1 × µl Rox and 1.25 µl of enzyme. Previously described primers QNIF4 (da Silva et al. 2007), NV1LCR (Svraka et al. 2007) and probe TM9 (Hoehne & Schreier 2006) were used for NoV GI analysis, and primers QNIF2 (Loisy et al. 2005), COG2R (Kageyama et al. 2003) and probe QNIFS (Loisy et al. 2005) used for NoV GII analysis. For FRNA bacteriophage GA analysis, previously described Levivirus genogroup II forward and reverse primers and probe were used (Wolf et al. 2008). Duplicate 5-µl aliquots of sample RNA were added to adjacent wells of a 96-well optical reaction plate in addition to no template controls and a standard curve. RT-qPCR inhibitors were controlled for each virus as previously described (Flannery et al. 2012).

NoV GI and GII reaction conditions were; initial incubation at 55°C for 60 min followed by 95°C for 5 min and then 45 cycles of 95°C for 15s, 60°C for 1 min and finally 65°C for 1 min. For FRNA bacteriophage GA, reaction conditions involved an initial incubation at 55°C for 30 min followed by 95°C for 5 min and then 45 cycles of 95°C for 15 s and 58°C for 1 min. NoV GI, NoV GII and FRNA bacteriophage GA were quantified by comparing the sample Cq values to the standard curves in copies per µl, and were then adjusted to reflect the volume of RNA analysed (expressed as genome copies g^{-1} DG). The LOD of the assay was determined as 20 detectable genome copies g^{-1} DG (gc g^{-1}).

2.7 Statistical analysis

Data was assessed for normality and virus concentrations were $\log_{10}$ transformed prior to correlation analysis and graphical representation using SPSS Statistics 20 (IBM Corp., Armonk, NY) and SigmaPlot software version 11 (Systat Software, Chicago, IL) respectively. Coefficients of variation were calculated from the untransformed data as CV = (SD x 100)/Mean.

2.8 Theoretical averages analysis

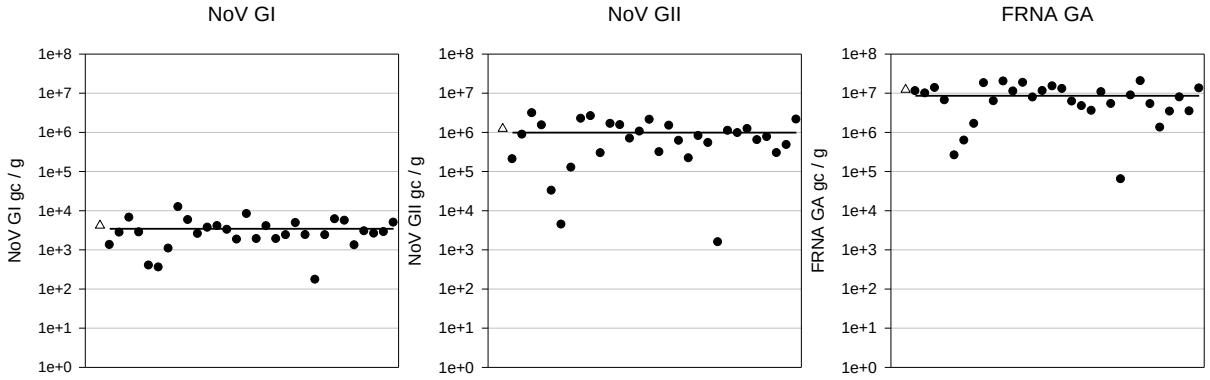
Ten individuals were randomly selected from the list of thirty and the average genome copies g^{-1} calculated until 5000 averages were available for each target. The distribution of these averages was compared with that of the thirty individuals and with the gc g^{-1} value determined for the pool of ten and graphically represented using SPSS Statistics 20 (IBM Corp., Armonk, NY).

3. Results

Concentrations of NoV GI, NoV GII and FRNA bacteriophage determined for a pooled sample of 10 mussels from the batch before bioaccumulation were <LOD, 1.12 and 1.20 log_{10} gc g^{-1}, respectively. After bioaccumulation, the concentrations of NoV GI, NoV GII and FRNA in the pooled sample of ten mussels were 3.62, 6.09 and 7.09 log_{10} gc g^{-1}, respectively.

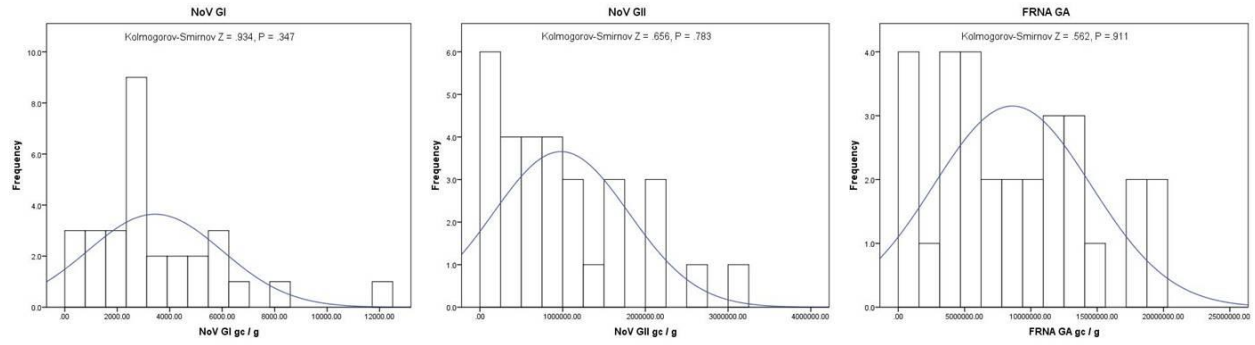
The inter-animal variation in NoV GI, NoV GII and FRNA Bacteriophage concentrations in bioaccumulated mussels (n=30) is shown in figure 1. The mean value for the thirty individuals and the value derived from the pool of ten mussels is also shown for each target. Kolmogorov-Smirnov tests accepted all three variables as consistent with normal distributions (P> 0.347 in all cases) (Figure 2). The variance for each target increased with the mean virus concentration (Figure 3).

Figure 1. Scatterplots showing gc g^{-1} values for thirty individual mussels and a single independent pool of ten mussels



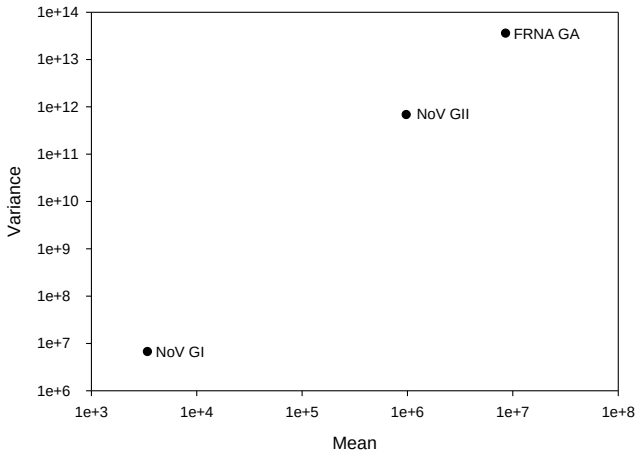
For each target, Solid dots show the gc g^{-1} value determined for individual mussels and solid lines shows their mean (n=30). Hollow triangles show the gc g^{-1} value for an independent pool of ten simultaneously accumulated mussels (n=1).

Figure 2. Histograms showing gc g^{-1} values for thirty individual mussels



For each target, histograms demonstrate the frequency distribution of concentrations in gc g^{-1}. Normal curve is shown for reference. According to Kolmogorov-Smirnov test, no target distribution is significantly different to the normal distribution (P> 0.347 in all cases).

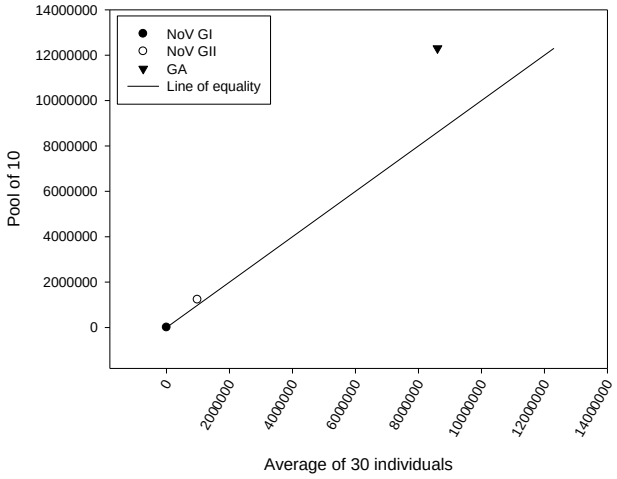
Figure 3. Comparison of mean gc g^{-1} values and variance for thirty individual mussels



The sample variance increased with increasing mean concentration of each target.

The viral concentrations derived for the pool of ten animals are compared with the mean of thirty individuals (Figure 4). The largest discrepancy between pool and mean values was observed for FRNA Bacteriophage, for which highest overall concentrations were observed. The pooled sample returned results 1.21-, 1.25- and 1.43-fold higher than the mean of thirty individuals, for NoV GI, NoV GII and FRNA Bacteriophage, respectively.

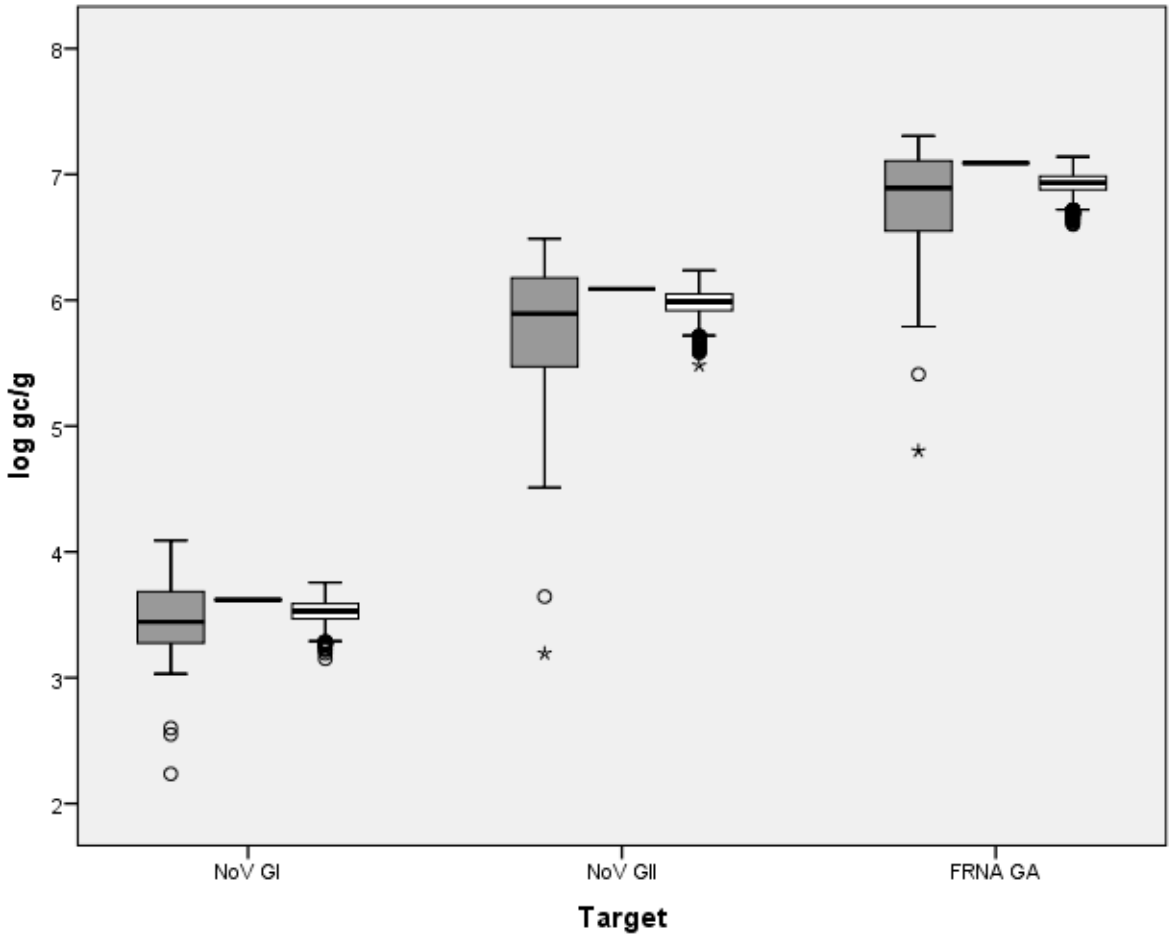
Figure 4. Comparison of gc g^{-1} values derived for a pool of 10 mussels with the mean of 30 individuals.



For each target, the concentration determined for a pool of 10 individuals is plotted against the mean of 30 individual results.

For each virus target, The $\log_{10}$ gc g^{-1} value determined for the pooled sample of ten individuals was compared with the distribution of data for the thirty individuals and with the distribution of 5000 averages generated for ten individuals (randomly selected from the same thirty individuals) (Figure 5).

Figure 5. Boxplots showing distributions for experimental and theoretical data for each target



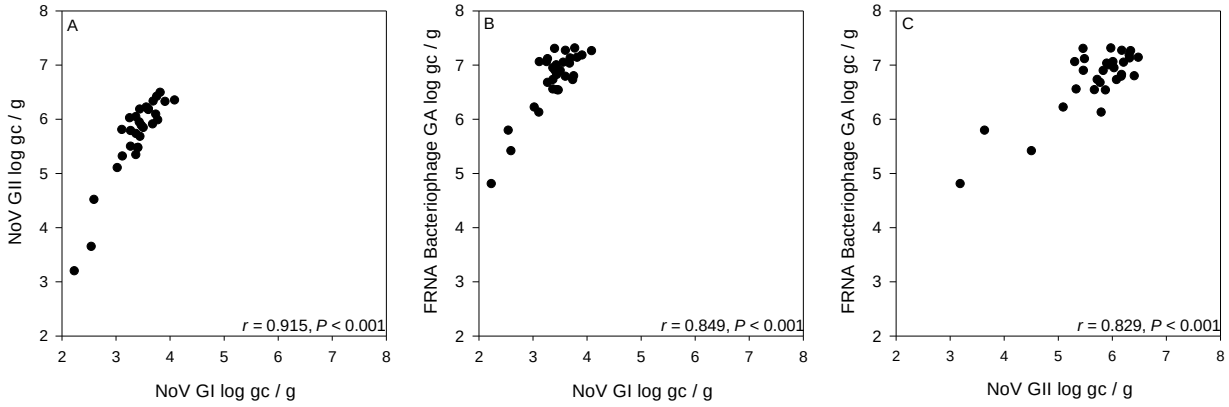
For each target, grey boxes show the distribution of data for the thirty individual mussels (n=30). Solid lines show the log_{10} gc g^{-1} value determined for the pooled sample of ten mussels (n=1). White boxes show the distribution of averages generated for ten log_{10} gc g^{-1} values randomly selected from the thirty individuals (n=5000).

For all targets, the data from individual glands (n = 30) approximates a log-normal distribution with outliers at the low end. For NoV GI, 10 % (n = 3) of the individuals were identified as outliers. For NoV GII and FRNA Bacteriophage, 6.7 % (n = 2) of individuals were identified as outliers. The same two individuals were identified as outliers for all targets. One further individual was an outlier of the GI concentration distribution. Figure 6 and Table 1 show that Pearson product-moment correlations indicate that highly significant positive associations exist between the target concentrations in the individual mussels. Concentrations of NoV GI, NoV GII and FRNA Bacteriophage derived for 30 individual mussels demonstrated CVs of 75%, 83% and 69% for raw (unscored) gc g^{-1} values, corresponding to log_{10}-Standard Deviations of 0.40, 0.76 and 0.57, respectively. Amongst 5000 randomly generated averages comprising ten from the list of 30, CVs were reduced to 19.4%, 21.7% and 17.9% for gc g^{-1} values, corresponding to log-standard deviations 0.09, 0.10 and 0.08, respectively. Some of the random selections of ten from the thirty continued to produce log_{10} gc g^{-1} averages which were outliers of the distribution at the low end, but their distance was much reduced.

NoV GI, NoV GII and FRNA Bacteriophage concentrations did not correlate with shell length (P = 0.163, P = 0.710, P = 0.494), shell width (P = 0.948, P = 0.765, P = 0.862), shell length × width (P = 0.466, P = 0.967, P = 0.777) or the mass of the gland (P = 0.380, P = 0.924, P = 0.570).

All samples passed extraction and RT-PCR efficiency action thresholds and efficiency values did not correlate with any virus target concentration (P > 0.466 in all cases).

Figure 6. Scatterplots of target correlation.



Comparisons of A) NoV GI and NoV GII, B) NoV GI and FRNA Bacteriophage C) NoV GII and FRNA Bacteriophage log_{10} gc g^{-1} concentrations in bioaccumulated mussels (n=30). Shown bottom right of each panel are highly significant results (P < 0.001 in all cases) of Pearson product-moment correlation.

Table 1. Pearson product-moment correlations between targets.

	NoV GI	NoV GII	Bacteriophage
NoV GI	-	-	-
	-	-	-
NoV GII	0.915**	-	-
	<0.001	-	-
Bacteriophage	0.849**	0.829**	0.779**
	<0.001	<0.001	0.002

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

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

Pearson product-moment correlations (r) and P-values.

4. Discussion

4.1 Experimental conditions

Initial concentrations of the target viruses in the mussels were low compared with those exposed to the viral accumulation. NoV GI was undetected before accumulation and increased by approx. 3.5 log_{10} gc g^{-1} following exposure. Environmentally accumulated concentrations of NoV GII and FRNA Bacteriophage were increased by approx. 5 and 6 log_{10} gc g^{-1} following exposure, respectively. Norovirus and phage titres correlated and were accumulated with similar efficiency. This supports the

use of FRNA bacteriophage as a potential indicator of viral contamination. Neish (2013) observed that NoV levels in oysters could increase during a period of time following bioaccumulation and attributed this to the movement of NoV through the oyster digestive tract prior to removing the digestive gland for analysis. For this reason, mussels were stored for 2 d at 4 °C prior to freezing. Animals used in this study originated from an area <1 m^2. Hence it is assumed that the observed variability between individuals is representative of uptake variability during the artificial accumulation and not significantly affected by variability in pre-accumulation concentrations. Byssal thread attachment occurred but the accumulation was brief, no clumping was observed and the water was well mixed, hence essentially equal exposure is assumed. The tank location of individuals did not provide any suggestion of positional effects because there was no discernible spatial pattern in the contamination levels of labelled mussels. There was no evidence of individual mussels accumulating exceptionally high levels of any virus. There was, however, evidence of a small number of animals accumulating only low levels, creating outliers at the low end. The potential for BMS in sub-optimal physiological condition to accumulate lower levels of NoV during exposure has been posited for *Crassostrea gigas* previously (Rangdale 2007; Lowther 2008). In the present study, low-level outliers occurred at a rate of ~10% which is consistent with findings reported by Lowther (2008) for *Crassostrea gigas* exposed in commercial oyster sacks. The poor accumulation of all three viruses suggests that these represent individuals in a poor physiological state. It is also consistent with the opinion that the pooling of ten animals should reduce the downward effect upon the pooled sample result to tolerable levels. This is demonstrated by the much smaller range determined for 5000 averages of 10 individuals from the population of 30, relative to the range for individual results. Nevertheless, the potential for a sample comprising animals which have been under physiological stress due to local conditions, to misrepresent a larger population should not be disregarded. In this study it was assumed that the average of ten individually processed mussels reflects the value which would be derived for a pooled sample. In the present study, the result determined for the pooled sample was slightly higher than the average of thirty individuals simultaneously accumulated, for all viral targets. For all targets, the result fell within the range described by 5000 averages of ten individuals, but was above the interquartile range of the corresponding distribution (Fig. 5). This may be explained by absence of animals containing unusually low concentrations amongst the pooled sample, or by technical advantages to processing the larger volumes of digestive glands which may be derived from pooled samples.

When the distribution of 5000 averages calculated for groups of ten from the 30 was plotted, some permutations continued to generate results which were labelled statistical outliers of the distribution on the low end (Fig. 5). These are permutations which contained one or several of the individuals with low virus concentrations but the effect upon the average was relatively small. Sample sizes less

than ten were not considered because there is a requirement to pool ten BMS for *E. coli* enumeration under Commission Regulation (EC) No. 2073/2005 (Anon 2005), because the ISO TS 15216-1:2013 method for quantification of norovirus in food stipulates ten BMS (Anon 2013) and because fewer than ten animals is likely to provide a smaller volume of digestive gland material than recommended by the ISO method (2 ± 0.2 g). According to Lobel (1986), a characteristic feature of metal uptake variability in mussels is that the variance shows strong positive correlation with mean metal concentration. A similar observation was made in this study which used different concentrations of each virus (Fig. 3). Therefore, variability has been described as percentage coefficient of variation (CV) to compare distributions with widely differing means. The variability between individual mussels exposed to virus in laboratory-simulated broadcast conditions in this study appears to be slightly less than that reported by Lowther (2008), for oysters in sacks. The data (Lowther 2008) shows that four sacks of 30 individual oysters returned CVs for NoV GI of 167%, 147%, 121% and 98%. In the present study, the CV for NoV GI concentrations in 30 individual mussels grown in laboratory-simulated broadcast conditions was 75%. For NoV GII in data from Lowther (2008) showed CVs for four commercial sacks of 93%, 134% 88% and 170%. In the present study, NoV GII CV was 83% for 30 individuals. Variability between individual broadcast cultivated mussels may be less than that between individual oysters grown in sacks but it is unclear whether this may be attributed to the species or the cultivation method simulated. Correspondingly, the CV for 5000 averages calculated for random selections of 10 individuals from 30 was less in the present study than that in Lowther (2008) after the same manipulation. However, the data should be compared cautiously not only because the simulated cultivation method and species both differ, but also because the mean concentrations of NoV GI and GII in individuals after accumulation were very much higher in the present study.

For all targets, the distribution of individual measurements was not significantly different to the normal distribution according to the Kolmogorov-Smirnov test. However, the data may be too few to properly reject the null hypothesis. Figure 2 shows the deviation from the normal distribution. When considering metal uptake in BMS, Lobel et al. (1989) noted that positive skewness is often observed in the frequency distributions of element concentrations in biological materials and that various authors have termed these distributions “log-normal”. Log transformation often makes distributions more normal and may reduce inequality of variance which is also important for many statistical tests. Lobel et al. (1989) found that conversion of data for individuals to logs rendered normal the frequency distribution for the majority of elements studied. Lowther (2008) was able to describe the data for concentrations of NoV GI and GII in individual *Crassostrea gigas* accumulated in sacks as “consistent with a log-normal distribution with outliers at the low end”. The same statement can be

made of the data generated in this study for NoV GI, GII and FRNA Bacteriophage concentrations in *Mytilus edulis* accumulated under simulated broadcast conditions.

4.2 Relationship to similar studies

Various studies have investigated virus uptake characteristics in batches of BMS. For example Enriquez et al. (1992) showed that the mussel *Mytilus chilensis* could concentrate Hepatitis A virus 100-fold from the surrounding water but only when food was present to stimulate filtration activity. Artificial bioaccumulations of virus by BMS are quite often performed for research purposes but variability in uptake between individulas has less often been studied. Intraspecies variability in NoV accumulation efficiency has been investigated in *Crassostrea gigas* by Rangdale (2007) and by Lowther (2008) and the results compared to the present study on *Mytilus edulis* previously. However, to the best of our knowledge, this is the first study to approach variability in virus accumulation between individual mussels.

BMS can accumulate a number of substances dangerous to human health and production and certification of a safe product is important for sustainable development (Saavedra et al. 2004). Numerous studies have measured the nature and causes of intra-animal variability in the accumulation of other waterborne contaminants in a range of shellfish species (Anandraj et al. 2002, Mubiana et al. 2006, Saavedra et al. 2004). Waterborne contaminants of bivalves may include pathogenic bacteria, viruses, metals and toxins originating from harmful algal blooms. However, the uptake and elimination of different contaminants are governed by widely variable physiological and biochemical processes and the results of studies concerning other contaminants may be of limited value in the context of this study. Furthermore, many studies have investigated individual variability in the context of biomonitoring, often with respect to metals in wild populations (e.g. the NOAA mussel watch programme). The underlying assumption of biomonitoring is that contaminant concentrations in bivalves can be related to bioavailable levels found in the surrounding environment (Wang 2001; Mubiana et al. 2006; Lobel et al. 1989). Less often have studies approached intra-species variability from a food safety perspective. In this context absolute levels in food product are more important than prediction of ambient levels in the environment. A dose-response for NoV RNA levels in shellfish has been suggested and absolute levels can be used to predict risk to human health (Lowther et al. 2012). The sampling needs and the complications faced by biomonitoring programmes may differ from those of a production area / end-product food safety programme. Saavedra et al. (2004) considered appropriate sampling strategies for monitoring metal levels in raft cultivated mussels for food safety. The authors were interested in the robustness of sampling strategies which pool animals, against problems associated with variability between individuals.

4.7 Caveats to the present study

The data presented here relates to a single tank-based bioaccumulation of 40 mussels and should be generalised with caution. Previous attempts to explore individual variability amongst mussels which were naturally contaminated in local broadcast-harvesting areas failed due to concentrations below the Limit of Quantification being detected (appendix I). Exploration of individual variability in replicate groups of mussels originating from several naturally contaminated sites will provide more conclusive information. The experimental system was designed to simulate the broadcast cultivation methods employed locally and it is not clear whether cultivation on rope, in sack or basket would differ. In the natural environment, other bathymetric, hydrodynamic or climatic factors may play a role in generating variability in virus uptake between individuals. Following bioaccumulation, NoV levels in oysters have been observed to rise during a period of time and this was attributed to movement of NoV through the digestive tract prior to removal of the digestive gland (Neish 2013). The rate of transport to the digestive gland may vary between individuals. It is not clear whether this phenomenon is significant in mussels. However, the mussels were stored live at 4 ºC for 2 d prior to freezing. The study used only one tank in which the animals were exposed simultaneously to NoV GI, GII and FRNA Bacteriophage. Hence, for each target there was only one exposure concentration. It would be valuable to repeat the experiment at a variety of tank concentrations for each target virus. This would help determine the potential for overlap in the range of concentrations recorded for individuals originating from different exposure levels and the ability of pooled samples to differentiate between contamination levels. Further research is also required to investigate the effect of virus association with other particles and the presence/absence of food upon uptake variability. There are also knowledge gaps regarding exposure periods, where factors leading to high variability may be particularly operative during brief exposure periods. For example, if transient perturbations of filtering activity can induce variability, then longer exposure periods may result in less variability than brief episodic exposure.

5. Conclusions

This study aimed to control environmental factors during viral accumulation in order to determine to what extent virus uptake variability between individual *Mytilus edulis* could compromise the quanitification of viral contamination in pooled samples. The experiment discovered a relatively wide quantity range for NoV concentrations accumulated amongst a batch of 30 market-size individuals after exposure to a controlled bioaccumulation. Further experimentation will be required to determine whether differential elimination rates for virus may also contribute towards individual variability in NoV loadings in BMS post-depuration.

The results do not support the presence of a significant effect of "hot mussels" containing high viral loads due to metabolic differences. The data suggest that batches for which samples comprising the digestive glands of ten *Mytilus edulis* are processed are unlikely to fail criteria in any future regulatory

context due only to the presence of few individuals containing unusually high levels. However, samples may contain animals in sub-optimal condition, having accumulated only low virus levels. Hence there is the potential for downward distortion of virus levels. But the occurrence of this phenomenon is likely to be rare. Animals determined to be in poor state should be excluded from sampling. This may be difficult, demonstrated by the fact that all animals in this study met the stated criteria and yet individuals accumulating low levels were present within the batch. However, the results of the study are consistent with previous work on *Crassostrea gigas*, where testing pools of ten effectively reduced the effect of a small proportion of animals accumulating very low levels. Although a relatively wide quantity range was observed for individuals, pooling the material from ten animals should minimise potential effect of animal-to-animal variation and give a representative result for a limited spatial area. A sample of ten *Mytilus edulis* should be suitable for surveillance and monitoring so long as 2 ± 0.2 g is provided. The Data also support the use of FRNA phage as an indicator of viral contaminantion by demonstrating similar uptake characteristics.

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CHAPTER 3

Use of *Mytilus edulis* biosentinels to investigate spatial patterns of norovirus and faecal indicator organism contamination around coastal sewage discharges

James B. Winterbourn,[†,*] Katie Clements,[‡] James Lowther,[§] Shelagh K. Malham,[‡] James E. McDonald, [‖] and Davey L. Jones[†]

[†]School of the Environment, Natural Resources & Geography, Bangor University, Bangor, Gwynedd, LL57 2UW, UK
[§]CEFAS, The Nothe, Barrack Road, Weymouth, Dorset, DT4 8UB, UK
[‡]School of Ocean Sciences, Bangor University, Bangor, Gwynedd, LL59 5AB, UK
[‖]School of Biological Sciences, Bangor University, Bangor, Gwynedd, LL57 2UW, UK

*Corresponding author. Mailing address: Environment Centre Wales, Bangor University, Deiniol Road, Bangor, Gwynedd, LL57 2UW, UK. Phone: +44 (0)1248 383052. E-mail: afpc58@bangor.ac.uk

Author contributions: JBW designed the experiment and conducted fieldwork and virological analysis. KC conducted bacteriological analysis using methods developed during her PhD. The Limit of Detection has not yet been determined. JBW performed spatial and statistical analysis and wrote the manuscript. JL provided standards and control materials for use in the norovirus assays. The manuscript was reviewed by all authors.

Abstract

Bivalve shellfish have the capacity to accumulate norovirus (NoV) from waters contaminated with human sewage. Consequently, shellfish represent a major vector for NoV entry into the human food chain, leading to gastrointestinal illness. Identification of areas suitable for the safe cultivation of shellfish requires an understanding of NoV behaviour upon discharge of sewage into coastal waters. This study exploited the potential of *Mytilus edulis* to accumulate NoV and employed the CEN method for quantification of NoV within mussel digestive tissues. To evaluate the spatial spread of NoV from an offshore sewage discharge pipe, cages of mussels were suspended from moorings deployed in a 1 km grid array around the outfall. Caged mussels were retrieved after 30 days and NoV (GI and GII), total coliforms and *E. coli* enumerated. The experimentally derived levels of NoV GI and GII in mussels were similar, with NoV spread from the outfall showing a distinct plume which matched very closely to a tidally-driven effluent dispersal model. A contrasting spatial pattern was observed for coliforms. These data demonstrate that coliform / *E. coli* concentrations do not accurately reflect viral dispersal in marine waters and contamination of shellfish by sewage-derived viral pathogens.

1. Introduction

Contamination of bivalve shellfish with norovirus (NoV) derived from human faeces represents a well-established human health risk (Lees 2000). According to the European Food Safety Authority (EFSA), production of shellfish in areas which are not faecally contaminated represents the most effective control measure for NoV, however, on-going microbiological monitoring regimes should still be implemented to ensure protection of consumers (EFSA Panel on Biological Hazards 2012). Traditionally, bacteria including coliforms and enterococci have been used to estimate the level of faecal contamination of water and / or shellfish and may be referred to collectively as Faecal Indicator Bacteria (FIB). In Europe, *Escherichia coli* (*E. coli*), a coliform species commonly found in the lower intestine of warm-blooded organisms, is adopted as the traditional indicator of faecal (sewage) contamination in shellfish and used for risk assessment and management (Anon 2004). However, studies have indicated that *E. coli* provides a poor indicator of the risk of NoV contamination. Reasons for this poor correlation include the different environmental persistence of viruses and bacteria in marine water and differences in their seasonal discharge pattern (Fong & Lipp 2005). *E. coli* may also be introduced to the environment from animal sources. Therefore, *E. coli* and NoV may originate from different sources and be conveyed into the marine environment via alternate routes, where they may be susceptible to different stresses. The current faecal indicator approach has repeatedly been demonstrated to inadequately reflect the risk from human viruses which are introduced from inadequately- or un-treated wastewater (e.g. adenoviruses, rotaviruses, hepatitis A virus; Ang 1998; Chalmers & McMillan 1995; Gill et al. 1983; Gerba et al. 1979; Griffin et al. 1999; Noble & Fuhrman 2001; Fong & Lipp 2005; De Donno et al. 2012).

Direct recovery and concentration of viral pathogens from coastal waters is problematic, often requiring large sample volumes and providing only a time-specific measure of contamination. However, bivalve shellfish have been shown to efficiently accumulate viral particles (Asahina et al. 2009; De Donno et al. 2012; Nenonen et al. 2008) and sensitive quantitative methods which detect NoV genomes in molluscan shellfish using molecular techniques (PCR) now exist (Lees & CEN WG6 TAG4 2010; Anon 2013). This offers the potential to use shellfish as an integrator of NoV pollution within both marine and estuarine environments. Further, due to their fixed location, they can be employed to provide a spatial map of viral pollutant flow from point sources.

The aim of this study was to improve our understanding of NoV behaviour upon discharge of sewage into coastal waters. Our first objective was to derive and compare the spatial contamination patterns for NoV genogroups one and two (GI and GII), *E. coli* and total coliforms about a long submarine offshore domestic sewage outfall (long sea outfall). Our second objective was to compare these field-derived spatial contamination patterns with those predicted from a tidally-driven effluent dispersal model. Beaches nearby to the long sea outfall are designated bathing waters and commercial

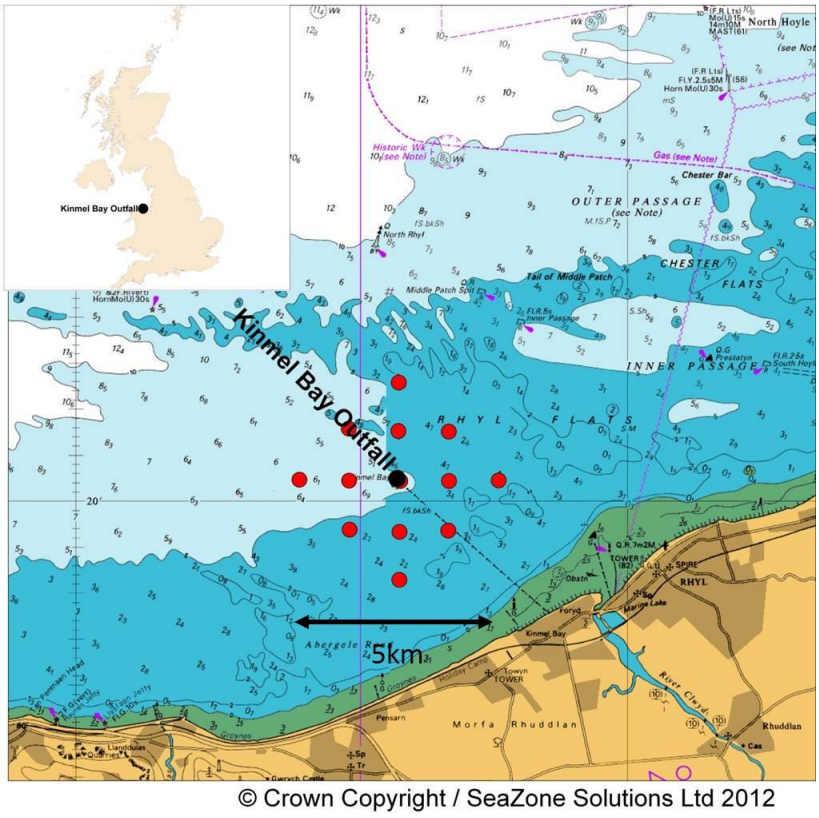
wind farms located offshore of the outfall have been identified by local industry for a potential shellfishery co-location. Therefore, our third objective was to relate findings to the suitability of the offshore wind farms for shellfish production and to nearshore bathing water quality. In lieu of EFSA advice to produce shellfish in waters which are not faecally contaminated and in lieu of studies suggesting that FIB may be a poor indicator of sewage-derived viral contamination; the specific intention was to detect any differences in the spatial contamination pattern for NoV, which might not be captured by the FIB approach.

2. Materials and Methods

Method overview. This study exploited the potential of the common (or blue) mussel *Mytilus edulis* (L.) to accumulate virions and bacterial cells from growing waters. This shellfish species was also chosen as it is commercially farmed on a large scale near the study area with the harvested product used solely for human consumption. NoV detection employed the European Committee for Standardization (CEN) approved method - a molecular method for quantification of NoV within mussel digestive tissues (Lees & CEN WG6 TAG4 2010). Culture methods were used for determination of bacterial Colony Forming Units (CFU) in line with the European Union Shellfish Water Directive (EU, 2006). In March 2012, an array of moorings was deployed, centred about the outfall of a wastewater treatment plant (WWTP). Caged mussels were re-sampled after 30 days.

Site selection. The offshore submarine sewage outfall pipe at Kinmel Bay, North Wales (53.336901N, 3.569200W (WGS84); Fig. 1), which serves a total population equivalent of 77,953 people, was selected for this study. The discharge is consented for up to 38,860 m^3/d with a dry weather flow not exceeding 15,941 m^3/d. Sewage released from the outfall receives only primary and secondary treatment (activated sludge). No ultraviolet (UV) or similar tertiary treatment is applied. Previous studies have indicated conventional activated sludge WWTP may achieve reductions for NoV GI and GII concentrations of less than one $\log_{10}$ genome copy (Flannery et al. 2012; Nordgren et al. 2009). In addition to treated effluent, under high flow conditions (i.e. stormflow) there are periods when storm water is discharged untreated into marine waters via this outfall, however, no such events were recorded during the duration of this trial. The outfall discharges into coastal waters of Liverpool Bay at 4 km offshore, in 6.9 m of water at Lowest Astronomical Tide, to achieve compliance with EU bathing water quality standards at proximate beaches. The conditions reported here are typical of many other discharge points around the UK coast. We hypothesized that these conditions could result in a significant release and persistence of potential human pathogens in marine waters. The impact of this outfall is of relevance to the local shellfish industry, being a point source proximate to an area identified for potential offshore shellfish production.

Fig. 1. Outfall Location and sample sites



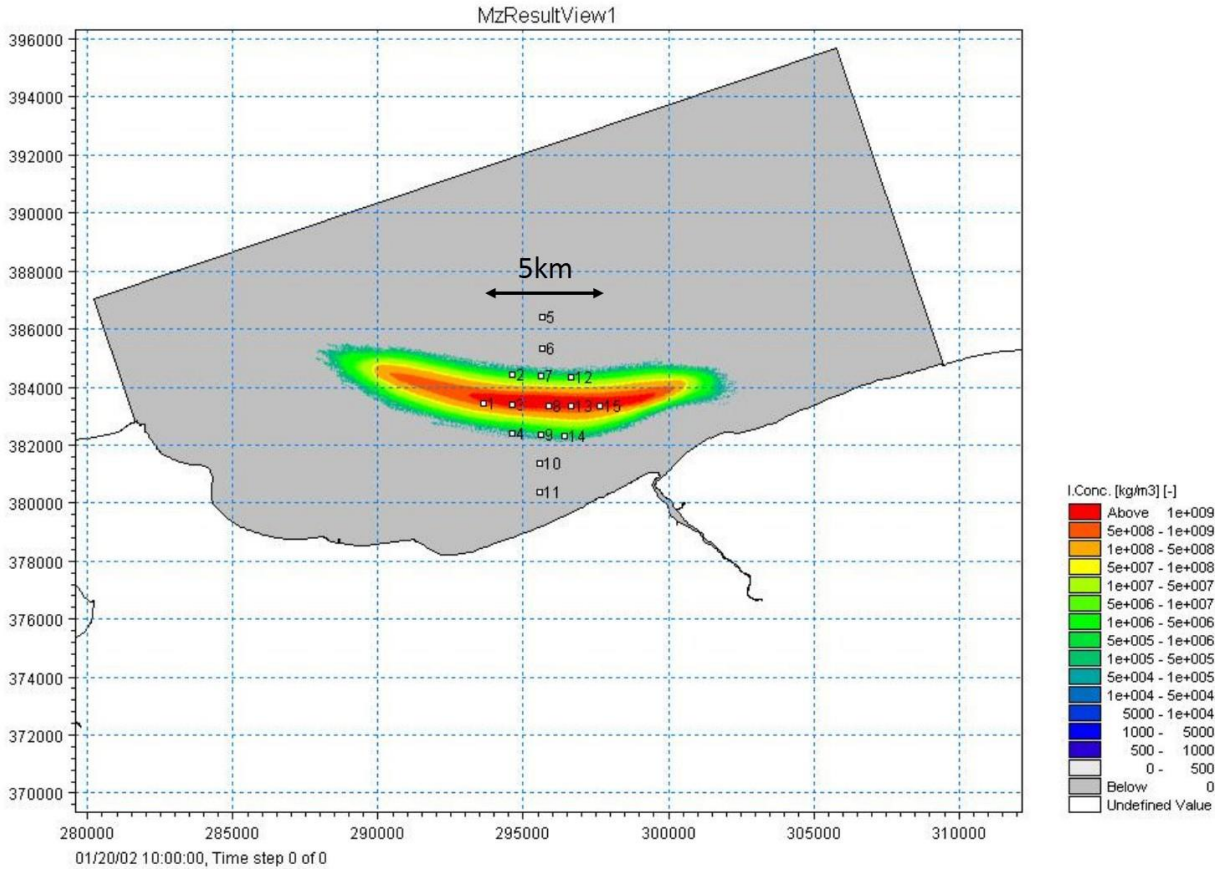


Figure 1. Outfall shown on N. Wales coast. Panel A (above) shows sample sites in diamond shaped array over local bathymetry. Panel B shows the summed concentration of a conservative pollutant which occurred in each cell over the duration of a model run.

Sampling Regime and Shellfish Biosentinels. A diamond-shaped array of 15 independent sampling points was selected based on model simulations of sewage plume behaviour (Fig. 1). The individual sample points were separated by 1 km in x and y dimensions. To minimise variability associated with growing conditions, *Mytilus edulis* were collected via a short trawl (<5 m) of broadcast-cultivated animals, from a commercial bed with an EU designated Class B (long term) classification. The animals were washed, size graded and 200 animals randomly selected for baseline enumeration of NoV and *E. coli* at time zero (T_0). Ten replicate samples of 10 animals were analysed for NoV and 10 replicate samples of 50 g shellfish flesh for coliforms and *E. coli*. Aliquots of 35 live animals of the same batch were then placed in net bags (300 × 300 mm). The net bags were placed in plastic cages and suspended at a sea depth of 1 m by attaching to a plough anchored Polyform A3 buoy. The cages were deployed on 12/03/12 and after 30 d the mesh bags containing shellfish were recovered.

Quantification of Norovirus in Mussels. NoV quantification in mussel digestive tissue was determined using quantitative reverse-transcription PCR (qRT-PCR) as described by Lowther et al. (2012a). Modification was made to the formation of the positive control and to the quencher used for the GII probe. In addition, aliquots of chopped mussel tissue were frozen (-20°C) and thawed once prior to Proteinase K digestion rather than being digested fresh or after short-term (24 h) refrigerated storage (4°C).

Briefly, homogenates were prepared by Proteinase K digestion of a 2 g aliquot of pooled digestive glands dissected from 10 animals, after Mengovirus vMC_0 was added as an extraction control. RNA extraction was performed with a Nuclisens® miniMAG® and magnetic extraction reagents (bioMérieux Inc., Durham, NC) following the manufacturer's protocol. The positive controls were derived from homogenates prepared as per the samples but after addition of 1 Lenticule® disc of Norovirus Reference Material for each genogroup (Public Health England, London, UK) to ten digestive glands. The animals used for the positive controls originated from extra animals placed within the experimental cages.

One-step qRT-PCR for Mengovirus (extraction control) and for both NoV genogroups, including plate layout, and reaction mixes, was performed exactly as described by Lowther et al. (2012a) but for the genogroup II assay, TAMRA was used as the quencher. The thermocycler used was an Applied Biosystems 7900HT (Life Technologies Ltd, Paisley, UK).

The use and treatment of a suite of qRT-PCR controls and all quantification steps also followed the same methods of Lowther et al. (2012a): Three aliquots of extracted RNA/sample were tested in each NoV genogroup-specific qRT-PCR assay, average quantities from three replicates giving overall quantity in detectable genome copies/g digestive gland (gen-c/g). Extraction efficiency and RT-PCR efficiency/inhibition were assessed using Mengovirus vMC_0 and RNA external controls, respectively. Retesting was undertaken according to action thresholds for extraction and RT-PCR

efficiencies of 1% and 25% respectively or due to failed positive/negative PCR controls. No adjustment for losses during processing or RT-PCR inhibition was made (uncorrected). This system was in agreement with the principles outlined in the draft Technical Specification developed by the joint CEN/ISO working group for standardisation of methods for detection of viruses in foodstuffs (Lees & CEN WG6 TAG4 2010).

Quantification of *E. coli* and Coliforms in Mussels. Bacterial colony forming units (CFU) were enumerated from shellfish flesh by direct plating onto selective agar as described in Clements et al. (2013). The method differs from the standard specified under legislation 853/2004 and is undergoing validation. The Limit of Detection is yet to be determined. Briefly, samples consisted of 5-10 individuals and only extant mussels were selected for analysis. Mussel samples were washed with sterile seawater to remove any residual sediment, debris and encrusting organisms before swabbing with 100% methanol to remove the shell surface biofilm. Samples were left for approximately 15 min to allow the methanol to fully evaporate. Mussels were opened aseptically and 50 g of flesh and intra-valvular fluid was obtained. Samples were homogenised for 60 s at 10,000 rev min^{-1} using a Bamix™ blender (Seal Rock Enterprises Ltd., Bishops Stortford, UK). From the resulting homogenate, 200 µL was plated onto Brilliance® selective agar (#CM0956; Oxoid Ltd, Basingstoke, UK) to determine *E. coli* and coliform counts. All plates were inverted and incubated at 37°C and bacterial CFU enumerated after 24 h. For T_0 n=10. In situ samples n=3 per site/month.

Statistical and geostatistical analysis. To ensure our data are comparable with survey data generated by the UK government National Reference Laboratory (Lowther et al. 2012a), samples returning "not detected" results for a particular NoV genogroup were assigned a score of 20 gen-c/g for that genogroup (half the limit of detection (LOD)). Samples giving positive results below the limit of quantification (LOQ; 100 gen-c/g) were assigned a score of 50 gen-c/g. Statistical analysis was carried out using IBM SPSS Statistics 20, graphs were prepared in Sigmaplot 12.3 and Geostatistical analysis and presentation was carried out in ArcMap 9.3.1 using the Spatial Analyst Extension.

Hydrodynamic Model. A hydrodynamic simulation model was already available which described the dispersion of the effluent plume from the Kinmel Bay offshore outfall. The model was prepared, using the Danish Hydraulic Institute (DHI)'s MIKE21 hydrodynamic and water quality modelling system, by Metoc (Intertek Ltd, Liphook, Hampshire) for Welsh Water (Dŵr Cymru Ltd, Treharris, UK) who are responsible for maintaining the Kinmel Bay WWTP and outfall. The model is property of Welsh Water and is used under permission. The model has a resolution of 45 × 45 m and encompasses 600 × 400 such cells. The model simulation was undertaken for a 3 day period, run under a calm wind scenario, with a model time step of 60 s and an output timestep of 10 min. The model predicted the effluent plume dispersal of a 1 m^3/s discharge released continuously over 12 h at a concentration typical of crude sewage (1×10^6 faecal coliforms/L). The model was designed to test

whether the offshore outfall had a significant impact on the bathing water at proximate beaches. As such the data used did not have a viral/bacterial decay rate associated with it, instead being run as a conservative pollutant. We considered this appropriate for our purposes as NoV is assumed to have a high environmental persistence. The summed concentration which occurred in each cell over the duration of the model run was recorded and graphically presented (Fig. 1). It describes the total number of bacteria predicted to pass through a cell over a model run. Therefore the measure is an amalgamation of all the modelled timesteps and does not denote a moment in time. The summed concentration for each cell which reflected the location of one of the experimental moorings was extracted and used as a predictor of relative exposure to contaminants originating from the plume.

3. Results

Baseline contaminant levels. Baseline levels for NoV GI and GII, *E. coli* and coliforms in mussels used to stock the experimental cages at T_0 are shown in Table 1. In 8 out of 10 replicates, GI NoV was detected at levels which were below the LOQ. These replicates were scored with 50 gen-c/g. GI was not detected in one out of the ten replicates, and was scored with 20 gen-c/g. Raw and scored NoV data are presented in supplementary information (Table S1).

Table 1. Baseline (T_0) Levels in Mussels Prior to Offshore Deployment.

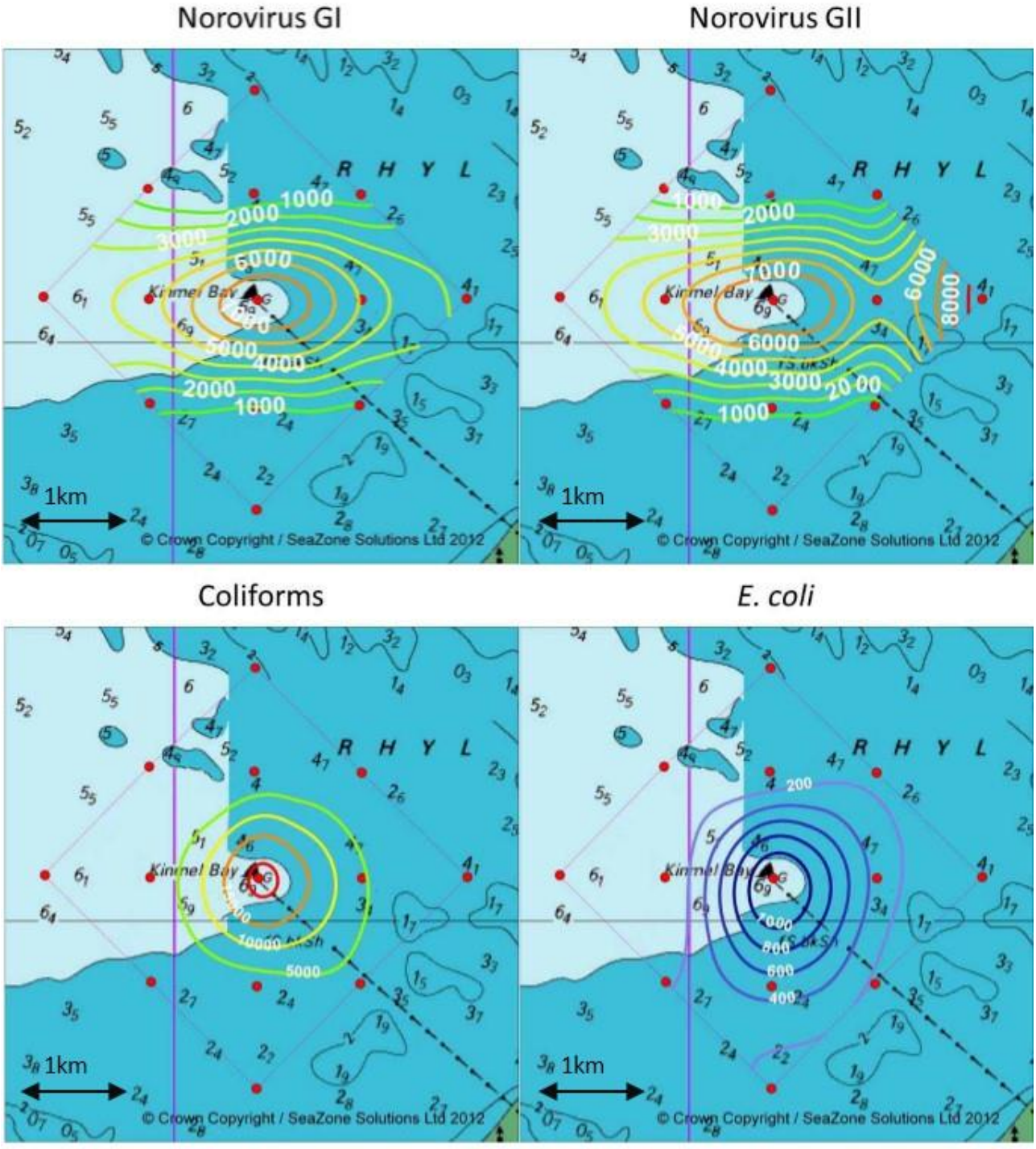
Target		Value
NoV GI	(gen-c/g)	52.2 ± 6.29
NoV GII	(gen-c/g)	3311 ± 167
E. coli	(CFU/100 g)	400 ± 163
Coliforms	(CFU/100 g)	3400 ± 670

NoV GI and GII are expressed as detectable genome copies/g digestive gland (gen-c/g). *E. coli* are CFU / 100g shellfish flesh and intravalvular fluid. Values represent mean and Standard Error of the mean n = 10 for all measures.

Norovirus and Bacteria in Mussels after 30 days. Levels of GI and GII NoV and indicator organisms in mussels after deployment around the outfall for 30 d are provided as on-line supplementary information (Table S2). After 30 d, GI NoV levels had increased from the T_0 baseline value of 52.2 gen-c/g at all sites except one at which it was not detected and two at which levels remained <LOQ. For GII NoV, levels increased from a T_0 value of 3312 gen-c/g at four adjacent sites within a row including the outfall site, and decreased at all other sites. Similarly, *E. coli* contamination of shellfish flesh increased in mussels directly over the outfall (approximately 3-fold from the T_0 value of 400 ± 163 to 1167 ±166 CFU/100 g) and decreased to undetectable levels at 5 sites. The coliform content of the mussels increased approximately 6-fold when placed directly over the outfall (3400 ±

670 to 20,833 ± 1764 CFU/100 g) and decreased at all but four sites where there was no significant change. The spatial patterns of NoV and coliforms / *E. coli* around the discharge point, however, were very different from each other with NoV showing much greater dispersion to the East and West and symmetry about the outfall. Contour plots were prepared to demonstrate spatial contamination patterns for NoV and FIBs in mussel samples (Fig. 2). The plots were prepared in ArcGIS. Each mooring was represented by a sample point with *x, y* location and a *z* value describing concentration level for each measured contaminant. The interpolation tools in ArcGIS Spatial Analyst were used to derive the intervening values, thereby creating a surface from sampled data i.e. interpolation predicted values at locations that lacked sampled points. Of seven interpolation tools offered by Spatial Analyst at ArcGIS v9.3.1 the spline method was chosen. Spline is a deterministic approach which generates a smooth surface passing exactly through the input points (Childs 2004). This surface was used to plot contours at user-defined intervals for each contaminant. The marine buoy at Site 5 was lost during the 30 d deployment period and therefore site 11 was omitted from the plot for symmetry.

Fig. 2. Norovirus and indicator organisms in mussel samples collected in April



For NoV GI and GII, contours represent genome copies / g digestive gland, *n*=1. The base contour is 1000 gc / g. For Coliforms and *E. coli*, contours represent mean CFU / 100 g shellfish flesh and intravalvular fluid, *n*=3. The base contours are 5000 and 200 CFU / 100g, respectively.

Both GI and GII NoV results showed a pattern of contamination elongated to the East and West of the outfall. For NoV GI, levels decreased with distance in all directions from the outfall. But for NoV GII, the highest contamination levels (9958 gen-c/g) were observed at the most Easterly sample point, 2 km to the East of the outfall. For both genogroups, levels declined more rapidly to the North and South of the outfall than to the East and West. However, significantly higher NoV contamination was observed South of the outfall (onshore) than to the North. The mean level for three adjacent sites South of the outfall (4, 9, 14) was significantly higher than for three adjacent sites to the North (2, 7, 12) for both GI and GII (t-test $P = 0.014$ and $P = 0.020$ respectively).

For *E. coli* and coliforms the spatial contamination pattern was different. *E. coli* was detected at highest levels directly over the outfall but was not detected within the transect to the West nor the North of the outfall, being skewed East and towards the shore. Total coliforms were also detected at highest levels over the outfall, were also skewed East and slightly towards shore, but were detected at all sites.

Using the data for all sites, Spearman's rank-order correlation coefficients (r_s) and their significance (P) were calculated for the four measures and are presented in Table 2. Scatter plots are shown in supplementary information, Figure S1. The data indicates a strong and significant positive correlation between NoV GI and GII concentrations. Total coliforms and *E. coli* concentrations were also correlated. Correlation between total coliform and NoV GI concentrations was weakly significant but correlation with GII was non-significant. *E. coli* did not correlate significantly with either NoV GI or GII.

Comparison of experimental results with hydrodynamic model predictions.

Data failed the assumptions for regression analysis but Spearman's rank-order correlation coefficients (r_s) and their significance (P) were calculated between the model prediction and experimentally derived levels of NoV GI, NoV GII, *E. coli* and total coliforms (Table 2). Both NoV GI and GII showed strong correlations with model predictions, which were highly significant. However, neither *E. coli* nor total coliforms showed any significant correlation with the model predictions.

Table 2. Spearman's rank-order correlations between measures and model predictions.

	E. coli	Coliforms	NoV GI	NoV GII	Model
Coliforms	0.747**	-	-	-	-
	0.003				
NoV GI	0.296	0.601*	-	-	-
	0.326	0.030			
NoV GII	0.220	0.543	0.905**	-	-
	0.470	0.055	<0.001		
Model	0.217	0.349	0.779**	0.752**	-
	0.477	0.242	0.002	0.003	

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

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

Spearman's rank-order correlations (r) and P-values.

Experimentally-derived levels were plotted and compared with predicted relative concentrations according to the model for North-South and West-East transects passing over the outfall (Fig. 3). The relative values extracted from the model were normalised to the value at site 8 (outfall) for each measure. NoV (GI and GII) results showed a good agreement with the model. To the West of the outfall, and particularly for GII, predictions and experimentally-derived levels matched very closely (Fig. 3c). The model, however, predicted slightly higher levels than were experimentally-derived for the site 1 km East of the outfall for both genogroups, and for GII, predicted lower levels at the Easternmost site (Fig 3ac). Higher levels than those predicted by the model were also found 1 km to the South of the outfall for both NoV GI and GII (Fig. 3bd). The model overestimated the relative levels for *E. coli* and total coliforms both to the East and to the West of the outfall (Fig. 3eg). However, higher levels than the model would predict were found to the South (onshore) of the outfall (Fig. 3fh).

FIGURE 3

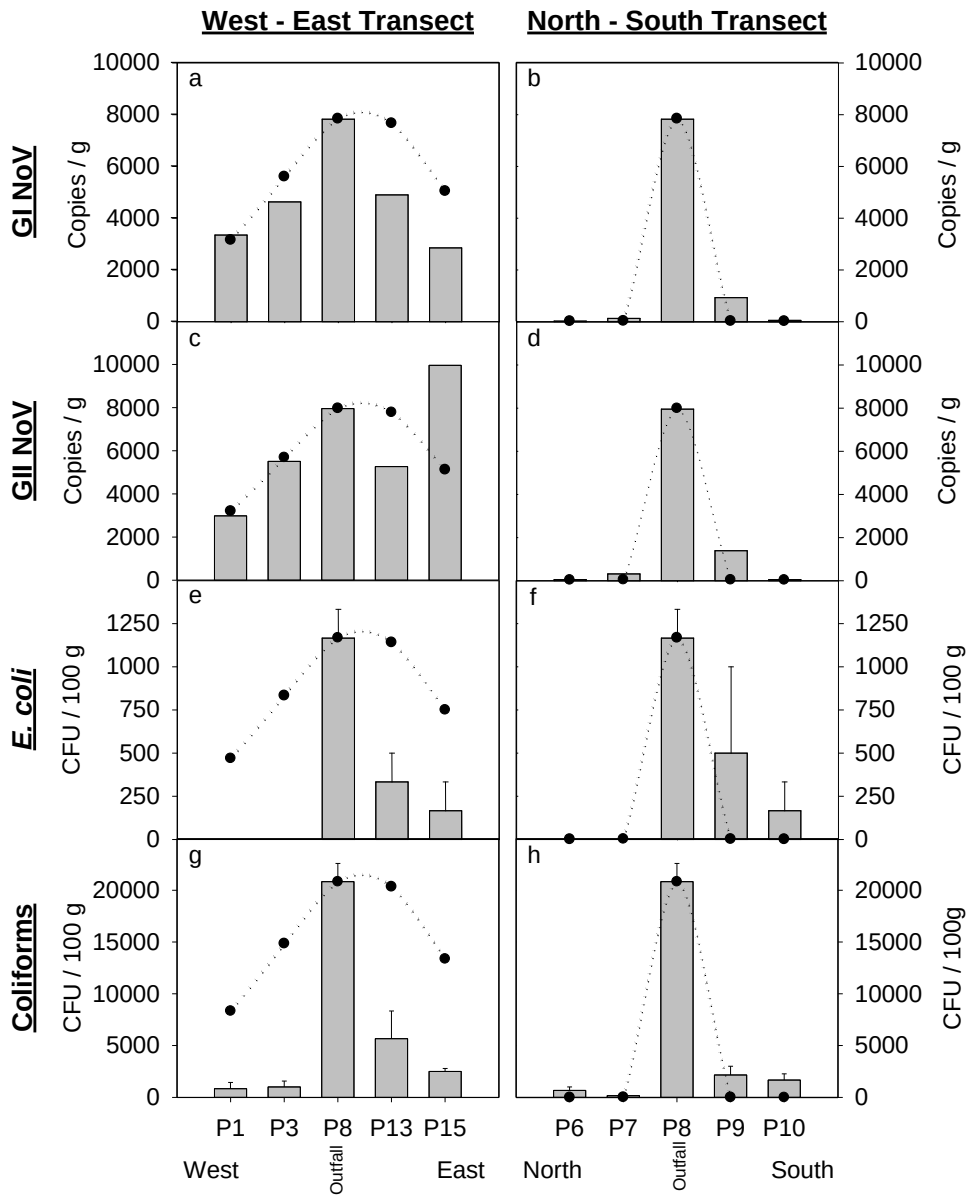


Figure 3. Distance between adjacent sites 1 km. For NoV GI and GII bars represent scored data as detectable genome copies/g (gen-c/g) digestive gland. Not detected scores = 20 gen-c/g. 1-100 (<LOQ) scores 50 gen-c/g. For *E .coli* and coliforms bars represent mean CFU / 100 g shellfish flesh and intravalvular fluid. Error bars represent Standard Error of the mean. *n* =3. Dotted lines show predicted relative concentrations extracted from the hydrodynamic model and normalised to the experimentally-derived value for site 8.

4. Discussion

Environmental context of study. It should be noted that the WWTP supplying the outfall selected for this study operates no UV or similar tertiary treatment and does not discharge directly into shellfish waters. Instead, the WWTP uses a long offshore submarine sewage outfall pipe to achieve compliance with the EU Bathing Water Quality Directive. This study investigated an older WWTP, operating only secondary treatment, to identify any impact upon an offshore area identified for potential shellfish production and particularly to illuminate any differences in the spatial contamination patterns for NoV and FIB. Therefore, the levels identified in experimentally deployed mussels should be interpreted within this context and may not be typical of levels which might accumulated by mussels in similar proximity to outfalls for tertiary treated effluents. However, it should also be noted that many coastal WWTP with tertiary treatment do periodically discharge untreated sewage under stormflow conditions, mostly in winter when the presence of NoV in wastewater is highest. From 2000-2005 the water companies investment programme, Asset Management Plan 3, included the microbial quality of shellfish waters as a specific driver for infrastructure improvements under the National Environment Programme. Improvements included UV disinfection of numerous continuous discharges. According to Campos et al. (2013) these investments have resulted in improvements to water quality in shellfish production areas.

Model. The simulation of hydraulics and hydraulic-related phenomena can assist research into problems which have become important and sensitive contemporary issues (Warren & Bach 1992). These include coastal engineering and pollution control. The model adopted by this investigation was constructed using MIKE 21 (DHI). The core component is the Basic Hydrodynamic Module (MIKE 21 HD), which simulates 2-dimensional free surface flows where stratification can be neglected, and provides the hydrodynamic basis for a range of modules built for specific purposes. These include Advection-Dispersion, Sediment Transport, Short Wave, Water Quality, Eutrophication and Heavy Metal modules. The combined value of these modules has been demonstrated in projects of great scope. As in the development of storm surge barriers to protect Venice from storm surges in the Adriatic Sea within a project legally required to have no detrimental effects on the lagoon ecosystem, navigation conditions or bathing waters at Lido and Cavallino (see Warren & Bach 1992).

The model output adopted for comparison with our experimentally-derived levels was used in the Rhyl Bathing Water Compliance Assessment which did not find the outfall to contribute significantly to non-compliance with the Bathing Water Directive, nor was it identified under solution options via asset improvement (METOC 2010). It used the advection-dispersion module MIKE 21 AD (DHI) and simulated the spread of the effluent plume, described by the concentration of a conservative pollutant discharged from the outfall and subject to forcing functions including tide and

river flows, and natural dispersion processes. It accounted for factors including local bathymetry and bed resistance coefficients. The concentration for each point of a grid for which currents and depths are provided by the Basic Hydrodynamic Module (Mike 21 HD) is calculated using the QUICKEST finite differences scheme (see Ekebjærg & Justesenu 1991). The correlation between model predictions and our experimentally derived pollutant levels is described below.

NoV contamination patterns. A relatively high T_6 value for GII NoV in mussels used to stock the experiment allowed for clear differentiation between sites where levels in resituated mussels increased (up to 3-fold) and sites where they decreased to levels below the LOQ (approx. 66-fold decrease; 3311 to <40 gen-c/g) suggesting that the pattern observed is representative of contamination in situ. Furthermore, spatial contamination patterns for GI and GII NoV were correlative except for a disparately high GII result at the easternmost site. Further work seeks to integrate the model presented here with that for the nearby Clwyd River (Fig. 1), into which sewage is also discharged. We speculate that the impact of the Clwyd River is of greater magnitude at Eastern sites and may contain a different GI/GII composition.

The most contaminated sites by either NoV genogroup all occupy the East-West transect through the centre point of the array, over the outfall, and concentrations declined steeply with distance both to the North and South. This finding is in visual agreement with hydrodynamic model predictions for the sewage discharge plume (Fig. 1) and coincides with a strong and significant rank order correlation between model predictions and experimentally-derived levels for NoV (both GI and GII). This finding contributes toward experimental validation of the existing hydrodynamic model of the effluent plume. Furthermore, it suggests that NoV is a persistent pollutant compared to FIB. Data failed the assumptions for linear regression. This is unfortunate because regression analysis might demonstrate that NoV levels can be predicted using either the sumplot or average effluent dilutions predicted by hydrodynamic models. Such a finding could help generate a very useful tool for determining shellfish production exclusion zones around other outfalls for which a hydrodynamic model is available e.g. a zone where mussels may be expected to accumulate >1000 NoV gc g^{-1}.

NoV impact upon nearshore bathing waters and offshore waters (identified for potential bivalve production) was not observed during the study period. Therefore, the pattern which was revealed supports continued investigation into the viability of production colocation with windfarms offshore of the study site which might allow for cultivation of NoV free mussels.

FIB Spatial Patterns. In contrast to NoV, agreement between model predictions and measured *E. coli* and coliform concentrations was less apparent; rank order correlations were weak and non-significant. Furthermore, whilst *E. coli* correlated with total coliforms and NoV GI correlated

strongly with NoV GII, the only statistically significant correlation between the FIB selected for enumeration and NoV was total coliforms with NoV GI and this association was not strong. Indeed NoV GI and GII were detected in mussels at very high levels at sites at which *E. coli* was not detected, notably to the West of the outfall. We are aware that the tidal current was flowing to the East at the time of sampling and therefore animals to the West are likely to have been less recently exposed to the effluent plume. This is consistent with evidence that FIB are an indicator of recent faecal contamination but NoV can persist longer in shellfish tissue. The water is deeper to the West of the outfall and a differential effect of water depth upon NoV / FIB behaviour is also plausible given potential association with particles and related sedimentation / resuspension phenomena. Importantly, all cages were suspended at 1 m below the surface rather than on the seabed.

Conversely, FIB were detected at sites at which NoV was not detected, with the distribution of FIB being somewhat more skewed towards the shore. We hypothesise that secondary non-point sources, which may be of animal origin, affect this pattern. Therefore, this study suggests that FIB indicate the presence of faecal contamination but may not accurately reflect persistent contamination by viral pathogens associated with human-sewage effluent.

NoV GI and GII ratios. T_0 baseline measurements of mussels used to stock the experiment showed that higher levels of GII than GI NoV had been accumulated from the production area prior to harvest. But after 30 d under experimental relocation, levels (in addition to spatial patterns) for GI and GII became strikingly similar at all sites excluding the easternmost. Influent/effluent samples were not available and unfortunately the explanation of this observation is outside the scope of the present study. However, potential factors affecting ratio include prevalence of GI:GII infection in the population, differential resistance to water treatment processes and environmental degradation, or differential shellfish accumulation efficiency by genotype. Therefore GI:GII ratios detected in the sentinels may be significantly different to those present in influent and effluent waters at the WWTP.

GI and GII NoV may have been received at the WWTP in influent at similar concentrations, undergoing comparable reductions and being discharged at similar concentrations during the period in which the caged mussels were in-situ. This scenario was observed temporarily during longitudinal monitoring by Flannery et al. (2012) and Nordgren et al. (2009). An alternative explanation is that GI and GII NoV were received at different concentrations but levels in treated effluent were similar due to differing resistance to the process: Da Silva et al. (2007) suggested that GI may be more resistant to WWTP processes than GII. Rajko-Nenow et al. (2013) reported a situation in which mean GII concentrations in influent were significantly greater than GI concentrations, but mean concentrations in effluent were not statistically different suggesting a greater reduction in GII concentration during treatment. Conversely, La Rosa et al. (2010) found NoV GI was more efficiently removed than GII. These observations suggest that GI:GII ratios may differ between influent and effluent and that

genogroup specific resistance may also depend upon treatment type and conditions. Other explanations relate to differential GI/GII behaviour post-discharge. Lysén et al. (2009) suggested GI NoVs may be more stable in the water environment. It has been shown that some NoV genotypes may accumulate more efficiently, in oysters, than other genotypes because of specific binding properties (Le Guyader et al. 2012). Human NoV infection is dependent upon Histo-Blood Group Antigen (HBGA) expression. The presence of similar ligands has been demonstrated in shellfish and potential influences on binding have been discussed. For example, GI.1 binds to A-like carbohydrate structures in the digestive gland of *Crassostrea gigas* and the presentation of these ligands may involve seasonal variation, whereas GII.4 accumulates much less efficiently and at sites where it might be more rapidly destroyed (Maalouf et al. 2010; Maalouf et al. 2011). Tian et al. (2007) demonstrated that multiple HBGAs are also expressed in mussel and clam gastrointestinal tissues. It is therefore possible that ligand specificities of NoV strains also result in strain dependent accumulation efficiencies in *Mytilus edulis*.

Implications for human health. Given that current regulations in Europe are based on concentrations of *E. coli* in shellfish flesh, mussels containing these levels of NoV could legitimately be sold for consumption following minimal treatment - potentially exposing consumers to an unacceptable risk of illness. It is possible that the method applied detected some inactivated NoV and may overestimate the amount of infectious virus present. However there is recent evidence that amount of genome detected is generally proportional to risk (Lowther et al. 2012b).

Much of the research concerning accumulation / elimination dynamics in shellfish has focussed upon oysters which are associated with more outbreaks than other species, possibly as a result of traditional raw consumption. However, with potential in Europe for virological standards applicable to all bivalve molluscan shellfish, similar data relating to *Mytilus edulis* (and other bivalves sold for consumption) is urgently required.

Notes

The authors declare no competing financial interest.

5. *Acknowledgements*

This study was funded by Bangor Mussel Producers, Welsh Water and the European Social Fund via the KESS Scholarships programme. We would like to thank Richard Dannatt and Samantha Mullan (Intertek) for assistance with model interpretation and Paul Henderson, Fergus O'Brien and Roger Cragg (Welsh Water Ltd) for outfall information, flow data and permission to use the model. We also thank Ian Harris (Bangor University) for help with the GIS; Gwynne Parry-Jones (Bangor University) and Trevor Jones (Bangor Mussel Producers) for boat time and skippering.

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Figure S1 – Scatterplots for measure and model predictions

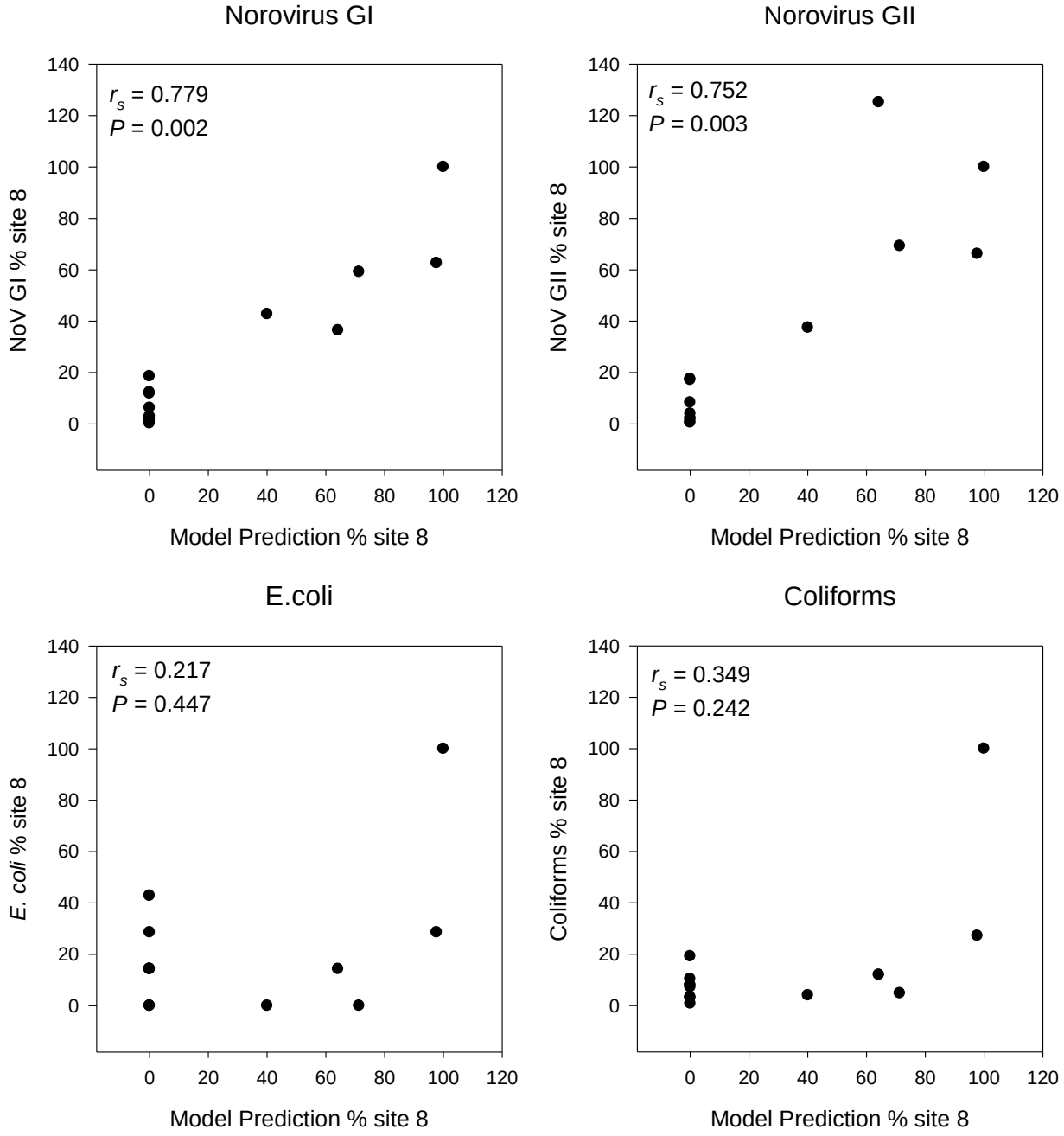

Figure S1 Scatter plots for measured and model predictions showing Spearman's rank-order correlation result for each measure. Data for model prediction and each measure is displayed as percentage of site 8 (outfall) value to standardise scales.

Table S1. Baseline (T_0) NoV detectable Genome Copies per Gram. Raw and Scored Baseline Levels

Replicate	1	2	3	4	5	6	7	8	9	10	mean	S.E.
GI	102	20	50	50	50	50	50	50	50	50	**52.2**	6.29
	[102]	[0]	[49]	[59]	[35]	[44]	[51]	[13]	[50]	[30]	[43]	[8.75]
GII	3909	3965	3216	3723	3122	3673	3304	2820	3127	2260	**3311**	167.01

Square brackets show unscored data. NoV of at least one genogroup was detected in all ten replicate samples (100%). GI NoV was detected in 9 (90%) and GII NoV in 10 (100%) of replicate samples. GI NoV was detected in 8/10 replicates at levels below the LOQ. These replicates were scored with 50 gen-c/g. GI was not detected in 1/10 replicates, which was scored with 20 gen-c/g.

Table S2. Norovirus and Indicator Organisms in Mussels Sampled After Deployment Around the Offshore Outfall for 30 d.

	Mooring number													
	1	2	3	4	6	7	8	9	10	11	12	13	14	15
NoV GI	3345	230	4631	1453	20 [0]	127	7825	928	50 [35]	50 [15]	490	4899	964	2853
NoV GII	2983	150	5508	1367	50 [11]	317	7954	1392	50 [75]	114	187	5264	662	9958
E. coli	0	333±333	0	167±167	0	0	1167±166	500±500	167±167	0	167±167	333±167	167±167	167±167
Coliforms	833±601	1500±289	1000±577	1667±333	667±333	167±167	20833±1764	2167±833	1667±601	1667±882	667±167	5667±2682	4000±500	2500±289

The mooring at site 5 was lost during the month. NoV GI and GII are expressed as detectable genome copies/g digestive gland. Quantitation based upon average of 3x 5µl aliquots of sample RNA. N=1. Un-scored, sub LOQ data shown in square brackets. *E. coli* and coliforms expressed as CFU / 100g shellfish flesh and intravalvular fluid. Mean and standard error shown $n = 3$.

CHAPTER 4

Assessment of the potential for offshore shellfish relaying to reduce bacterial and viral contamination

KATIE CLEMENTS,[1*†] JAMES B. WINTERBOURN,[2†] LUIS GIMÉNEZ,[1] DAVID L. JONES,[2] SHELAGH K. MALHAM[3] AND JONATHAN W. KING[3]

[1] *School of Ocean Sciences, Bangor University, Menai Bridge, Anglesey, LL59 5AB, UK;* [2] *School of Environment, Natural Resources and Geography, Bangor University, Gwynedd, LL57 2UW, UK;* [3] *Centre for Applied Marine Sciences, Bangor University, Menai Bridge, Anglesey, LL59 5AB, UK*

† Authors contributed equally.

* Corresponding author. E-mail: ktclements@hotmail.co.uk; ospc02@bangor.ac.uk

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This investigation was commissioned by the Low Carbon Research Institute (LCRI) in collaboration with the Centre for Applied Marine Sciences (CAMS) at Bangor University. This manuscript awaits compilation with other studies to be published as an LCRI desk study.

Both KC and JBW contributed to the experimental design after the concept was initiated by JWK. JBW conducted fieldwork. Bacterial analysis was conducted by KC using methods developed during her PhD which are not yet fully validated. Virological analysis was conducted by JBW using standard methods. KC and JBW conducted data analysis and manuscript production by section.

Abstract

Bivalve shellfish have the capacity to accumulate human pathogens including norovirus (NoV) when grown in water contaminated with human faecal matter. As such, they represent a vector for pathogen transmission into the human food chain. Measures to eliminate pathogens from shellfish, such as depuration and relaying, have been shown to be effective for the the reduction of bacteria but ineffective for NoV elimination. In the case of oysters, the European Food Safety Authority has suggested relocation of production operations to alternative areas which possess very low faecal contamination. Production of common mussels (*Mytilus edulis*) routinely takes place within inshore waters where faecal contamination can be high; however, relaying shellfish offshore may offer an alternative mitigation strategy against shellfish contamination. The objective of this study was to identify the effect of relocation to distances offshore on shellfish contamination levels within a potential production area. A single linear 12 km transect was established in March 2012 off the North Wales coast, consisting of 5 monitoring points at 1, 2, 4, 8 and 12 km offshore. At each monitoring point, caged mussels collected from a local inshore production area were suspended 1 m below the sea surface. The monitoring points were sampled 3 times over a 4 month period. Faecal indicator bacteria (FIB) and NoV concentrations in shellfish tissues were determined using standard methods. The results from this study were limited due to equipment loss and a high frequency of NoV results below the Limit of Quantification (LOQ). As such, no statistically significant effect of distance offshore upon contamination levels could be determined. However, upon relocation of commercially-grown mussels to the study area, FIB concentrations were reduced, to low or undetectable levels and NoV concentrations were reduced, mostly to levels below the LOQ for the selected assay. Levels of both FIB and NoV remained low or undetectable throughout the study period. We conclude that offshore deployment of mussels offers an alternative mitigation strategy to reduce the amount of shellfish-associated human pathogens entering the food chain.

KEY WORDS: *Mytilus edulis,* human pathogens, offshore relaying, norovirus, *E. coli,*

1. *Introduction*

Human population growth has placed increasing pressure on global resources and on the oceans to provide affordable and nutritious food for human consumption (Naylor et al. 2000). It is currently estimated that 16 kg of seafood is consumed annually per human inhabitant (Teplitski et al. 2009). Shellfish represent an ancient human food source, evidenced by prehistoric shell middens found worldwide, and their importance has been widely recognised since Roman times (Potasman et al. 2002). Production has increased dramatically over the previous 30 years (Potasman et al. 2002) partly due to consumers recognising the nutritional benefits of shellfish consumption (Børresen 2008).

Despite the positive attributes of shellfish for human nutrition, bivalve shellfish can accumulate human pathogens when grown in areas contaminated with human faecal matter. Therefore they represent a vector for pathogen transmission into the human food chain (Lees 2000). Norovirus (NoV) is the leading cause of shellfish-vectored gastro-enteric illness in humans worldwide and contamination of bivalve shellfish with NoV represents a well-established human health risk (Lees 2000). Research into the health risk posed by shellfish consumption has largely focused on oysters as they are traditionally consumed either raw or lightly cooked and have been implicated in the highest number of cases of shellfish vectored illness in humans (Le Guyader et al. 2012). However, *Mytilus edulis* (and other bivalves sold for human consumption) have also been implicated in outbreaks of human viral illness (Prato et al. 2004). This has led for calls to introduce a Europe-wide virological standard (EU Regulation) applicable to all bivalve molluscan shellfish which would help ensure consumer safety. As our knowledge of the factors regulating contamination (and decontamination) of *Mytilus edulis* are lacking (particularly for NoV), data is urgently required in this area to help guide the formulation of European microbiological shellfish standards.

The European Food Safety Authority (EFSA) suggests that production of oysters in areas which are not faecally contaminated is the most effective control measure because current depuration and relaying practices are ineffective for elimination of NoV (EFSA Panel on Biological Hazards 2012). The same best practice recommendations, which are dependent upon identification of clean waters, are likely applicable to the production of other bivalve species.

In Europe, *Escherichia coli* are utilised as the Faecal Indicator Bacteria (FIB) to quantify faecal contamination in shellfish and are routinely used for risk assessment and management (EU 2004). However, studies have shown that *E. coli* provides a poor indicator of the risk of viral contamination (Gerba et al. 1979, Gill et al. 1983, Chalmers and McMillan 1995, Ang 1998, Griffin et al. 1999, Noble and Fuhrman 2001). Differential environmental persistence of viruses and bacteria in marine waters, in addition to differential seasonal discharge patterns, may explain the poor correlation between FIB concentrations (*E. coli* and total coliforms) and viral contamination levels in shellfish

(Fong and Lipp 2005). Therefore, *E. coli* enumeration may be insufficient to safeguard consumers against the risk of shellfish-vectored viruses and unsuitable for the determination of sufficiently clean waters.

Recent advances have led to the development of reliable methods to detect and quantify NoV genomes in molluscan shellfish using molecular based techniques (Lees 2010, Anon 2013). These advances enable direct evaluation of NoV contamination in shellfish, being the most frequently identified aetiological agent in shellfish-vectored illness, and as a potential indicator for other sewage-derived viral contamination in shellfish. This enables shellfish to be used as accumulation matrices and avoids the need for sampling large volumes of water, which only give time-specific information.

Offshore based shellfish cultivation has been shown to be a successful method of increasing production capacity in many countries worldwide (Buck et al. 2005, Cheney et al. 2010). In addition, concentrations and infectivity of sewage-derived pathogens are assumed to reduce with distance from shore-based discharges. This is explained by physical factors including dilution, dispersion and sedimentation of contaminants originating from diffuse or point-sources at or near shore, and increased exposure to physico-chemical stressors including temperature, UV and salinity (Maalouf et al. 2010). Therefore, offshore based cultivation or relaying of shellfish may provide a means of meeting an increasing consumer demand and simultaneously mitigating against contamination issues, to provide a product which is safe for human consumption.

The primary aim of this study was to assess the suitability of an area previously identified by industry as a potential relaying area, particularly with regard to water quality. Within this, we had four key objectives: *Objective 1* was to determine concentrations of selected FIB and NoV present within common mussels (*Mytilus edulis*) experimentally relayed across a linear transect originating near-shore, bisecting the potential production area, and terminating 12 km offshore. It was hypothesised that concentrations of selected FIB and NoV accumulated in mussels may be related to the distance from shore at which they are relayed due to greater potential for re-contamination near-shore; *Objective 2* was to detect any differential behaviour between FIB and NoV which may be present within environmentally contaminated mussels at T_0 (time-zero). It was hypothesised that any FIB existing in mussel tissues at T_0 would be eliminated more rapidly than any NoV, subject to sufficiently clean waters existing within the transect. This is because NoV has been suggested to pose greater challenges for elimination than FIB under depuration or relaying regimes (Schwab et al. 1998, Le Guyader et al. 2006, Ueki et al. 2007, Le Guyader et al. 2008, Savini et al. 2009, Lees et al. 2010, Richards et al. 2010); *Objective 3* was to detect any differential behaviour between FIB and NoV accumulated within shellfish tissues *in situ*. It was hypothesised that, should the experimental location be subject to any sewage effluent contamination, a more pronounced effect of distance from shore would be observed for concentrations of FIB in mussels, than for NoV. This is because NoV has

been suggested to have greater environmental persistence (Loisy et al. 2005), potentially enabling detection of an impact at greater distance from potential sources than for FIB. *Objective 4* was to assess the suitability of the selected location for offshore relaying of *Mytilus edulis* with respect to survival and growth. These four objectives were ultimately designed to help guide the optimal location for offshore relaying in terms of balancing shellfish biomass with pathogen elimination potential.

2. Materials and methods

Sampling Location

A single, linear transect, was established in February 2012, running 12 km north from the Great Ormes Head (North Wales, UK; Fig. 1). Monitoring points were established at pre-determined distance intervals from shore; 1 km (53.3518 Longitude, -3.86957 Latitude), 2 km (53.3610 Longitude, -3.86957 Latitude), 4 km (53.3785 Longitude, -3.86957 Latitude), 8 km (53.4141 Longitude, -3.86957 Latitude) and 12 km (53.4503 Longitude, -3.86957 Latitude) due north from the shore. Continuous discharge point-sources were identified at Ganol and Penamaenmawr wastewater treatment plants (WwTP) to the east and west of Great Ormes Head. The Conwy river carries effluent from several WwTPs within its catchment.

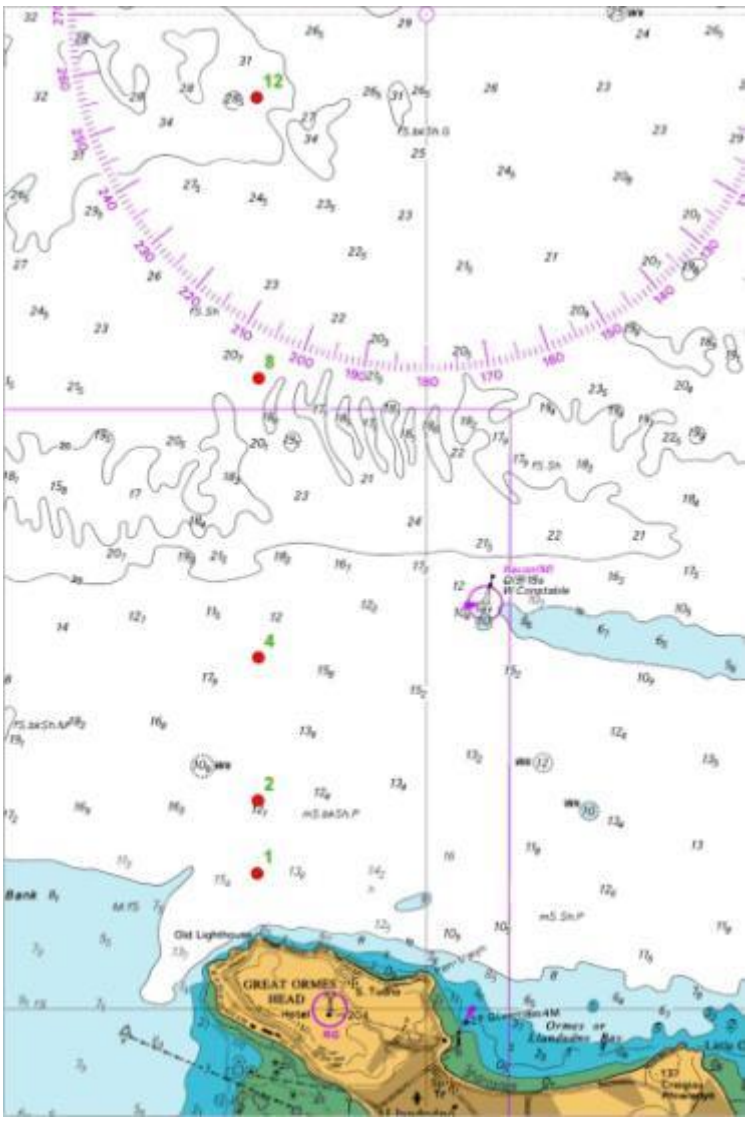

Fig. 1 Map showing the location of the offshore transect. Sampling points are represented by filled circles and numbered according to their respective distance offshore (in km).

Establishment of monitoring points

Common mussels (*Mytilus edulis*) were collected from a near-shore, sub-littoral mussel bed that is routinely monitored for bacterial contamination and has been classified as "Class B" (containing between 230 – 4,600 *E. coli* per 100 g) in accordance with regulation EC/854/2004 (EU 2004). To minimise variability associated with growing conditions, a single, short trawl (approximately 10 m) was used for collection. Only extant mussels were selected for future analysis whilst mussels with open or damaged shells were discarded. The collected mussels were rinsed with seawater to remove any residual debris and 'fouling' organisms prior to hand grading to ensure that only mussels of marketable size (>45 mm length) were utilised. From the resulting pool of mussels, 200 animals were randomly selected to provide a (time-zero) T_0 measure of selected FIB and NoV contamination at the time of harvesting. These animals were transported at 4°C and subsequently processed within 6 h of collection. The remaining animals were placed into 300 mm x 300 mm polymesh bags (20

mussels per bag). Fifteen polymesh bags were placed into a single SEPA oyster basket (SEPA, Edwardstown, South Australia), which were suspended within 6 h of sorting from a plough-anchored polyform buoy at each of the designated monitoring points at a depth of 1 m below the sea surface.

Monitoring protocol

Prior to the establishment of monitoring points across the entire transect, a single monitoring point was established at a distance of 4 km offshore to assess the suitability of the equipment and the methodology described above. The trial monitoring point was established on 25th February 2012 and remained *in situ* until 28th March 2012 (32 days).

The full scale experimental design, as described above, was deployed on the 28th March 2012 and remained *in situ* for 4 months, with samples being collected after 49 days (16th May 2012), 61 days (28th May 2012) and 134 days (9th August 2012) respectively.

At each sampling collection event, the oyster baskets were opened and three of the polymesh bags containing the mussel samples were randomly selected, per monitoring point. The selected bags were transported to the lab at 4°C and processed within 6 h. Non-selected bags were immediately returned to the oyster basket and the basket re-situated.

Determination of bacterial concentrations in mussels

Bacterial colony forming units (CFU) were enumerated from shellfish flesh by direct plating onto selective agar as described in Clements et al. (2013). The method differs from the standard specified under legislation 853/2004 and is undergoing validation. The Limit of Detection is yet to be determined. Only live mussels were selected for analysis. Approximately 5 to 8 individual mussels were removed from each polymesh bag and subsequently processed to determine bacterial concentrations. Mussel samples were surface swabbed with 100% methanol to eliminate the shell surface biofilm. The methanol had completely evaporated after 10 min at room temperature. Once dry, the mussels were aseptically opened and 50 g (wet weight) of flesh and extra cellular fluid was obtained. Mussel samples were homogenised for 60 sec at 10,000 rev min^{-1} using a Bamix® blender (Seal Rock Enterprises Ltd., Bishop's Stortford, UK). From the resulting homogenate, 200 µL were plated onto Brilliance® selective agar (#CM1046; Oxoid Ltd, Basingstoke, UK) to determine both *E. coli* and total coliform counts. All plates were inverted and incubated at 37°C and bacterial colony forming units (CFU) enumerated after 24 h.

Determination of Norovirus concentrations in mussels

Only live animals were selected for analysis. The initial (T_0) measurement consisted of ten replicate samples of ten mussels. For subsequent sample collection events, ten mussels were removed from each of the three polymesh bags, providing three replicate samples, and subsequently processed to determine concentrations of both NoV genogroup I (GI) and NoV genogroup II (GII).

NoV concentration in mussel digestive tissue was determined using quantitative reverse-transcription PCR (qRT-PCR) as described by (Lowther et al. 2012). Modification was made to the formation of the positive control and to the quencher used for the genogroup II probe (TAMRA) and

in addition, aliquots of chopped digestive glands were frozen (-20°C), within 6 h of collection, and thawed once prior to Proteinase K digestion rather than being digested fresh or after short-term (24 h) refrigerated storage (4°C). The positive extraction controls consisted of homogenates prepared as per samples after the addition of 1 lenticule® disc of NoV Reference Material for each genogroup (HPA) to ten digestive glands. Thus the positive extraction control was positive for both genogroups and of the appropriate matrix.

Homogenates were prepared by Proteinase K digestion of pooled digestive glands, dissected from 10 mussels, after Mengovirus vMC0 was added as an extraction control. RNA extraction from these homogenates was performed with a Nuclisens® miniMAG and magnetic extraction reagents, following the manufacturer's protocol.

One-step qRT-PCR for Mengovirus (extraction control) and for both NoV genogroups, including plate layout, and reaction mixes, was performed exactly as described by (Lowther et al. 2012) except, for the genogroup II assay, where TAMRA was used as the quencher. The thermocycler used was an Applied Biosystems 7900HT.

The use and treatment of a suite of qRT-PCR controls and all quantification steps also followed the same methods of (Lowther et al. 2012) including assessment of extraction efficiency and RT-PCR efficiency/inhibition (using RNA external controls), calculation and reporting of results in genome copies/g digestive gland, retesting action thresholds for extraction and RT-PCR efficiencies or due to failed controls, and no adjustment for losses during processing or RT-PCR inhibition was made (uncorrected). Average quantities enumerated from three aliquots of extracted RNA/replicate sample give overall quantities in detectable genome copies g^{-1} digestive gland.

This system for NoV quantification was in agreement with the principles outlined in the draft Technical Specification developed by the joint CEN/ISO working group for standardisation of methods for detection of viruses in foodstuffs (Lees 2010). The Mengovirus vMC_0 tissue culture supernatant and plasmids carrying the GI and GII target sequences, required to generate log10 dilution series (standard curves), were supplied by Dr. James A. Lowther, CEFAS, UK.

Samples returning "not detected" results for a particular NoV genogroup were assigned a score of 20 copies g^{-1} for that genogroup (half the estimated nominal limit of detection (LOD)). Samples giving positive results below the limit of quantification (LOQ; 100 copies g^{-1}) were assigned a score of 50 copies g^{-1}. This is consistent with the approach of the National Reference Laboratory and with UK survey data (Lowther et al. 2012).

Determining the growth and mortality rates of offshore relayed mussels

Mussel shell length was used to provide an indicator for growth. Individual mussel shells were measured, in mm, from shell umbo to shell tip using digital vernier callipers (± 0.02 mm). The mussel

shells were retained from the bacterial and viral determination protocol. 100 individual mussel shells were measured both pre- and post-deployment for the trial monitoring point. Prior to full scale deployment of the monitoring points a further 100 individual mussel shells were measured as described above. Mussel shells collected from the monitoring points were retained and measured, post bacterial and viral processing, with a total of 60 mussel shells measured per monitoring point.

Mussel flesh weight was also utilised as a measure for growth within the full scale investigation only. At T_0; ten animals were shucked and the wet weight of the flesh was recorded. Post collection; on May 28th 2012, five animals from each of the monitoring points were shucked and the wet weight of the flesh was recorded for comparison.

Mussel mortality was assessed by observing both the 'gape' of the shell and shell integrity, any mussels with gaping or damaged shells was discarded prior to deployment. For samples recovered from moorings, all mussels were examined individually post collection for mortality, defined as gaping by more than 2 mm and not responding to percussion, or not responding when the tissues were touched. Frequencies were recorded.

Statistical Analysis

Data were analysed using IBM SPSS Statistics 20 (IBM Corp., Armonk, NY). Prior to analysis data were assessed for normality using a one sample Kolmogorov-Smirnov test ($P \leq 0.05$). Bacterial data were analysed using a Kruskall-Wallis test, any significant differences ($P \leq 0.05$) were investigated further using Mann-Whitney U test with fixed factors of either date (2012) or distance offshore (km). Relationships between environmental bacterial reservoirs were investigated using Spearman rank order correlations (spearman's *rho* = r_s) to determine both the association and the significance of the relationship. Quantitative analysis of viral data could not be performed due viral concentrations below the LOQ being present.

Mussel growth was assessed using a series of One Way Analysis of Variance (ANOVA) and any significant differences ($P \leq 0.05$) were investigated using the least significant difference (LSD) test.

3. Results

Trial monitoring point (4 km offshore)

Results from the trial monitoring point supported the implementation of the full scale experimental protocol. The equipment deployed remained *in situ* for the duration of the trial, demonstrating its suitability for the full scale experimental protocol. After the 32 day trial period bacterial contamination levels within the mussels were significantly reduced to below detectable limits (Table 5.1). Over the same period, NoV GI and GII levels were reduced from 164 ± 18 (mean ± SE) and 6540 ± 1021 respectively to below quantifiable limits for both GI and GII in 9 out of 10 replicate

samples. GI was detected < LOQ in 7 out of 10 replicates and not detected in 3 out of 10 replicates. GII NoV was not detected in 3 out of 10 replicates and could be quantified marginally above the LOQ (135 gc g^{-1}) in 1 out of 10 replicate samples only (Table 1).

Table 1 Bacterial and viral concentrations from mussels determined both pre-deployment (T_0) and post-deployment (T_{28}) of the trial monitoring point 4 km offshore. In all cases n = 10 ± Standard Error (SE).

	Length of Deployment	
	(T_0)	**(T_{28})**
Bacterial concentrations		
E. coli (CFU / 100g)	**1400** ± 470.2	**N.D.**
Total Coliforms (CFU / 100g)	**13350** ± 2751.8	**N.D.**
Viral concentrations		
NoV GI (gc / g)	**163.59** ± 17.57	100% <LOQ (33% N.D)
NoV GII (gc / g)	**6540** ± 1021.0	90% <LOQ* (33% N.D)

N.D. = Not Detected. LOQ = Limit of Quantifcation. For the bacteriological methods the Limit of Detection (LOD) and LOQ are not yet determined. For each NoV assay the LOQ is 100 gc g^{-1} and the LOD is 40 gc g^{-1}.

* 1 replicate quantifiable at 135 gc/g

Mussel shell length measurements, as a proxy for growth, showed a weakly significant increase (P = 0.05; Independent samples t- test) in shell length between pre- and post-trial (data not shown). Mussel mortality was assessed post trial, to determine the effect of cage-based culture on survival. Overall, mortality was found to be low, with five fatalities out of a total of 300 mussels, equating to 1.66% mortality after 32 days *in situ*.

Full scale offshore investigation

Results from the full scale investigation were severely hampered by equipment losses. Of the five monitoring points initially deployed on 28th March 2012, only three remained *in situ* after 49 days. The monitoring points at both 8 km and 12 km offshore could not be located on 16th May 2012. The monitoring point at 4 km offshore remained *in situ* for 61 days, however, could not be located 134 days post-deployment. Potential reasons for loss include equipment theft, collisions with marine traffic or damage by wave activity.

Bacterial concentrations in mussels

Initial (T_0) concentrations of *E. coli* within mussel tissue (100 ± 67 CFU 100 g^{-1} mussel flesh) were below the maximum threshold for 'Class A' classification (230 *E. coli* 100 g^{-1} mussel flesh) in accordance with EC/854/2004 (EU 2004). Concentrations of *E. coli* within mussel tissue decreased significantly at all distances offshore relative to the initial *E. coli* concentration ($P \leq 0.001$ for all sites; Fig. 2a). *E. coli* was not detectable at any distance offshore after 28th March 2012 (Fig. 2a).

In contrast to the observed *E. coli* concentrations, total coliform concentrations within mussel tissue did not differ significantly between the initial (T_0) total coliform concentrations and the concentrations observed on 28th March 2012 or 28th May 2012 at all offshore distances ($P \geq 0.05$ in all remaining moorings; Fig. 2b). A significant difference in total coliform concentrations was observed between the initial total coliform concentration and total coliform concentration on 9th August 2012, which showed coliform concentrations to be undetectable at all offshore distances ($P = 0.023$ in all cases; Fig. 2b.). No significant difference in total coliform concentrations were observed between the remaining monitoring points at 1, 2 and 4km offshore (16th May 2012: $P = 0.102$, 28th May 2012: $P = 0.105$) or between 1 and 2km offshore (9th August 2012: $P = 1.000$; Fig. 2b).

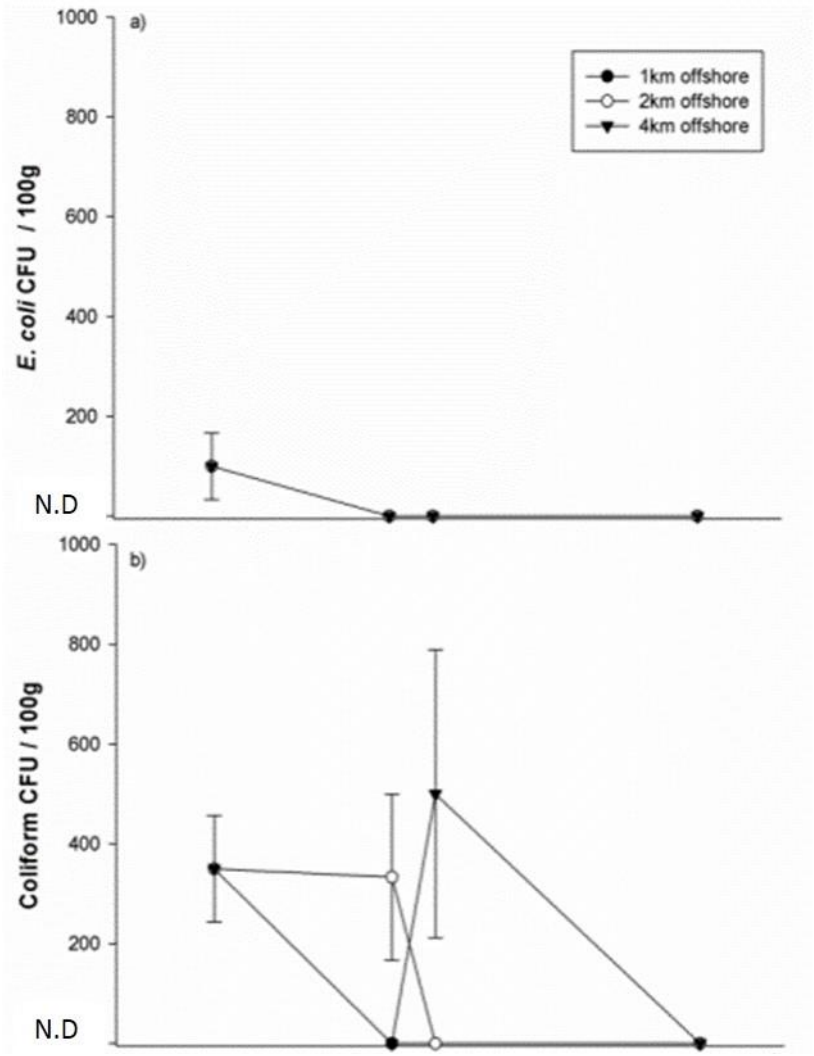

Fig. 2 Concentrations of (a) *E. coli* and (b) total coliforms observed in offshore relayed mussels from differing distance offshore over 124 days. The bottom line of the *y-axis* represents not-detected (N.D.) where the Limit of Detection has not yet been determined. In all cases $n = 3$ where the data points represent the mean ± Standard Error (SE).

5.3.4 Viral contamination of mussels

Pre-deployment (T_0) concentration of NoV genogroup I (GI) within mussel digestive tissue was found to be below the LOQ (i.e. < 100 gc g^{-1}) in all ten replicate samples. In two of these replicates (20%), NoV GI was not detected (i.e. samples contained < 40 gc g^{-1}). Hence, according to the scoring convention, the mean concentration was 44 gc g^{-1}. Post deployment, all samples (24/24) analysed for NoV GI returned results that remained below the LOQ. In 33.3% (8/24) of these samples, NoV GI was not detected.

T_0 concentrations of NoV genogroup II (GII) were quantifiable in all ten replicate samples with a mean ± SE of 830 ± 92 gc g^{-1}. Post deployment, 91.67% (22/24) of samples analysed returned

results that were below the LOQ, including 29.17% (7/24) of samples in which NoV GII was not detected.

At low levels the qRT-PCR amplification begins to behave stochastically and may show greater variability between replicate samples i.e. the confidence interval becomes wider. Therefore the nominal LOQ represents an accepted level below which results should be considered tentative. Notwithstanding this, trends may be observed below the LOQ and interpreted cautiously (Fig. 3.)
Prior to the sample collection 28th May 2012, sample points at 1 km and 2 km offshore may have been exposed to a spike in NoV GI levels which appeared to have less impact at 4km offshore (Fig 3.a). In the previous collection (16th May 2012) NoV GI had not been detected in any replicate at 4km offshore, whilst it was detected more frequently, albeit at levels below the nominal LOQ, nearer the shore. On this date, NoV GI could be detected in 2 of 3 replicates at 1 km offshore, and 1 of 3 replicates at 2 km offshore. Similarly, on 16th May, non-detection of NoV GII occurred exclusively at 4 km offshore and did so in 3/3 replicate samples, whilst it was detectable in all three replicates at 1 and 2 km offshore (Fig 3.b,c). NoV GII could be detected at levels above the nominal LOQ in one of three replicates at both 1 and 2 km offshore, with levels of 146 gc g^{-1} and 114 gc g^{-1}, respectively. For the sample collection of 28th May 2012 Non-detection of GII NoV on this date occurred in 2/3 replicate samples at the 4 km mooring but was detected in the third replicate at the 4 km mooring and in all replicate samples from 1 and 2 km moorings (Fig. 5.3b).

The mooring at 4 km could not be located on 9th August 2012. At this point the seasonality observed in NoV prevalence is likely to have affected levels at all moorings, with low community and environmental prevalence being well documented. There appeared to be no difference in either NoV GI or GII levels between the remaining moorings at 1 and 2 km offshore, although both NoV GI and GII remained detectable at low levels.

No statistical analysis was undertaken to support trends observed in tentative, sub nominal LOQ data.

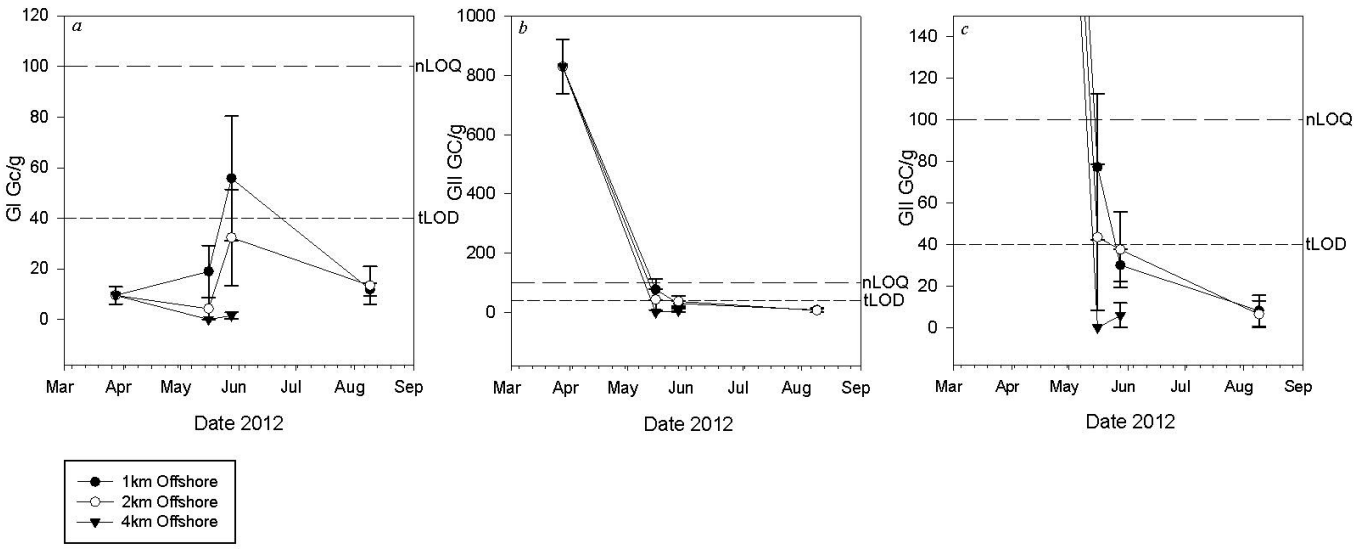

Fig. 3. Results for (a) norovirus GI and (b) norovirus GII detected in replicate mussel samples from different offshore distances for each time point. (c) shows NoV GII with *y-axis* zoom to post-deployment data. In all cases n = 3. The nominal Limit of Quantification and theoretical Limit of Detection are shown on each plot.

Growth and mortality of offshore relayed mussels

Shell length of the mussels was measured to determine the suitability of offshore cage based culture with respect to mussel growth. Mussel shell length significantly increased between 28th March and 9th August 2012 ($P \leq 0.05$; Fig. 4) at all offshore locations. Shell length had also significantly increased (at all offshore distances) by 16th May 2012 compared to initial (T_0) shell length measurements ($P \leq 0.01$; Fig. 4). Offshore distance also had a significant effect on mussel shell length. Mussels located 4 km offshore showed significantly greater growth than mussels located 2 km offshore ($P = 0.024$; Fig. 4). No significant difference in shell length was noted for mussels located at 1 km and 4 km offshore ($P = 0.347$) or mussels at 1 km and 2 km ($P = 0.139$; Fig. 4).

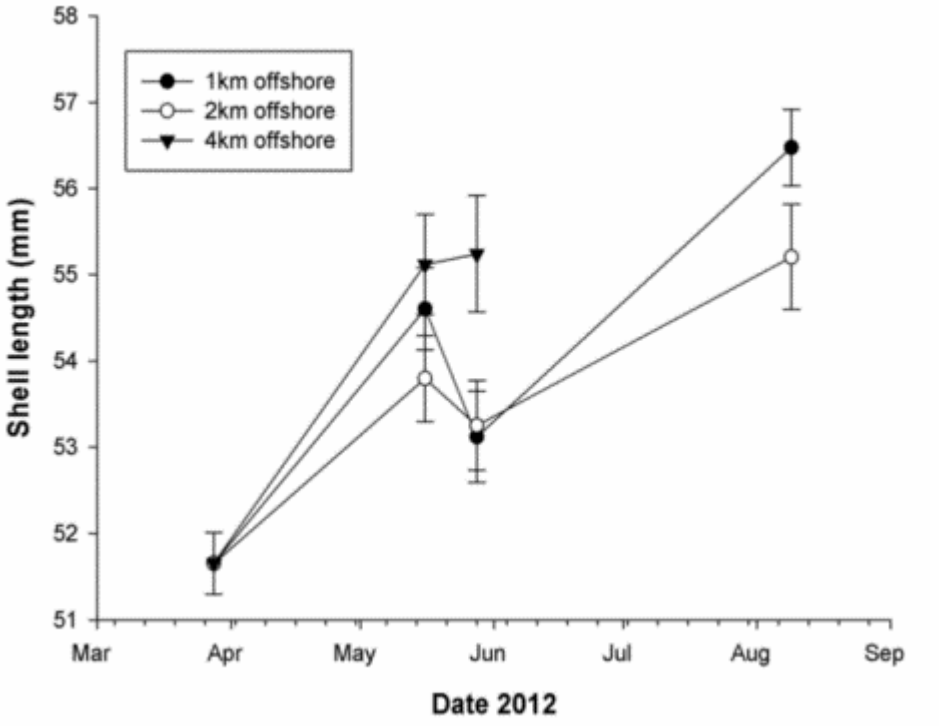

Fig. 4. Mussel shell length as observed in offshore relayed mussels from differing distance offshore over 124 days. n = 60 (with the exception of 28th March 2012 where n = 100). The data points represent the mean ± Standard Error (SE).

Mussel flesh weight analysis demonstrated no significant difference between the initial (T_0) flesh weight and the flesh weight of mussels held at 1 km and 2 km offshore on the 28th May 2012 ($P \geq 0.05$; Fig. 5). In contrast to shell length, mussel flesh weight showed a significant decrease in mussels held 4 km offshore relative to their initial flesh weight ($P \leq 0.05$; Fig. 5).

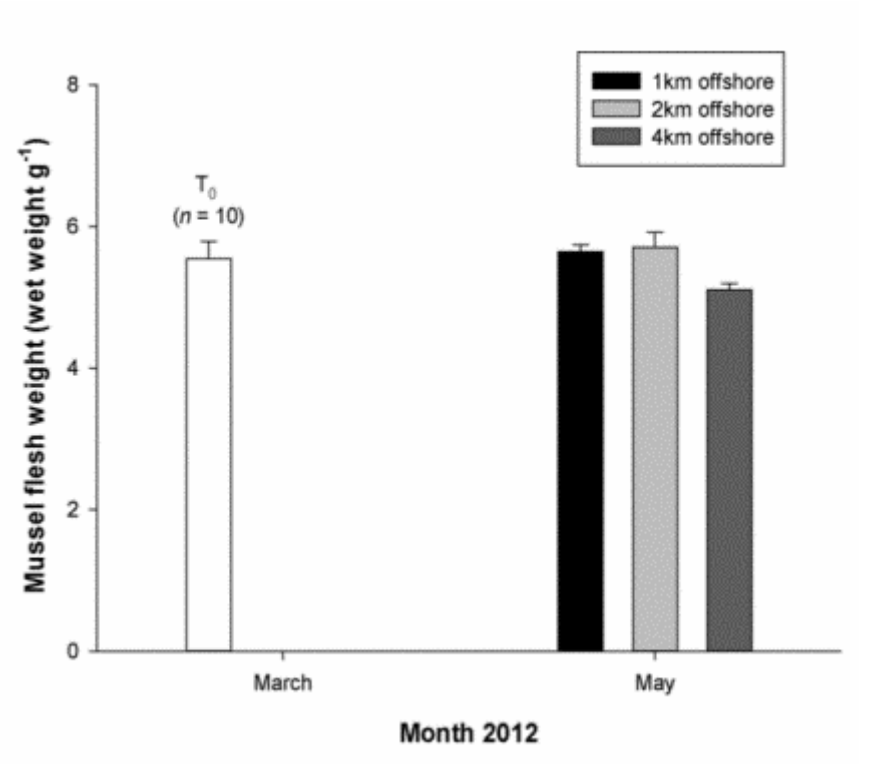

Fig. 5. Mussel flesh weight measured pre-deployment on 28th March 2012 and post- deployment on 28th May 2012 over differing distance offshore. In all cases $n = 5$ except at T_0 where $n = 10$. Data points represent the mean ± Standard Error (SE).

At all points along the transect, mussel mortality was low (<15%) until 28th May 2012 (Fig. 6). However, high cumulative mortality was evident at remaining moorings (1 and 2 km) on 9th August. This may be explained by the observation of numerous crabs which had become trapped inside the cages after growth.

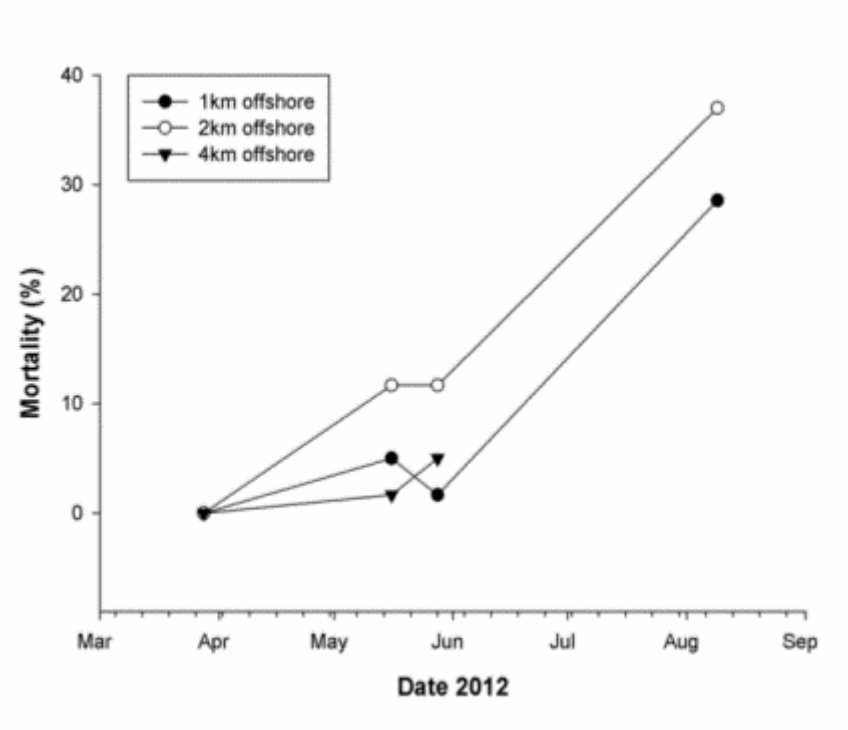

Fig. 6. Percentage mortality observed in offshore relayed mussels from differing distance offshore over 124 days. In all cases n = 20 (with the exception of 28th March 2012 where n = 100). The data points represent the mean ± Standard Error (SE).

4. *Discussion*

Levels of NoV and coliforms in mussels after offshore deployment

The results of this pilot study were unable to determine any significant effect of distance from shore on *E. coli* or total coliform concentrations in experimentally deployed mussels: *E. coli* was not detected in shellfish tissues collected from any monitoring point within the transect and total coliform concentrations were not significantly different between monitoring points.

The results of this study were also unable to determine any significant effect of distance from shore upon NoV concentrations in mussels experimentally deployed within the potential relaying area. Levels were frequently below the LOQ. GI NoV was never detected at levels above the LOQ in animals relocated to the area. GII was only detected at quantifiable levels in 1/3 replicates each at 1 and 2 km moorings on 16th May after 49 days. GII was never detected at quantifiable levels 4 km offshore, or at any mooring at subsequent time points. This finding should be interpreted with caution due to the subtle distinction between "not detected" and "negative". Under the approach of the National Reference Laboratory, samples returning a result in which NoV was not detected are assigned a score of 20 gc g^{-1}, which is half the estimated Limit of Detection and samples in which any NoV is detected below 100 gc g^{-1} (estimated limit of quantification) are scored 50 gc g^{-1}. The stochastic behaviour of the assay below this level requires that these results be treated with caution. The difference in levels between any sample returning a sub LOQ result for a given genogroup and one in which it was not detected may be marginal.

Despite these seemingly inconclusive findings, this study demonstrated reduction of NoV after relocation to the potential production area. Firstly it showed, in the trial mooring, that NoV levels of approximately 6700 gc g^{-1} (sum GI and GII) in mussels relocated from a nearshore commercial production area to the potential offshore production area located 4 km offshore were reduced to levels below the LOQ of the method in 9/10 replicates during the 32-day trial period. The only quantifiable result was marginally above the LOQ for GII only, in 1/10 replicates. Secondly, it showed that NoV concentrations of approximately 900 gc g^{-1} (Scored GI data + GII data) were reduced to <LOQ levels for GI and levels around the LOQ for GII in 49 days at points located 1 and 2 km offshore. At 4 km offshore, neither NoV genogroup could be detected at this time point. Levels were <LOQ or not detected for both genogroups at all monitoring points at subsequent time points. However, the study is not able to attribute the reduction in NoV levels solely to the relocation of the animals, due to uncontrolled factors: The study commenced in Spring, subsequent rising seawater temperatures and reduced prevalence of NoV infection in the community may contribute towards lower NoV levels in the environment in general.

An observation which supports the hypothesis that the waterbody investigated is less faecally contaminated than the inshore production area from which mussels were sourced for both the trial

mooring and the full experiment is as follows: On 28th March, mussels recovered from the trial mooring showed reduction from approximately 6700 gc g^{-1} (GI+GII) present in animals harvested from the production area, to levels in which 90% of replicate samples were <LOQ. Mussels also harvested on 28th March from the supplying area showed less reduction: Levels of approximately 900 gc g^{-1} remained, suggesting the relocated mussels were exposed to less NoV contamination. This observation should be treated with caution because no control mooring, stocked with the batch of mussels used in the experimental area, was deployed in the production area: The figure of 900 gc g^{-1} is derived from the same bed but of a different trawl and so conclusions based on direct comparison are inappropriate. Secondly, the experiment does not account for potentially different epidemiological patterns of NoV infection within the different communities proximate to the respective waterbodies. It is therefore possible that NoV recontamination of mussels would have been detected within the potential offshore production area in the event of a local outbreak. NoV epidemiology is dynamic and this observation relates to a single time point. It is possible that the sampling regime missed the occurrence of lower levels within the supplying area and higher levels within the potential offshore production. Notwithstanding this, FIB are assumed to be discharged in sewage continuously: That *E. coli* and total coliforms were reduced to levels below detection thresholds during the 32 day trial, and were not detectable at any monitoring point after 49 days in the full experiment, despite being detectable in both supply batches prior to deployment, does support the suggestion that these waters are cleaner than the waters from which mussels were supplied.

M. edulis productivity after offshore deployment

Assessments on survival and growth of offshore relocated *M. edulis* indicated low percentage mortality (until predation by trapped crabs) and continued growth suggesting that offshore cultivation may be considered a suitable alternative to near shore cultivation. However, it is important to view these findings with caution, as the substantial equipment losses incurred during the course of this experiment prevent firm conclusions from being drawn. Further, the sample size used for these analyses was small and further research is recommended in this area to confirm the findings over repeated annual cycles. It is important to note that this study only focused on the viability of offshore shellfish cultivation in relation to bacterial and viral contamination of shellfish. This study did not attempt to ascertain the economic viability of offshore shellfish cultivation. Offshore cultivation may be one approach to meeting EFSA recommendations to produce shellfish in waters which are not faecally contaminated, resulting in a 'safer' or more marketable shellfish product, but the approach may not prove economically viable or be well accepted by industry.

Significance of the results for North Wales M. edulis shellfisheries

Common mussels are commercially harvested in near shore environments off the North Wales coast. The North Menai Strait Mussel Fishery has been awarded Marine Stewardship Council (MSC) certification as an "enhanced" fishery producing a sustainable harvest of 8-10,000 metric tonnes annually (SAGB 2010). This industry contributes to approximately 39,000 metric tonnes produced in the UK per annum. All commercial mussel beds off the North Wales coast have been assigned "Class B" classification (between 230 – 4,600 *E. coli* per 100 g^{-1} mussel flesh) in accordance with EC legislation EC/854/2004 (EU 2004) and must be purified prior to retail so as to meet "Class A" standards (< 230 *E. coli* 100 g^{-1} mussel flesh). Post-harvest purification not only increases production costs, but it may also limit the total shellfish harvest as the purification stage allows only for batch harvesting rather than continual harvesting. In addition, the final (purified) shellfish product is determined to be safe for human consumption based upon bacterial (*E. coli*) concentrations. It has been shown that viral contamination may persist in shellfish after bacterial end-product standards are met (Doré and Lees 1995, Schwab et al. 1998, Lees 2000, Richards et al. 2010, EFSA Panel on Biological Hazards 2012). The behaviour of NoV, during depuration specifically, has been difficult to assess until recently as quantitative methods were not available. The majority of work since has focused on oysters, where persistence of NoV through the depuration process has been demonstrated (Nappier et al. 2008, Le Guyader et al. 2009, McLeod et al. 2009, Neish 2013). Consequently, alternative options for shellfish production are being considered to reduce bacterial and viral contamination of shellfish and to reduce the occurrence of shellfish vectored illness. Offshore shellfish production is routinely practiced in many countries worldwide as a means of either establishing a new production area or enhancing a pre-existing industry (Goulletquer and Le Moine 2002, Buck 2007) and the results presented here support its use in North Wales shellfisheries.

This pilot study utilised experimentally caged mussels to monitor contamination levels of FIB and NoV. Whilst cage-based culture is frequently used for oysters and may be an option for offshore mussel production, it is likely that alternative methods such as rope culture would be more suitable. This study makes no attempt to compare different offshore shellfish culture methods with respect to FIB and NoV accumulation / elimination rates and further research in this area is required. Caged adult mussels were employed in this experimental design as this allowed us to establish monitoring points in a timely manner and to control various ecological factors such as population density and predator exclusion which may have a negative impact on the physiological state of the caged mussels (Nakaoka 2000, Nunes et al. 2011).

Further work

The findings from this study can only be regarded as preliminary. To obtain more detailed information regarding the effect of distance offshore on both FIB and NoV contamination of mussels

during relaying, a further in-depth study would need to be conducted. Specifically, this would increase both the number of sampling points and the frequency of sampling. As the feeding rate of mussels is generally accepted to be a key determinant for the accumulation of both FIB and NoV (Hawkins et al. 1996, Burkhardt and Calci 2000), this would also need to be incorporated into any future experimental designs. An assessment of the food levels present (perhaps using turbidity as a proxy) would further enhance any future experimental design.

Deployment of both the trial mooring and the full experimental apparatus were delayed by unsafe conditions for the necessary boat work. The study was intended to commence in Autumn but did not do so until Spring. The seasonality exhibited by NoV has been well documented (Lopman et al. 2003, Lowther et al. 2008, Rohayem 2009, Lowther et al. 2012). The study could be improved with deployment of NoV negative mussels in Autumn, suspended from robust moorings and tested regularly through Winter. Samples which are subsequently found to contain detectable NoV are then known to be qualitatively different to the baseline and this may provide improved information regarding the areas which remain consistently uncontaminated. Earlier deployment of mussels may allow NoV to accumulate during winter months to levels in excess of the LOQ, allowing quantitative analysis to detect any effect of distance and improve long term comparison of the differential behaviour between NoV and FIB. The experimental design could also better reflect the proposed method of cultivation to be conducted within the area. This study only examined the effect of cage-based culture on relocated adult mussels and these results may not be comparable with other culture methods using juvenile mussels.

In addition, the economic viability of offshore shellfish relaying must be assessed. Whilst offshore cultivation may mitigate against both bacterial and viral contamination of shellfish, it may not be economically viable to relocate an entire industry. Further consideration therefore must also be given to both the shellfish industry's requirements and also to the wider environmental implications of potential offshore relocation.

In conclusion, the findings from this study indicate that offshore relaying of mussels in the selected area may aid reduction in both FIB and NoV contamination accumulated from growing waters. However, substantial equipment losses coupled with low detection frequencies of both NoV and FIB did not allow for a full quantitative assessment to be undertaken. These findings do, however, tentatively suggest that the selected area may be considered suitable for offshore shellfish relaying following further research. No effect of distance offshore could be accurately determined and identification of critical distances would be useful to the industry (although this may be geographically very context specific). Offshore relocation appeared to show little negative impact on the growth rate of mussels. Mussel mortality was shown to remain relatively low (<15%) over all offshore distances,

until a spike in percentage mortality was observed across all distances in August 2012; we ascribe this to predation, an additional area that requires further work.

5. *Acknowledgements*

This work was supported by the European Social Fund (ESF) through the European Union's Convergence program administered by the Welsh Government in association with Bangor Mussel Producers and Deepdock Ltd. The authors would also like to thank Trevor Jones (Bangor Mussel Producers) and Gwynne Parry Jones (Bangor University) for their assistance with the deployment and maintenance of the experimental equipment offshore. KC and JBW contributed equally in the experimental design and in the preparation of this manuscript.

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CHAPTER *5*

Fate of norovirus during commercial and laboratory simulated depuration of naturally contaminated mussels

JAMES B. WINTERBOURN,[1*] KATIE CLEMENTS,[2] DAVID L. JONES,[1] JAMES MCDONALD[5], JAMES LOWTHER[4] AND SHELAGH K. MALHAM[3]

[1]*School of Environment, Natural Resources and Geography, Bangor University, Gwynedd, LL57 2UW, UK;*

[2]*School of Ocean Sciences, Environment Centre Wales, Bangor University, Bangor, Gwynedd, LL57 2UW, UK;*

[3]*Centre for Applied Marine Sciences, Environment Centre Wales, Bangor University, Bangor, Gwynedd, LL57 2UW, UK;*

[4]*CEFAS, The Nothe, Barrack Road, Weymouth, Dorset, DT4 8UB, UK;*

[5]*School of Biological Sciences, Bangor University, Bangor, Gwynedd, LL57 2UW, UK*

*Corresponding author. E-mail: afpc58@bangor.ac.uk

Author Contributions: JBW designed and conducted the experiment. KC performed bacterial analysis using methods described in Clements et al. (2013). The method differs from the standard specified under legislation 853/2004 and is undergoing validation. The Limit of Detection is yet to be determined. JBW performed virological and data analysis and wrote the manuscript reviewed by DLJ and SKM. JL provided standards and control materials for use in the norovirus assays.

ABSTRACT

Bivalve shellfish such as mussels and oysters have the capacity to accumulate human pathogens from water contaminated with human sewage. To reduce the health risks associated with this contamination, shellfish are typically depurated in clean seawater. The subsequent suitability of shellfish for sale is then determined by compliance with bacterial end-product standards. Whilst depuration is effective in reducing the occurrence of shellfish-vectored illness of bacterial aetiology, it may fail to eliminate human pathogenic viruses even when bacterial standards are met. As norovirus (NoV) now represents the principal agent of shellfish-associated gastroenteric illness, there is a critical need to improve the efficiency of NoV removal from shellfish. The experiments reported here aimed to determine the efficacy of a commercially operated depuration system to eliminate NoV from naturally contaminated and commercially harvested blue mussels (*Mytilus edulis*). Two commercially harvested batches of mussels were tested for NoV at harvest and immediately pre- and post-depuration, using the ISO standard quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) method. A subsample of the first batch was also depurated under laboratory conditions to allow for time-series sampling and enumeration of *E. coli* by culture methods in addition to NoV by qRT-PCR. Rapid elimination of *E. coli* was observed in our laboratory system. However, the lack of statistically significant differences in NoV levels between pre- and post-depuration samples in either of two commercially-depurated, or one laboratory-depurated, batch indicated that neither the commercial nor lab-scale system was able to significantly reduce naturally accumulated NoV contamination in live *Mytilus edulis*. We conclude that transport and refrigerated storage has minimal impact on NoV concentrations and that current commercial depuration practices are insufficient to protect consumers from exposure to NoV.

KEY WORDS:

Norovirus, depuration, food poisoning, microbiological standards, risk assessment

1. *Introduction*

Bivalve molluscan shellfish (BMS) consumption is growing worldwide (Soto 2009) and BMS can accumulate human pathogens when their production areas are contaminated with human faecal matter. Contamination of BMS with norovirus (NoV) represents a well-established human health risk with NoV being the primary aetiological agent of shellfish-vectored gastro-enteric illness in humans worldwide (Lees 2000). Bacteriological criteria are often employed to assess the impact of sewage on BMS and their growing waters. *Escherichia coli* (*E. coli*) is used as an indicator of faecal contamination within hygiene frameworks implemented both in Europe and the US (Anon 2004a; US/FDA 2011). In the US, legislation classifies production areas according to levels of thermotolerant bacteria in seawater (WHO 2010). In Europe, regulations 854/2004 (Anon 2004a) require that harvesting areas are classified according to levels of *E. coli* in shellfish tissue. These regulations specify acceptable levels of the indicator bacteria and dictate the level of processing (e.g. depuration) required to decontaminate shellfish from a particular production area.

Depuration in clean seawater has been employed for over a century as a method of extending the filter feeding process of bivalves (Herdman & Scott 1896). It was further developed in the 1920s in order to purge microbiological contaminants. This process has the advantage that shellfish processed by depuration can be sold live. The process is currently subject to legal control in the E.U. under Directive 853/2004 (Anon 2004a). The legislation stipulates that depurated shellfish comply with the end-product standard for shellfish sold live. I.e. bivalve shellfish harvested from class 'B' areas (230 ≤ 4,600 *E. coli* per 100 g flesh) must be purified in an approved depuration system and the end products must comply with the EC 854/2004 microbial standards of ≤230 *E. coli* per 100 g flesh and absence of salmonella in 25 g; Anon 2004a; Anon 2004b). In England and Wales commercial depuration systems must meet the conditions of approval determined by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS). There are several generally approved systems which all employ UV irradiation for disinfection of recirculated water within a closed system (Lee et al. 2008). Disinfection of recirculated water serves to inactivate any pathogens purged from the animals preventing re-uptake or cross-contamination (Doré 2003). The organoleptic characteristics of the shellfish remain unaffected (Lees & CEN WG6 TAG4 2010). While this framework has reduced the occurrence of BMS-vectored bacterial illness (Lees 2000), it may not be effective against human viral pathogens due to the poor correlation between bacterial and viral contaminants in both water and shellfish (Mena & Gerba 2009; Fong & Lipp 2005; De Donno et al. 2012). Lees and the working group CEN WG6 TAG4 (2010) report that the control of virus risk using faecal indicator bacteria is complicated by differential behaviour of bacteria and viruses both within the environment and during post-harvest depuration and storage.

The removal of viruses by BMS depuration can be challenging as enterically transmitted viruses are frequently eliminated and inactivated more slowly than most bacteria (Ueki et al. 2007; Nappier et al. 2008; Conn 2010; Jaykus et al. 1994; Dore & Lees 1995; Richards et al. 2010). Importantly, compliance with bacterial standards after depuration may not reflect virological safety (Dore & Lees 1995; Schwab et al. 1998; EFSA Panel on Biological Hazards 2012). Consequently, many outbreaks of viral illness have been linked to depurated BMS which have been deemed safe for sale (i.e. compliance with regulatory *E. coli* standards following depuration in approved plants and after production in officially classified waters; Grohmann et al. 1981; Perrett & Kudesia 1995; Ang 1998; Conaty et al. 2000). For example, depurated and compliant mussels from Italy and Greece have tested positive for infectious Hepatitis A (Chironna et al. 2002) while an oyster-vectored outbreak in Scandinavia caused NoV gastroenteritis in at least 356 patients despite oysters complying with all European faecal coliform standards (Christensen et al. 1998).

Both raw and cooked mussels have been implicated in outbreaks of human viral illness (Prato et al. 2004) and depuration studies have identified a variety of contaminating viruses present in *Mytilus edulis* (Bosch et al. 1995; Abad et al. 1997; Enriquez et al. 1992; De Medici et al. 2001).

Our laboratory has intermittently detected NoV of genogroup I and genogroup II in batches of mussels originating from a local shellfishery using the quantitative method described under ISO TS 15216-1:2013 (Anon 2013). In mussels procured for other experimental purposes, higher levels of NoV have most often been detected during winter. This pattern is consistent with oyster surveillance of the UK National Reference Laboratory (Lowther et al. 2008; Lowther et al. 2012) and the USA (Burkhardt & Calci 2000; Woods & Burkhardt 2010). However, the laboratory used by the commercial depurator and wholesaler has never reported detection of NoV in any mussel batches originating from the fishery. Therefore an investigation was initiated on behalf of the industrial partner.

The primary aim of this case study was to assess, using standardised methods, the fate of NoV naturally accumulated in broadcast-cultivated mussels, during their transport and depuration. The first objective was to intercept and track NoV levels in a commercial batch of mussels at the point of harvest, through export and after a commercially operated depuration regime applied after export. The second objective was to shed further light on the differential behaviour of *E. coli* and NoV during depuration of mussels.

2. Materials and Methods

Collection of Mussels

Mussels (*Mytilus edulis*) were collected from a near-shore, sub-littoral mussel bed during a full-scale commercial harvesting operation. It was ensured that mussels for the experiment were collected from one short dredge (<10 m) to minimise the potential variability which might be present across the production area. The bed is routinely monitored for bacterial contamination and has been classified as "Class B" (containing between 230 – 4,600 *E. coli* per 100 g) and therefore requiring depuration of product in accordance with regulation EC/854/2004 (EU 2004a). Extant mussels >45 mm in shell length were selected for the investigation; mussels with open or damaged shells were discarded.

Shellfish Transport and Commercial Depuration Protocol

A batch of ~700 mussels was collected as described above. To investigate the fate of NoV in exported and then commercially depurated mussels, 170 animals were selected from this wider population. Fifty animals were randomly selected and assigned to five replicate samples each comprising ten mussels. These were frozen immediately and stored (-20°C) to later provide a baseline (T_0) measure of NoV (GI and GII) contamination at the time of harvesting, for comparison with samples which would be frozen after their export and after subsequent commercial depuration. A further 120 mussels were assigned to replicates of 10 mussels. Each of the 12 replicate samples was loosely contained in a clearly labelled mesh bag. These mesh bags were evenly distributed within the haulier's refrigerated (4°C) transport container, although they were placed atop of the bales comprising the remainder of the commercial shipment. The shipment was transported for 860 km lasting 36 hours. Five of these bags were labeled with the instruction "freeze immediately upon receipt". The commercial depurator collected these mesh bags, placed them within a sealed plastic bag and transferred them to a freezer (-20°C) on arrival. Seven mesh bags were provided for samples undergoing depuration to allow wider distribution within the tank. These were labelled "distribute evenly within system during depuration of corresponding batch and freeze immediately after". After the process, the depurator also placed these mesh bags into a sealed plastic bag before freezing (-20°C). The exported and depurated mussels were collected and returned to our laboratory packed in dry ice in two boxes, one each for pre- and post-depuration samples. To ensure similar treatment of all samples, the 5 replicate baseline samples held at our laboratory were transferred into a similar box containing the same amount of dry ice for the duration of the journey. Upon receipt, all samples including the baseline were simultaneously replaced into a -20°C freezer for later analysis.

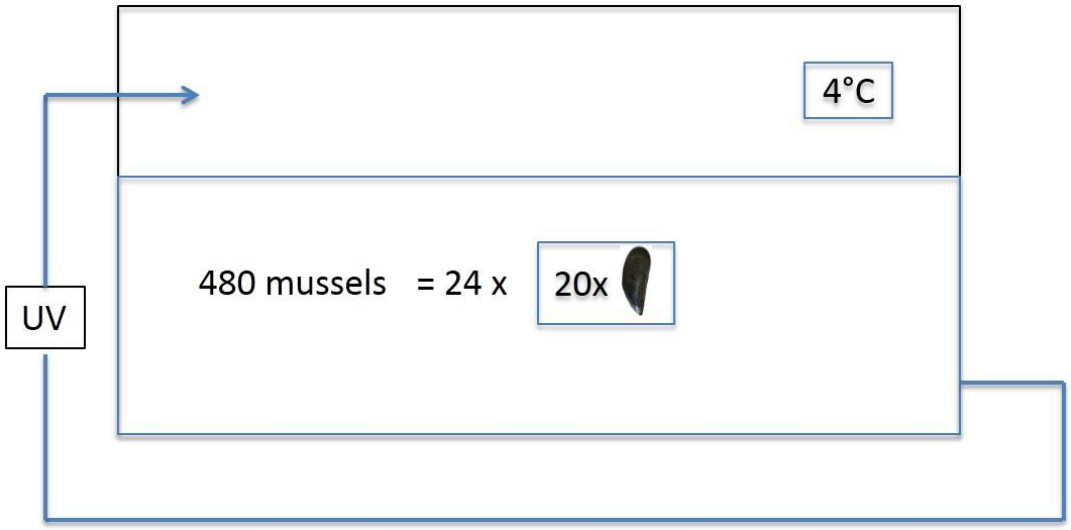
The commercial depuration process consisted of treating 4500-5500 kg of mussels per container/tank in a layer 70-80 cm deep above a raised mesh floor, allowing voided material to sink

below the animals. Seawater at ambient temperatures (4°C at the time of this study) was pumped into the containers and recirculated via UV light and ozone treatment without heating. The temperature was maintained at 4°C with depuration lasting 24 h.

In the experiment described above there was a delay (19 h) in the shipment of mussels to the depurator due to bad weather. As this may have impacted upon the depuration process, a second batch was tracked using the same methodology described above except that 7 replicates were processed for all time points and an additional 4 replicates processed fresh after harvest for comparison with corresponding frozen samples. The travel time for batch 2 was 17 h, a period typical for the exports from this fishery to this depurator.

Laboratory-Simulated Depuration Protocol

A scaled down version of the commercial depuration system containing approximately 500 L of seawater was used for shellfish depuration.



To simulate the commercial depuration, the system was installed in a temperature controlled room (4°C). The water temperature was equilibrated to 4°C to match the Dutch commercial system. In the UK, CEFAS stipulate a minimum depuration temperature of 5°C for *Mytilus edulis* in commercial plants (CEFAS 2010). Dissolved oxygen was measured using a standard probe and always exceeded 7.9 mg L^{-1} (~76% saturation at 4°C). The minimum dissolved oxygen value currently used by Cefas in assessing the oxygenation capabilities of depuration systems is 5mg L^{-1} (CEFAS 2010). Salinity was measured using a standard refractometer and levels were maintained at 35 parts per thousand. The minimum specified salinity for commercial depuration of mussels is 19 ppt. (CEFAS 2010). Water sterilisation was achieved via UV disinfection at a rate of 10 mJ cm^{-2}, equal to the minimum doseage required for commercial depuration (CEFAS 2010). We were unable to install

ozone treatment for safety reasons. The water recharge flow rate was maintained at 30 L m^{-1} which exceeds the minimum (20 L m^{-1}) prescription (CEFAS 2010). Water quality was tested at 12 h intervals for nitrate, nitrite, ammonium and pH using an API saltwater master test kit™ (Mars Fishcare, Chalfont, USA). Mussels were loaded into the system in raised baskets containing 20 mussels which had been pre-rinsed with sterile seawater. The baskets were widely perforated on all sides, allowing free flow of water and permitting voided material to drop out of the basket. Baskets were removed carefully to avoid re-suspension of voided material, according to the time points described below.

To compare differential NoV and *E. coli* behaviour in a laboratory scale simulation, 480 mussels originating from the same batch described in the transport study were used. Eighty mussels were randomly selected and assigned to eight replicate samples of ten mussels. Four replicate samples were used for determination of baseline NoV (GI and GII) contamination and four replicate samples for *E. coli* and total coliform enumeration. These animals were transported to the laboratory at 4°C and subsequently processed fresh within 4 h of collection. NoV levels determined for these samples should correspond with those frozen for the commercial depuration investigation. A further 400 mussels were assigned to five groups of 80 mussels. This allowed for four replicate measures of both NoV and *E. coli* / coliform concentrations at each of five further time points.

Attempt was made to time-align the actual and laboratory-simulated depuration regimes i.e. for the simulation, a sampling time point was determined to coincide with the commencement of the commercial depuration. All samples in our laboratory were held at the same temperature used in export (4ºC) until this time. After the baseline at harvest, the first sample was taken halfway through the anticipated transport period for the commercial batch. The second immediately prior to depuration, the third after 12 h depuration the fourth at the end of 24 h simulated depuration. We were able to extend the simulated depuration beyond the commercial, to a time-point after 48 h. At each time point during depuration, four baskets of 20 mussels were randomly selected and carefully lifted from the system. Each basket provided one sample for bacterial analysis (10 mussels) and one for viral analysis (10 mussels).

Determination of Bacterial Concentrations in Mussels

Bacterial colony forming units (CFU) were enumerated from shellfish flesh by direct plating onto selective agar as described in Clements et al. (2013). The method differs from the standard specified under legislation 853/2004 and is undergoing validation. The Limit of Detection is yet to be determined. Only live mussels were chosen for evaluation. Ten individual mussels were removed from each basket and processed to determine bacterial concentrations. Mussel samples were rinsed with sterile seawater to remove any residual sediment and debris before surface swabbing with 100%

methanol to eliminate the shell surface biofilm. Methanol was completely evaporated at room temperature (ca. 10 min). Once dry, the mussels were aseptically opened and 50 g (wet weight) of flesh and intravalvular fluid was obtained. Mussel samples were homogenised for 60 s at 10,000 rev min^{-1} using a Bamix® blender (Seal Rock Enterprises Ltd., Bishop's Stortford, UK). From the resulting homogenate, 200 µL were plated onto Brilliance® selective agar (#CM1046; Oxoid Ltd, Basingstoke, UK) to determine both *E. coli* and total coliform counts. All plates were inverted and incubated at 37°C and bacterial colony forming units (CFU) enumerated after 24 h and expressed as CFU $100g^{-1}$.

Determination of Norovirus Concentrations in Mussels

NoV concentration in mussel digestive tissue was determined using quantitative reverse-transcription PCR (qRT-PCR) as described by Lowther et al. (2012). Modification was made to the formation of the positive extraction control and to the quencher used for the genogroup II probe (TAMRA). It should also be noted that samples derived from the laboratory depuration were processed fresh (<6 h, 4°C) but samples originating from commercial export and depuration were frozen (-20°C), transported on dry-ice, returned to -20°C and thawed once prior to dissection and Proteinase K digestion. The positive extraction controls consisted of homogenates prepared as per samples after the addition of 1 lenticule® disc of NoV Reference Material for each genogroup (HPA) to ten digestive glands. Thus the positive extraction control was positive for both genogroups and of the appropriate matrix.

Briefly, homogenates were prepared by Proteinase K digestion of pooled digestive glands, dissected from 10 mussels, after Mengovirus vMC0 was added as an extraction control. RNA extraction from these homogenates was performed with a Nuclisens® miniMAG and magnetic extraction reagents following the manufacturers' protocol. One-step qRT-PCR for mengo virus (extraction control) and for both NoV genogroups, including plate layout, and reaction mixes, was performed exactly as described by Lowther et al. (2012) except, for the genogroup II assay, TAMRA was used as the quencher. The thermocycler used was an Applied Biosystems 7900HT.

The use and treatment of a suite of qRT-PCR controls and all quantification steps also followed the same methods of Lowther et al. (2012) including assessment of extraction efficiency and RT-PCR efficiency/inhibition (using RNA external controls), calculation and reporting of results in genome copies g^{-1} (gc g^{-1}) digestive gland, retesting action thresholds for extraction and RT-PCR efficiencies or due to failed controls, and no adjustment for losses during processing or RT-PCR inhibition was made (uncorrected). Average quantities enumerated from three aliquots of extracted RNA/replicate sample give overall quantities in detectable gc g^{-1} digestive gland. This system was in agreement with the principles outlined in the draft Technical Specification developed by the joint

CEN/ISO working group for standardisation of methods for detection of viruses in foodstuffs (Lees & CEN WG6 TAG4 2010) and is in agreement with the now published standard (Anon 2013).

Results which were below the theoretical Limit of Quantification (LOQ; 100 gc g^{-1}) were not scored in the way described by Lowther et al. (2012). The raw data values are shown relative to the LOQ in all plots. This shows that to score the few results <100 gc g^{-1} at 50 gc g^{-1} could move them further from the distribution.

Statistical Analysis

Data were analysed using IBM SPSS Statistics 20 (IBM Corp., Armonk, NY) and plots were prepared using SigmaPlot v12.3 (Systat Software, Inc., San Jose California USA). Kolmogorov-Smirnov test was used to assess normality, Levene's test for equality of variance. The results of these determined the appropriate test for differences as ANOVA or Kruskal-Wallis. Where t-tests were used, *P* values were adjusted where homogeneity of variance could not be assumed. Samples deemed <LOQ for NoV were not excluded from the statistical analysis.

3. Results

Commercial Depuration

Poor weather conditions delayed the transport of the commercial batch, which remained in refrigerated transport (36 h, 4ºC) for slightly longer than expected. The quantifiable levels of NoV GI and GII in replicate samples which were frozen whole are displayed in figure 1: Immediately upon harvest (0 h), immediately prior to commercial depuration (36 h), or immediately after 24 h commercial depuration (60 h). 1 out of 7 of the post-depuration replicates returned a value below the LOQ for NoV GI with a raw-data value of 76 gc g^{-1}.

Mean values at harvest and post depuration were 187 ± 19 and 123 ± 11 gc g^{-1} for NoV GI and 345 ± 65 and 318 ± 25 gc g^{-1} for NoV GII (mean ± SEM). These figures are approximately 0.27 and 0.09 $\log_{10}$ higher than the nominal method LOQ for NoV GI and 0.54 and 0.50 $\log_{10}$ for NoV GII.

FIGURE 1

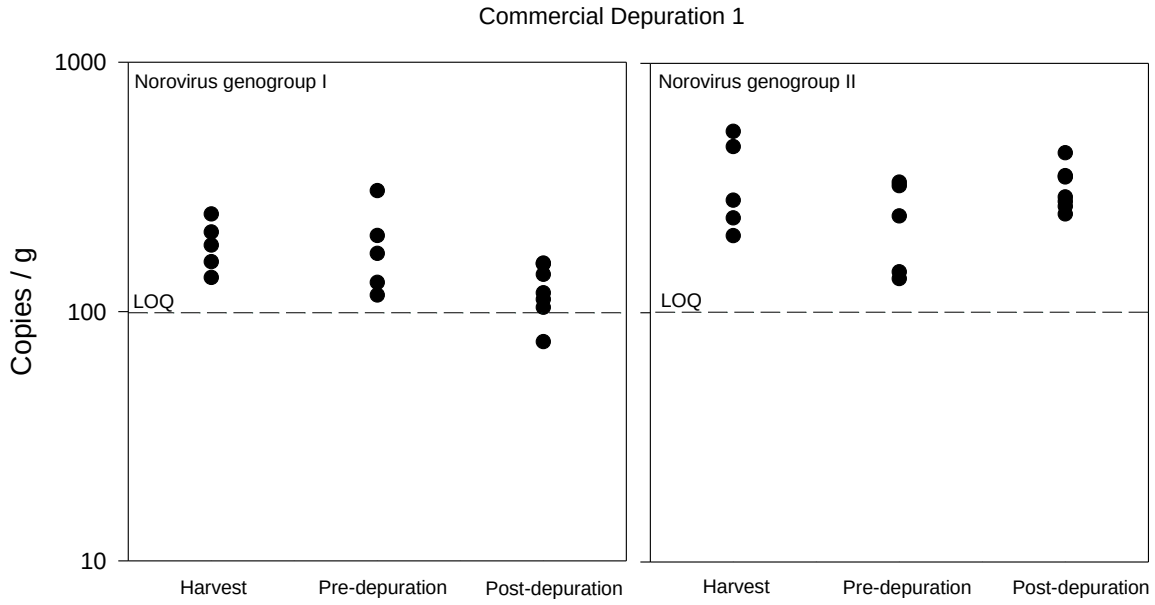


FIGURE 1. Detectable NoV (gc g^{-1}) in mussel Batch 1 at harvest, immediately prior to depuration and post-depuration, panelled by genogroup I and II. Note the y-axis is on a log scale. Dotted line represents Limit of Quantification (LoQ) for each assay (100 gc g^{-1}). Note one post-depuration replicate is sub-LOQ for GI NoV. For harvest and pre-depuration, n=5. For post-depuration, n=7.

For NoV GI, one-way ANOVA demonstrated no significant difference between the replicate samples taken at the three stages of the production chain ($F(2,14) = 3.235$ $P = 0.070$). For GII, data failed the assumptions for ANOVA test. Levene's test indicated that homogeneity of variance could not be assumed ($P = 0.027$). Kruskal-Wallis test demonstrated no significant difference in GII levels between the replicate samples taken at the three stages of the production chain ($P = 0.329$).

Figure 2 shows summed GI and GII levels for each replicate sample taken over the three sampling occasions. The single post-depuration replicate returning a < LOQ value of 76 gc g^{-1} for GI (Fig. 1) was included in the analysis and was not scored. Mean levels for the summed GI and GII data were 532 gc g^{-1} at harvest and 442 gc g^{-1} after 24 h depuration.

FIGURE 2

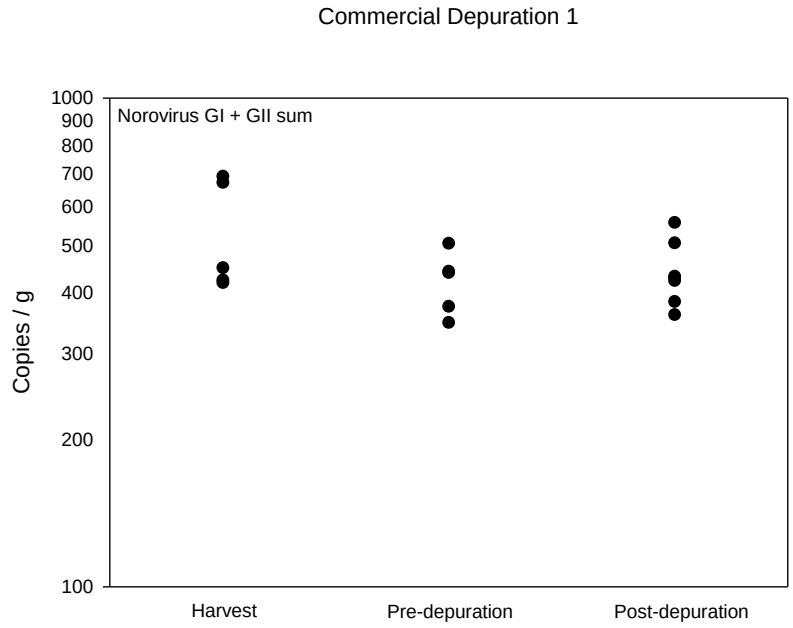


FIGURE 2. Summed NoV genogroup I and II (gc g^{-1}) in mussel Batch 1 at harvest, immediately prior to depuration and post-depuration. Note the y-axis is on a log scale. Note one sub-LOQ NoV GI replicate was included unscored in the sum. For harvest and pre-depuration, n=5. For post-depuration, n=7.

In the second batch of mussels sent for commercial depuration (i.e. with no delay in transportation), no significant difference was observed in NoV GI or GII levels between this and the previous batch harvested from the same production area (P = 0.646; P = 0.746 respectively). In addition, there was no significant difference in NoV GI or GII concentrations between fresh or frozen samples (P = 0.136; P = 0.802 respectively). The quantifiable levels of NoV GI and GII in the second batch of samples at harvest, immediately prior to depuration and after depuration are shown in Figure 3. One out of seven replicates at harvest was <LOQ for NoV GI with a raw data value of 93 gc g^{-1} and 3/7 pre-depuration replicates were also <LOQ for GI, with raw data values of 83, 74 and 45 gc g^{-1}. One out of seven post-depuration replicates was <LOQ for GII with a raw-data value of 89 gc g^{-1}.

Mean GI and GII levels at harvest were 205 ± 29 and 320 ± 35 respectively (mean ± SEM). These figures equate to 0.31 $\log_{10}$ and 0.51 $\log_{10}$ higher than the estimated nominal method LOQ for each genogroup, respectively. After depuration, mean GI and GII values were 211 ± 36 and 326 ± 61, equating to 0.32 $\log_{10}$ and 0.51 $\log_{10}$ higher than the LOQs, respectively.

FIGURE 3

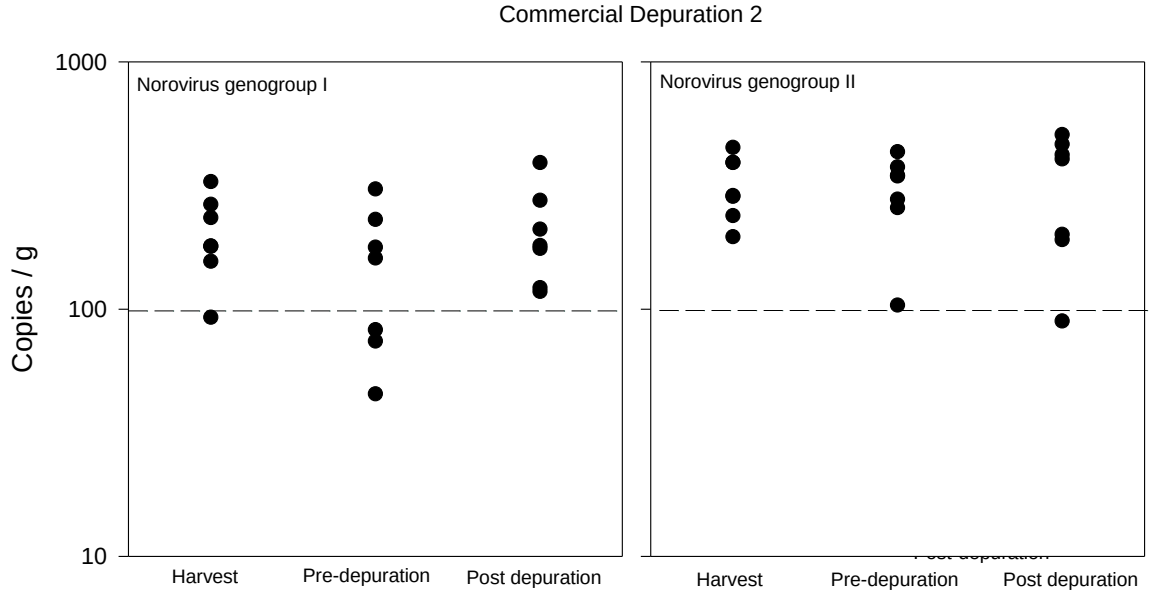


FIGURE 3. Detectable NoV (gc g^{-1}) in mussel Batch 2 at harvest, immediately prior to depuration and post-depuration, panelled by genogroup I and II. Note the y-axis is on a log scale. Dotted line represents Limit of Quantification (LoQ) for each assay (100 gc g^{-1}). All treatments, n=7.

One-way ANOVA demonstrated no significant difference in GI or GII levels between the replicate samples taken at the three stages of the production chain (F = 0.854, P = 0.442; F= 0.049, P = 0.952, respectively).

Figure 4 shows summed GI+GII levels for the three sampling occasions. Mean levels for the summed GI and GII data were 526 gc g^{-1} at harvest and 537 gc g^{-1} after depuration. One pre-depuration replicate sample with a sum-value of 149 gc g^{-1} appeared to be outside the distribution of other data but was not considered an outlier by Grubb's test. This sample returned the lowest result, both for GI (45 gc g^{-1}, <LOQ) and GII (104 gc g^{-1}), for this time point. This may have resulted from a single suboptimal vRNA extraction. Extraction efficiency according to Mengovirus recovery, for this replicate, was the lowest for this timepoint (10%). However, the extraction efficiency of this sample was not considered an outlier by Grubb's test, nor did it fail CEN criteria for extraction efficiency and it was not excluded from the analysis.

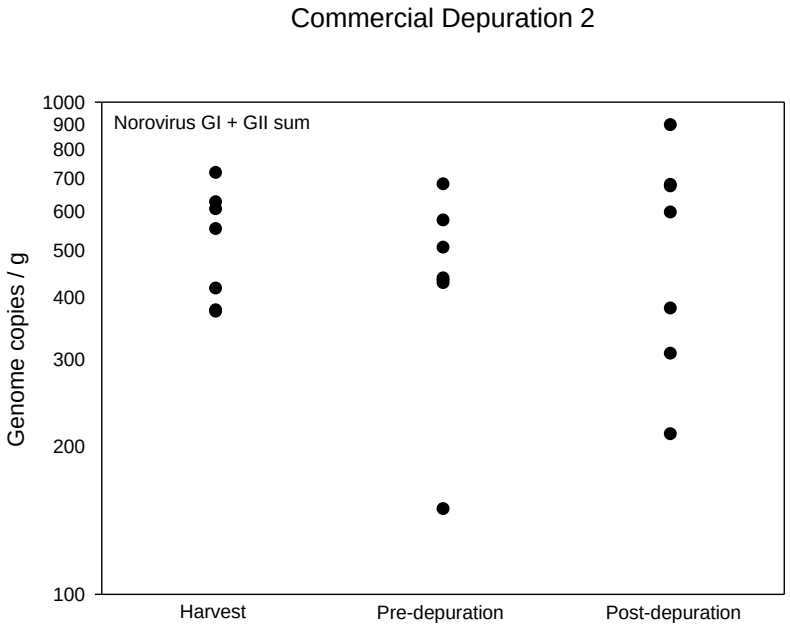


FIGURE 4. Summed NoV genogroup I and II (gc g^{-1}) in mussel Batch 2 at harvest, immediately prior to depuration and post-depuration. Note the y-axis is on a log scale. All treatments, n=7.

Laboratory Depuration Simulation

Figure 5 displays the quantifiable levels of NoV (GI and GII), *E. coli* and coliforms in replicate samples which were processed immediately upon harvest (0 h), 12 h prior to laboratory depuration, immediately prior to depuration, and after 12, 24 and 48 h of laboratory depuration.

One out of four replicate samples processed during storage, 1/4 replicates at commencement of depuration, 1/4 replicates after 12 h depuration and 1/4 replicates after 48 h depuration were sub-LOQ for NoV GI, with raw data values of 69, 99, 81 and 25 gc g^{-1} respectively. These samples were not excluded from analysis. Mean levels at harvest and after depuration were 186 ± 17 and 105 ± 27 for NoV GI and 433 ± 49 and 521 ± 72 for NoV GII (mean ± SEM). These equate to 0.27 and 0.02 $\log_{10}$ for NoV GI and 0.64 and 0.71 $\log_{10}$ for NoV GII, higher than the nominal method LOQ.

FIGURE 5

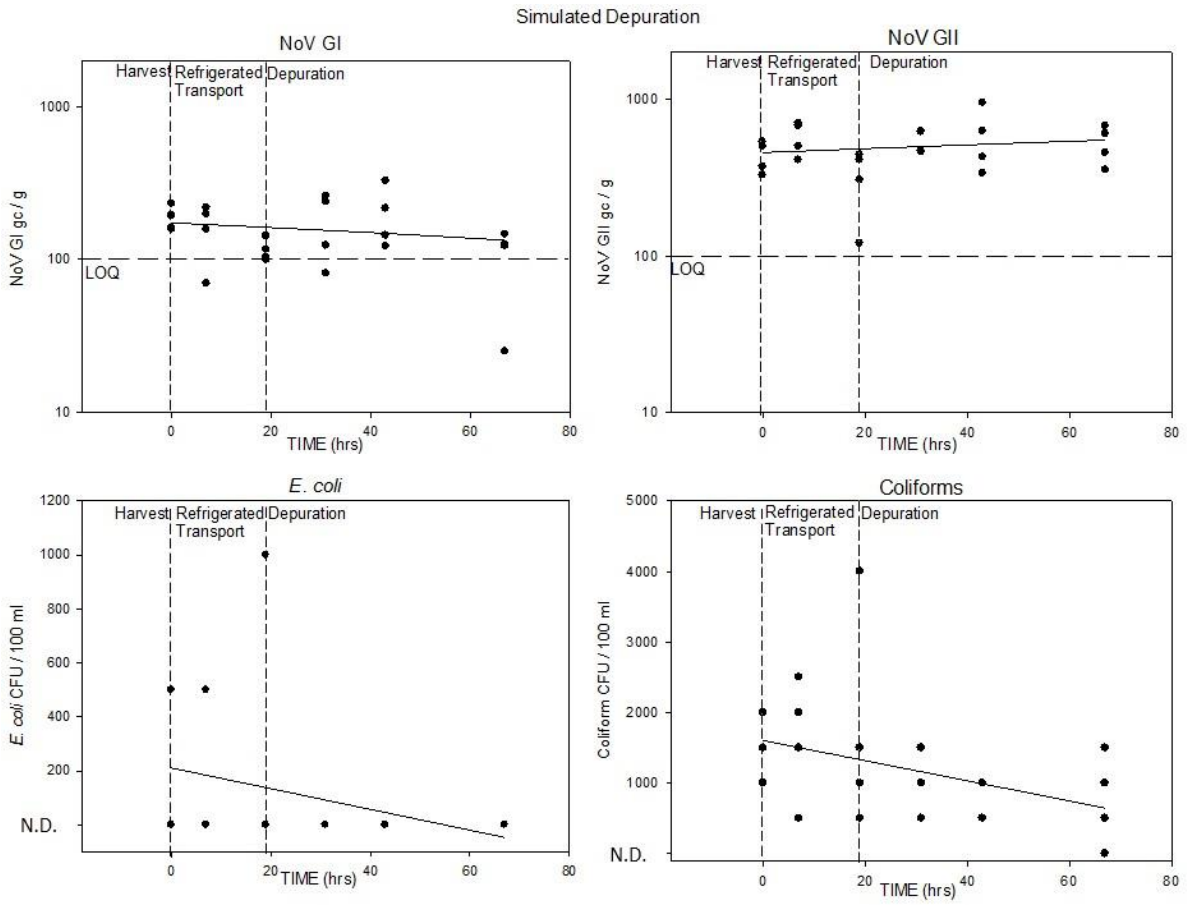


FIGURE 5. Viral and bacterial concentrations detected in mussels during the laboratory-simulated depuration. For NoV GI and GII the nominal method LOQ (100gc g^{-1}) is shown. For *E. coli* and total coliforms the *y-axis* base represents not detected (N.D.) where the limits of detection and quantification are not yet determined.

For NoV GI, there appeared to be a slight downward trend from harvest, through the refrigerated transport period, and 48 h depuration. However, one sub-LOQ result of 25 gc g^{-1} after depuration should be interpreted with caution as its true position could be much closer to the other replicates. ANOVA found no significant difference between time points (P = 0.244). For NoV GII ANOVA, found no significant difference between time points (P = 0.208).

For *E. coli*, some variability was present in the dataset. A mean concentration of 250 CFU 100 g^{-1} at harvest reduced to 125 CFU 100 g^{-1} during 7 h storage but this was not significant (P = 0.537). Immediately prior to depuration the mean *E. coli* concentration was 250 CFU 100 g^{-1} but was detected in only one of four replicates, at 1000 CFU 100 g^{-1}. The same sample provided a relatively high result for total coliform bacteria. *E. coli* could not be detected in any replicate samples after 12, 24 or 48 h but the limit of detection has not yet been determined. An increase in total coliform concentration of 1375 to 1750 CFU 100 g^{-1} during storage was not statistically significant (T-test P =

0.661). Similarly, the reduction from 1750 to 750 CFU 100 g^{-1} during 48 h depuration also proved non-significant (P = 0.280).

Animals used in the laboratory depuration originated from the same trawl as above but were dissected and digested with proteinase K immediately rather than frozen. Independent sample T-tests demonstrated no significant difference between T_0 values determined from frozen whole animals for the commercially depurated subsample, and T_0 values determined for the immediately dissected and digested samples used in the laboratory simulation (NoV GI P = 0.970; NoV GII P = 0.336 ; NoV GI+GII sum P = 0.313).

The data for harvest and post-depuration time-points for all experiments is summarised in Table 1.

Table 1: Norovirus concentration at harvest and after depuration including significance of the Log_{10} Reduction.

		Mean concentration at harvest (gc g^{-1})	Mean concentration after depuration (gc g^{-1})	Log_{10} reduction	Significance	Test
Commercial depuration Batch 1	NoV GI	187 ± 19	123 ± 11	0.18	$P = 0.070$	ANOVA
	NoV GII	345 ± 65	318 ± 25	0.04	$P = 0.329$	Kruskal-Wallis
Commercial depuration Batch 2	NoV GI	205 ± 29	211 ± 36	-0.01	$P = 0.442$	ANOVA
	NoV GII	320 ± 35	326 ± 61	-0.01	$P = 0.952$	ANOVA
Lab-Simulated Depuration	NoV GI	186 ± 17	105 ± 27	0.25	$P = 0.244$	ANOVA
	NoV GII	433 ± 49	521 ± 72	-0.08	$P = 0.208$	ANOVA

Note that negative Log_{10} reductions indicate a rise in mean levels but that no changes were determined to be statistically significant. Kruskal-Wallis test was used when data failed the assumptions for ANOVA.

4. Discussion

Use of Naturally Contaminated Mussels in Commercial and simulated Depuration

It is possible that the behaviour of NoV may differ in naturally contaminated shellfish in comparison to those exposed to a pulse addition of NoV under artificial laboratory conditions. For example, NoV exposure dose and the duration of accumulation may impact upon the subsequent depuration of NoV from shellfish tissues. Both Neish (2013) and Ueki (2007) have noted increasing NoV levels in BMS during a period following artificial exposure to contaminated water. Neish (2013) attributed this uptake lag to transport through the digestive tract prior to removal of the digestive gland for analysis. According to Neish (2013), this complicates interpretation of depuration data because it may contribute to observed lack of reduction during depuration. To overcome this, Neish (2013) incorporated a 7 d uptake period for oysters, rather than a concentrated spike, prior to undertaking depuration experiments in oysters. Naturally contaminated mussels, as used in this experiment, represent the preferred experimental system and in this situation the uptake of viral particles may be largely associated with solid particles (e.g. sorbed to unicellular algae, Bosch et al. 2005). It is highly likely that the degree of physical protection may affect the dynamics of NoV depuration.

Some depuration studies have adopted depuration methodologies which may not correspond to commercial systems (Lee and Younger 2002). In this study, naturally contaminated mussels, harvested specifically for human consumption, were intercepted and their NoV concentrations measured at time points at harvest and immediately before and after a commercial depuration procedure. Information received from the depurator of the commercial batch was that the batch would not be mixed with others before or during depuration. Hence it is not expected that cross-contamination affected these results although it is recognised that the cohort with which the samples were depurated originate from a larger geographic production area and this has the potential to affect results. Notwithstanding this, results for the laboratory simulation were similar and these animals originated from a dredge of <10 m and were not recombined with a larger batch.

Efficacy of Depuration

Norovirus

In these experiments, log reductions between harvest and after depuration of between -0.08 and 0.25 were observed (i.e. the mean level of NoV GI or NoV GII in replicate samples was slightly higher after depuration than at harvest in some cases). None of these changes, however, proved to be statistically significant. Hence it is concluded that neither of the two commercial 24 h depurations, nor the laboratory-simulated 48 h depuration (all at 4°C), was able to reduce concentrations of naturally accumulated NoV in *Mytilus edulis.*

This finding is not surprising given the difficulties in eliminating NoV (and HAV) from BMS reported by other authors. Schwab et al. (1998) only observed a 7% reduction in NoV concentrations in oyster digestive gland after 48 h of depuration. NoV persisted in oysters for 10 d at 10 °C (Ueki et al. 2007) while HAV persisted in oysters in an infective state for three weeks, and was detectable by PCR for six weeks (Kingsley & Richards 2003). But findings are not always consistent between species; For example, Abad et al. (1997) demonstrated a 98.7% reduction in infectious HAV after a 96 h depuration of *Mytilus edulis.* Similarly, the response may not be comparable between studies with the same species; Enriquez et al. (1992) and De Medici et al. (2001) demonstrated persistence for 168 and 120 h respectively also in *Mytilus edulis*. These varied findings may reflect the complexity of viral elimination from BMS. Further, the comparison of prior studies is complicated by the lack of a standard method for NoV/HAV quantification in BMS.

Recently, the European Food Safety Authority have described depuration and relaying practices as "ineffective for elimination of NoV" from oysters (EFSA Panel on Biological Hazards 2012). However, various studies have been conducted in an attempt to optimise depuration to reduce NoV. Early work by Jaykus et al. (1994) provided evidence that depuration process temperature may be an important factor for virus elimination. Reduction of NoV in oysters from a contaminated site to low levels in 4-8 d at 15-17 °C was shown to be possible after relaying to reduce initial levels (Doré et al. 1998). Further, increasing depuration tank temperatures to 18-20°C was found to be optimal for removal of FRNA bacteriophage from *C. gigas* (Doré 2003). Extending the depuration time period is also recommended for enhancing removal of viruses (Doré & Lees 1995; Muniain-Mujika et al. 2002). Increases to process temperature and time have since been recommended to depurators in the UK by the Food Standards Agency and the Shellfish Association of Great Britain to reduce NoV, but the evidence for improved elimination remains inconclusive (Neish 2013). In the present study, NoV was not significantly reduced during any commercial or simulated depuration period investigated. It is not surprising that depuration at 4°C did not reduce NoV because this is consistent with Neish's (2013) work on *C. gigas* in which there was no significant reduction in NoV over 14 d at 8 °C, reduction was more apparent at 16 °C but 59% of the initial concentration remained after 14 d. The minimum approved depuration temperature for *C. gigas* is 8 °C (CEFAS 2010). But according to Neish (2013) depuration at this temperature is ineffective in reducing norovirus levels even over a prolonged period. Other work has suggested that elevated depuration temperature may not be a viable solution for viral elimination: Norovirus and Hepatitis A Virus persisted in oysters after 23 h depuration at 20 °C, whilst significant depletion of Poliovirus was observed in the same period (Mcleod et al. 2009). NoV persisted for 29 d at 20 °C (Nappier et al. 2008) and 10 d at 10 °C (Ueki et al. 2007). Whilst Neish (2013) determined the elimination rate at 16 °C to be significantly greater than at 8 °C, the effect was "smaller than anticipated". Further experiments may show higher temperatures

to be more effective, possibly in combination with novel treatments, but it also needs to be determined whether such heating could be economically viable. In this study, the commercial depuration system used an ozone treatment. We were unable to replicate this in our simulation but neither system proved effective suggesting that ozone may have limited efficacy. Neish (2013) determined that neither ozone disinfection of water, nor direct contact with oysters, significantly improved removal of NoV from oysters compared to UV alone. Souza (2013) suggested that the two main problems for depuration, as a strategy to improve food safety, are: The UV dose required to inactivate some viruses may be high (Ko et al. 2005); some viruses (e.g. NoV) may be very hard to release from the shellfish tissues such that they are not exposed to the UV (Mcleod et al. 2009). Some of the difficulty in eliminating NoV from BMS may be explained by the specific binding of NoV to shellfish tissues (Tian et al. 2007; Le Guyader et al. 2012; Maalouf et al. 2010).

The 24 h commercial depuration investigated here was relatively short and conducted at low temperature (4°C). In the UK, this depuration procedure would not have met the CEFAS protocol for inspection and approval of depuration systems, which specifies a minimum temperature of 5°C for *Mytilus* spp and that purification must be for a minimum continuous period of 42 h without disturbance (CEFAS 2010). Unsurprisingly, no significant difference in relatively low, naturally accumulated NoV GI or GII concentrations was detected during either the commercial, nor laboratory-simulated depuration.

Faecal Coliforms

E. coli and total coliform bacteria were only enumerated from the fresh samples available in the laboratory-simulated depuration. Though considerable variability was present in the data prior to depuration, no *E. coli* was detected at the first depuration time-point (12 h depuration) or any subsequent time-point. This is not especially surprising because other workers in this research group have observed elimination of naturally accumulated *E. coli* in mussels after 6 h experimental depuration (Clements et al. 2014).

It is somewhat surprising to note, however, the absence of any significant reduction in the concentration of total coliforms during 48 h depuration. This may be explained by the very low temperature and short period but it is unclear why *E. coli* would be eliminated much faster than other coliforms.

Viral Standards

It has been noted in this investigation, that neither NoV genogroup was significantly reduced by depuration, but in our laboratory-simulation, total absence of *E. coli* was observed in <12 h. According to Neish (2013), although removal rates for *E. coli,* FRNA Bacteriophage and NoV could

all be increased by raising process temperature, only marginal removal of NoV was reported regardless of the experimental conditions investigated, whilst *E. coli* elimination was always rapid and complete. Schwab et al. (1998) showed minimal (7%) vs efficient (95%) reduction in NOV and *E. coli,* respectively, within the digestive diverticula of oysters after 48 h depuration. It is now widely noted that NoV is purged from BMS more slowly than *E. coli* during depuration. In addition, it is now quite widely accepted that BMS can be successfully purged of *E. coli* and marketed in compliance with legislation whilst continuing to contain harmful levels of other potential pathogens (Power and Collins 1989, Muniain-Mujika et al. 2002, Nappier et al. 2008, Barile et al. 2009, Nappier et al. 2010). Le Guyader et al. (2008) reported upon a shellfish-associated outbreak which occurred after heavy rain and flooding, when gastroenteritis was at a peak in the population. Prolonged depuration was instigated by IFREMER to satisfy regulations based upon *E. coli,* but failed to avoid 205 shellfish-vectored cases of infection by various enteric viruses. Doré et al. (1998; 2000) found significant numbers of commercially depurated oysters, sampled at the point-of-sale, were contaminated with NoV, despite 100% sample compliance with the regulatory *E. coli* standard. This disparity makes the use of *E. coli,* as an indicator of end-product hygiene compliance, questionable. Indeed, the use of faecal coliforms to indicate production area hygiene is also complicated because shellfish with acceptable levels of bacterial contamination at harvest may contain harmful levels of enteric virus due to the longer environmental persistence of some viral pathogens and their low infectious doses (Richards et al. 2010). It is likely that other possible bacterial indicators e.g. faecal streptococci would have similar disadvantages (Lees 2000). FRNA Bacteriophage has also shown promise as a useful viral indicator (Doré 2003) but Neish (2013) noted that its elimination kinetics were different to NoV and it could be effectively eliminated by extended depuration at an increased temperature where NoV could not. Neish (2013) notes, and I also accept, that the PCR method can detect the presence of genomic material originating from inactivated virus, and there is some potential for recontamination with signal-generating RNA persisting after recirculation. FRNA Bacteriophage does have the advantage that its infectivity can be determined using standard culture-based methods (Anon. 1995) whereas PCR may underestimate reductions in infectious virus. For example, RNA is damaged by UV but the PCR amplicon is relatively short so its presence in partially degraded genomes is possible. Capsid integrity is also important for viral infectivity and is not differentiated by PCR. However, it seems likely that depuration fails to dissociate NoV from BMS tissues because this would explain outbreaks resulting from depurated BMS, and total retention of NoV signal observed in this study.

Diversity in the pathogens and harmful contaminants which may be accumulated by BMS, and differences in their behaviour, complicate efforts to improve the safety of BMS (Kay et al. 2004). The depuration method is optimised for removal of bacteria and may be ineffective for contemporary

and emerging viral / protozoan pathogens (da Fonseca et al. 2006). Lees in (2000) suggested that improvements in methods for detecting virus in BMS be applied to the re-evaluation of depuration practices. NoV is quite readily detected and can be monitored (Henshilwood et al. 1998; Le Guyader et al. 1998). Whilst virological analysis is expensive and difficult (Pinto & Bosch 2008), it is widely noted that current regulation does not assure protection of the consumer (nor reassurance to the producer). A Europe-wide virological standard (EU Directive) has been considered and recently standardised methods (Anon 2013) are now suitable for adoption into legislation. Standardised methods will also improve the rate at which scientific knowledge can be acquired regarding accumulation / elimination of virus by BMS and other aspects of BMS-viral safety.

NoV Levels and Limits of Quantification and Detection

Mean concentrations of NoV GI or GII at time points during these experiments occurred in a range just 0.02 to 0.71 $\log_{10}$ higher than the nominal method LOQ for the respective assays and some time-points included replicate measures which were technically <LOQ raising complications in the interpretation of data. However, these levels fall at the lower end of a wide range of concentrations detected by our laboratory for this production area, and are not unusual. Indeed, this investigation was, in part, initiated because of a discrepancy between our results and those of the wholesale depurator's chosen commercial laboratory. We frequently detect NoV, often at levels well in excess of our adopted method's (ISO TS 15216) LOQ, in this production area whilst the depurator claims to possess negative test results for all depurated batches derived from it. This generated interest because the literature regards depuration as generally ineffective for NoV elimination and we showed this to be applicable to the process investigated. Whilst the falsification of test results is a possibility, it is likely that the laboratory used by the depurator applies one of several less sensitive assays to detect NoV. Such assays may be insensitive to NoV levels representing infectious doses.

It is not clear whether the relatively low, and method LOQ-proximate, levels which were observed to persist through depuration in this study, represent a significant threat to human health. This highlights difficulties faced by authorities in determining a limit which is protective of human health and to which compliance can be reliably determined. Low concentrations of human viruses in shellfish represent a health risk (Pinto & Bosch 2008) but recent research has suggested that the 50% human infectious dose for NoV may not be as low as it was previously thought (Atmar et al. 2014). However, there are complications in converting between PCR-detectable gc g^{-1} of digestive gland, the tissue targeted; and infectious virions consumed per food portion. These include variables including portion size and the amount of virus which may be present outside the digestive gland. As fore-mentioned, the standard quantitative method also suffers from an inability to differentiate between infectious/inactivated virus. However, Lowther et al. (2010) report that evidence for the existence of a

dose-response for RNA levels in BMS is supported by the fact that the geometric mean levels in outbreak samples (1,048 gc g^{-1}) are significantly greater than in non-outbreak related samples (121 gc g^{-1}). Further, NoV outbreaks are relatively few compared with the frequency of NoV detection in shellfish products. Importantly, recent research (Flannery et al. 2014) has shown that cooking shellfish can effectively eliminate concentrations of infective virus.

Effect of Freezing

No significant effect of freeing upon detectable NoV concentrations was detected in this investigation suggesting that freezing effectively preserves NoV within shellfish tissues. This can facilitate the logistics of larger experiments and when tracking BMS which may be traded globally. However, until further research properly tests whether freezing of shellfish or shellfish samples can significantly affect PCR-detectable gc g^{-1}, caution should be taken in the comparison of fresh and frozen samples. Use of freezing should be included in methodological statements.

5. Conclusion

This investigation has clearly demonstrated failure of a commercial depuration system to reduce NoV concentrations in *Mytilus edulis* harvested from a sewage contaminated production area. However, our knowledge of the factors regulating the elimination of NoV from mussels remains limited and further experimental studies are urgently required in this area. We conclude that after depuration the mussel shipment investigated could pose a health risk if not thoroughly cooked prior to consumption.

6. Acknowledgements

This work was supported by the European Social Fund (ESF) through the European Union's Convergence program administered by the Welsh Government in association with Bangor Mussel Producers and Deepdock Ltd. Bacteriological analysis of mussel samples was conducted by KC and the data provided to JBW.

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CHAPTER 6

Effectiveness of cooking to reduce norovirus and infectious F-specific RNA bacteriophage concentrations in *Mytilus edulis*

Manuscript published in Journal of Applied Microbiology

Author contributions: JBW conceived the experiment to complete this thesis. JBW, JF and PRN contributed to the experimental design. JF propagated FRNA bacteriophage and provided norovirus positive faecal suspension. JBW arranged and conducted the bioaccumulation. JBW, JF and PRN performed the cooking experiments and subsequent dissections. JF performed the bacteriophage plaque assay. JBW and PRN performed the qRT-PCR assays. JF performed data analysis. JBW, JF and PRN contributed to the manuscript reviewed by all authors.

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Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Effectiveness of cooking to reduce Norovirus and infectious F-specific RNA bacteriophage concentrations in *Mytilus edulis*

J. Flannery*, P. Rajko-Nenow*, J.B. Winterbourn*, S.K. Malham and D.L. Jones

Bangor University, Bangor, UK

Keywords
cooking, FRNA bacteriophage, mussels, norovirus, risk management, RT-qPCR.

Correspondence
John Flannery, Bangor University, Bangor, Gwynedd LL57 2UW, UK.
E-mail: j.flannery@bangor.ac.uk

*These authors contributed equally to this study.

2014/0423: received 27 February 2014, revised 7 April 2014 and accepted 24 April 2014

doi:10.1111/jam.12534

Abstract

Aims: The aim of this study was to determine if domestic cooking practices can reduce concentrations of norovirus (NoV) and F-specific RNA (FRNA) bacteriophage in experimentally contaminated mussels.

Methods and Results: Mussels ($n = 600$) contaminated with NoV and FRNA bacteriophage underwent four different cooking experiments performed in triplicate at ~70°C and >90°C. Concentrations of infectious FRNA bacteriophage (using a plaque assay) were compared with concentrations of FRNA bacteriophage and NoV determined using a standardised RT-qPCR. Initial concentrations of infectious FRNA bacteriophage (7·05 $\log_{10}$ PFU g^{-1}) in mussels were not significantly reduced in simmering water (~70°C); however, cooking at higher temperatures (>90°C) reduced infectious FRNA bacteriophage to undetected levels within 3 min. Further investigation determined the time required for a 1-log reduction of infectious FRNA bacteriophage at 90°C to be 42 s therefore a >3-log reduction in infectious virus can be obtained by heating mussel digestive tissue to 90°C for 126 s.

Conclusions: Domestic cooking practices based on shell opening alone do not inactivate infectious virus in mussels, however, cooking mussels at high temperatures is effective to reduce infectious virus concentrations and the risk of illness in consumers.

Significance and Impact of the Study: The data will contribute towards evidence-based cooking recommendations for shellfish to provide a safe product for human consumption.

Introduction

The contamination of shellfisheries with enteric viruses arising from wastewater discharges represents a health risk to consumers as shellfish can bioaccumulate these viruses from the surrounding waters (Lees 2000). Human norovirus (NoV) of the family *Caliciviridae* is the most common cause of non-bacterial gastroenteritis transmitted by bivalve molluscan shellfish (Bellou *et al.* 2013). NoV represents a genetically diverse genus of positive-sense RNA viruses of which NoV genogroup I (GI) and genogroup II (GII) are frequently implicated in outbreaks of shellfish-borne illness (Bellou *et al.* 2013). Regulations to mitigate the risk of illness from the consumption of faecally contaminated shellfish are often based on *Escherichia coli* monitoring. However, *E. coli* has been shown to be a poor indicator of NoV contamination in shellfish (Doré *et al.* 2003; Flannery *et al.* 2009) and outbreaks of NoV illness associated with shellfish consumption continue to occur worldwide (Bellou *et al.* 2013).

Currently, the detection of NoV in environmental samples relies on molecular techniques and a standardised RT-qPCR method for the detection of NoV in foodstuffs has been recently published (Anonymous 2013). While RT-qPCR does not distinguish between infectious and non-infectious virus particles, it has been used to demonstrate that the risk of NoV illness associated with oyster consumption increases with greater concentrations of NoV genome copies present (Lowther *et al.*, 2012). The standardized RT-qPCR method, therefore, can provide a

suitable index of the risk of NoV illness to oyster consumers, and is recommended by the European Food Safety Authority (EFSA) to provide an additional control to mitigate the risk posed by NoV-contaminated shellfish (EFSA 2012). However, the risk posed by NoV-contaminated shellfish may vary since oysters are commonly consumed raw, while other bivalves such as mussels may be cooked prior to consumption.

An early study investigating virus inactivation in clams showed that cooking at temperature of >85°C for 3 min was sufficient to inactivate poliovirus and hepatitis A virus (HAV; Millard *et al.* 1987). This informed recommendations by the UK Ministry of Agriculture, Fisheries and Food that the cooking of bivalve molluscs should achieve a minimum internal temperature of 90°C maintained for at least 90 s (Waterman 2001). Few studies concerning *Mytilus edulis* exist (Croci *et al.* 2005) and much data have been extrapolated from other species such as cockles (Millard *et al.* 1987), clams (Toffan *et al.* 2014) or mussel species not farmed in the United Kingdom (Hewitt and Greening 2006; Croci *et al.* 2012). In 2011, EFSA recommended that further investigation be carried out to determine the effectiveness of processing for shellfish species (EFSA 2011).

This study aims to investigate whether domestic cooking practices can effectively reduce infectious virus concentrations in NoV-contaminated mussels. The standardized RT-qPCR assay ISO15216 was used to determine concentrations of NoV GI, NoV GII. In the absence of a cell-culture method for NoV (Papafragkou *et al.* 2013) F-specific RNA (FRNA) bacteriophage of the family *Leviviridae* would be used to provide information on infectious virus reduction during cooking. In mussels, we directly compared the concentration of a human-specific Levivirus (FRNA bacteriophage GA) determined by RT-qPCR with those determined using a plaque assay to characterize the potential overestimation of infectious virus during cooking experiments. The data should go towards evidence-based recommendations for cooking mussels to provide a safe product for consumption.

Materials and methods

Preparation of virus stocks

FRNA bacteriophage GA was propagated in *Salmonella enterica serovar Typhimurium* WG49 host by broth enrichment (Anonymous 1995). Briefly, following 6 h incubation, host cells were centrifuged at 2000 *g*, and the supernatant stored at 5°C until use (within 2 h). Concentrations of FRNA bacteriophage GA in the aliquots were 7·32 $\log_{10}$ plaque forming units (PFU) ml^{-1} and 7·56 $\log_{10}$ genome copies ml^{-1} determined using the plaque assay and RT-qPCR assay respectively. For NoV, two separate stool samples, one positive for NoV GI and the other for NoV GII, were diluted 20% w/v with PBS to a volume of 20 ml, and contained 3·59 $\log_{10}$ and 6·76 $\log_{10}$ genome copies ml^{-1} for NoV GI and NoV GII respectively.

Bioaccumulation experimental set-up

A total of 600 blue mussels (*Mytilus edulis*) collected from a commercial harvesting area were acclimatized in two aerated tanks each containing 30 l of UV disinfected seawater, located in a darkened, temperature controlled room at 10 ± 1°C. The seawater was recirculated using a submersed water pump (Micro-Jet MC450; Aquarium Systems, Sarrebourg, France). To each acclimatization tank, 3·5 g yeast extract (Oxoid, UK) was added as a source of food for the mussels ($n = 300$) as used by Millard *et al.* (1987). Following acclimatization (48 ± 2 h), a sample of 10 mussels was analysed as a negative control. The remaining mussels ($n = 590$) were divided evenly between two 30-l tanks containing fresh seawater and 3·5 g yeast extract each. The same batch of seawater (salinity 35 ppt, dissolved oxygen 8·2 mg l^{-1}) was used during acclimatization and bioaccumulation. The two bioaccumulation tanks were spiked with 10 ml of previously prepared NoV faecal material suspensions and 10 ml FRNA bacteriophage GA culture each. Mussels were allowed to bioaccumulate for 24 ± 2 h at 10 ± 1°C.

Sampling set-up for mussel cooking studies

Following bioaccumulation, a sample of eight mussels was removed from each tank (tank 1 and tank 2) and processed for viral analysis immediately to determine viral concentrations prior to the cooking experiments. Mussels ($n = 204$) from tank 1 were used in experiments A and B, and mussels ($n = 204$) from tank 2 were used in experiments C and D.

The cooking and sampling parameters for each of the experiments (A, B, C and D) are shown in Table 1. Each experiment was performed in triplicate and each replicate consisted of 34 virus-contaminated mussels placed into a 35 cm diameter domestic cooking pan containing 400 ml of molecular biology grade water so that all mussels were fully submerged. Two contaminated mussels per replicate experiment were used for temperature monitoring during the experiments. The temperature of a closed mussel, an open mussel and the cooking water was recorded every 15 s for the duration of each cooking experiment using thermocouples (960 mm K-type wire; Maplin, UK) that were connected to digital multimeters (Philex Electronic, Bedford, UK). A 2 mm hole was drilled into the shell of

Table 1 Experimental design of the mussel cooking experiments

Experiment	Cooking power used	Purpose	Initial temperature of water	Sampling interval after 50% open mussels (t_0)
A	Low	Lightly simmering	Ambient (20°C)	1 min
B	Low	Lightly simmering	Preheated (~70°C)	1 min
C	High	Boiling	Ambient (20°C)	2 min
D	High	Boiling	Preheated (>90°C)	2 min

a mussel through which a thermocouple was placed into flesh of the mussel and the valves were kept closed using a plastic cable tie. A second thermocouple was placed within the digestive tissue (DT) of an open mussel and was secured with a cable tie. A third thermocouple was placed within the cooking liquid (molecular biology grade water).

Two different cooking practices that involved cooking mussels in pre-heated water or from water at ambient temperatures to a pre-determined final temperature were investigated (Table 1). Using a low or high setting on the cooking hob, final water temperatures of either ~70°C or >90°C were achieved to simulate inadequate or adequate cooking temperatures respectively. Throughout each of the cooking experiments, the pan was covered with a lid except when mussels were removed per sampling point. For experiments A and C, turning on the hob to the pre-determined low or high setting was considered the commencement of the experiment. For experiments B and D, the mussels were added to the water once it had reached the pre-determined temperature achieved by the high or low setting and this was considered the start. During the cooking experiments, the first mussel sample consisting of eight animals was removed from the pan and immediately placed on ice when 50% of the mussels had opened (t_0). Subsequent mussel samples were removed at time intervals t_1, t_2 and t_3 respectively (Table 1). Immediately after collection, the eight mussels were placed on ice and divided into two samples consisting of four mussels each. At each sampling point, one mussel sample was analysed for NoV GI, NoV GII, and FRNA bacteriophage GA using the RT-qPCR assay, whereas the second mussel sample was analysed for FRNA bacteriophage using the plaque assay.

Virus inactivation within digestive tissues during cooking

Following bioaccumulation, the DT from the remaining mussels ($n = 150$) were dissected, finely chopped and then pooled together. One gram of DT was placed into each of forty-three 1·5 ml sterile micro-centrifuge tubes to be used in the inactivation experiment. One tube containing 1 g of non-heat-treated DT was used to determine the initial virus concentrations of the pooled DT. Heat treatment of the mussel DT was carried out in a heating block (Fisher Scientific Ltd., Loughborough, UK) set to 90°C. Prior to experiments, the thermal homogeneity and heat-up times of the heating block was verified using a thermocouple that was placed into 1·5 ml tubes containing 500 μl water in the wells of the heating block. The average time required for the 500 μl water to reach 90°C was determined to be 120 ± 5 s. For each replicate experiment, 14 tubes containing 1 g of DT each were placed into the heating block. One tube was fitted with a thermocouple probe and was used to measure the internal temperature of the DT throughout the experiment. After turning on the heating block, one tube was removed at 60 s, another at 120 s and the remaining eleven tubes were removed at 20-s intervals. All tubes were immediately placed on ice to prevent further virus inactivation, and were then split into two aliquots of 500 mg DT for infectivity and RT-PCR analysis. The heat treatment experiment was performed on three occasions.

Infectious FRNA bacteriophage GA analysis

For infectious FRNA bacteriophage GA analysis, the DT from four mussels per sampling point in the cooking experiments were dissected and finely chopped. The DT from mussels used in the cooking and heat block experiments was diluted 1 : 3 with 0·1% (wt/vol) neutralized bacteriological peptone (Oxoid, UK) and centrifuged at 2000 *g* for 10 min. The supernatant was retained for analysis within 24 h using a double agar layer method (Anonymous 1995). Briefly, host cells of *S.* Typhimurium WG49 were grown at 37°C to yield >10^6 CFU ml^{-1} in tryptone yeast-extract glucose broth. Cells were mixed with 2·5 ml of tryptone yeast-extract glucose 1% agar and 1 ml of appropriately diluted mussel DT supernatant. This mixture was poured onto the hard agar (tryptone yeast-extract glucose 2% agar) and incubated overnight at 37°C. FRNA bacteriophage GA were identified as semi-transparent plaques that were then counted and multiplied by the dilution factor to obtain the titre in PFU g^{-1}. The theoretical limit of detection (LOD) of the assay was determined to be 10 PFU g^{-1}.

Virus extraction from mussel DT for RT-qPCR analysis

From the cooking experiments, the total DT from the four mussels per sampling point was dissected, weighed and finely chopped. From the heating block experiments,

the total 500 mg DT was used for virus extraction. An equal volume to gram of proteinase K solution (100-μg ml^{-1}; 30 U mg^{-1}; Sigma-Aldrich, Gillingham, UK) was added to the mussel DT. Ten microlitres of Mengo virus strain MC_0 was added to the DT/proteinase K mix to act as an internal positive control (IPC) virus to control for extraction efficiency. The sample was then incubated at 37°C with shaking at 300 rev min^{-1} for 1 h followed by incubation at 60°C for 15 min. Following centrifugation at 3000 *g* for 5 min, the supernatant volume was recorded and stored at 4°C until RNA extraction was carried out (within 24 h).

Viral RNA extraction

Viral RNA was extracted from 500 μl faecal material suspension, FRNA bacteriophage GA stock and mussel DT proteinase K extract using the NucliSENS® miniMAG® and magnetic extraction reagents (bioMérieux, Marcy lÉtoile, France) following the manufacturer's instructions. Molecular biology grade water was processed alongside samples to act as a negative RNA extraction control. RNA was eluted into 100 μl of elution buffer that was stored at −80°C until RT-qPCR analysis was undertaken (<1 week).

RT-qPCR assay for NoV and FRNA bacteriophage GA

A Quantstudio six instrument (Life Technologies, Paisley, UK) was used for RT-qPCR analysis. Twenty microlitres of the NoV GI, NoV GII or FRNA bacteriophage GA reaction mix was prepared using RNA Ultrasense one-step qRT-PCR system (Invitrogen, Paisley, UK) containing 1× reaction mix, 500 nmol l^{-1} forward primer, 900 nmol l^{-1} reverse primer, 250 nmol l^{-1} probe, 1× μl Rox and 1·25 μl of enzyme. Previously described primers QNIF4 (Da Silva *et al.* 2007), NV1LCR (Svraka *et al.* 2007) and probe TM9 (Hoehne and Schreier 2006) were used for NoV GI analysis, and primers QNIF2 (Loisy *et al.* 2005), COG2R (Kageyama *et al.* 2003) and probe QNIFS (Loisy *et al.* 2005) used for NoV GII analysis. For FRNA bacteriophage GA analysis, previously described Levivirus genogroup II forward and reverse primers and probe were used (Wolf *et al.* 2008). Duplicate 5-μl aliquots of sample RNA were added to adjacent wells of a 96-well optical reaction plate in addition to no template controls. IPC extraction efficiency using mengo virus strain MC_0 and RT-qPCR inhibitors using NoV RNA transcripts were controlled for each virus as previously described (Flannery *et al.* 2012). Samples with an EC RNA amplification efficiency of >25% and an IPC extraction efficiency of >1% were considered acceptable and results were not adjusted for losses during processing or RT-PCR inhibition.

NoV GI and GII reaction conditions were as follows: an initial incubation at 55°C for 60 min followed by 95°C for 5 min and then 45 cycles of 95°C for 15 s, 60°C for 1 min and finally 65°C for 1 min. For FRNA bacteriophage GA, reaction conditions involved an initial incubation at 55°C for 30 min, followed by 95°C for 5 min and then 45 cycles of 95°C for 15 s and 58°C for 1 min. NoV and FRNA bacteriophage GA were quantified by comparing the mean Cq value to the dsDNA standard curves in copies μl^{-1}, and then adjusted to reflect the volume of RNA analysed (expressed as genome copies g^{-1} DT). The LOD of the assay was determined as 20 detectable genome copies g^{-1} DT.

Calculation of $\log_{10}$ reduction value during cooking

To determine the $\log_{10}$ decrease in detectable virus during the cooking experiments, initial virus concentrations in the non-heat treated mussels were expressed as C_0. The concentrations of detectable virus at all subsequent times were expressed as C_{t0}, C_{t1}, C_{t2} and C_{t3}. The log reduction in detectable virus was described by the formula $-\log_{10}\left(\frac{C_{t(n)}}{C_0}\right)$. Data were assessed for normality and virus concentrations were $\log_{10}$ transformed prior to statistical analysis using SigmaPlot software ver. 11 (Systat Software, Chicago, IL).

Results

Virus concentrations in mussels

Concentrations of NoV GI and NoV GII in the mussels before bioaccumulation were <LOD and 1·12 $\log_{10}$ genome copies g^{-1} DT respectively. FRNA bacteriophage concentrations in these mussels were <LOD and 1·20 $\log_{10}$ genome copies g^{-1} DT using the plaque assay and RT-qPCR assay respectively. Following bioaccumulation, concentrations of NoV GI (2·81 $\log_{10}$ genome copies g^{-1} DT) and NoV GII (6·18 $\log_{10}$ genome copies g^{-1} DT), infectious FRNA bacteriophage (6·85 $\log_{10}$ PFU g^{-1} DT) and FRNA bacteriophage using RT-qPCR (6·87 $\log_{10}$ genome copies g^{-1} DT), were detected in the mussels.

Temperatures and virus reduction during cooking experiments

Figure 1 shows the temperatures of the cooking water, a closed mussel and an open mussel recorded during the four cooking experiments. Experiments A and B were carried out using lightly simmering water, while experiments C and D used boiling water. The time required for 50% of the mussels ($n = 16$) to open (t_0) and associated water temperature was $t_0 = 7·75$ min; 70 ± 3°C

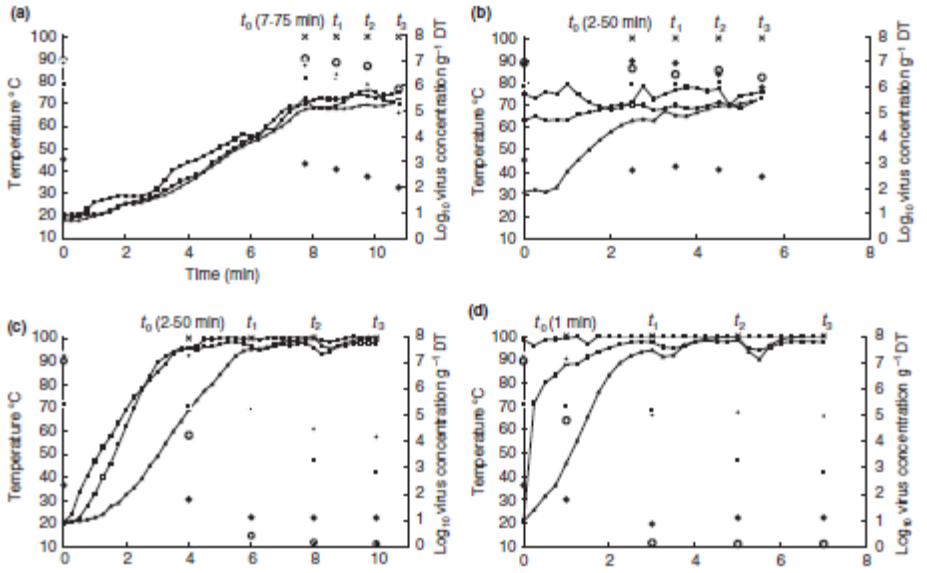

Figure 1 Temperature and sampling time points in the cooking experiments. Mean temperatures recorded in the cooking water (■), an open mussel (□) a closed mussel (▲) and mean $\log_{10}$ concentrations of norovirus (NoV) GI (♦), NoV GII (■) and F-specific RNA bacteriophage detected using the RT-qPCR assay (●) and plaque assay (○) are shown in figures a–d for experiments a–d respectively. The time required for 50% of mussels to open (t_0) is indicated (○) as are subsequent sampling times (t_1–t_3).

(experiment A) and t_0 = 2·50 min; 71 ± 4°C (experiment B). The internal temperature of a closed mussel at t_0 was 68 ± 3°C and 64 ± 3°C in experiments A and B respectively. Experiments C and D used a higher hob power setting, and a mean water temperature of 90 ± 3°C was reached after 3·25 min in experiment C and had been reached prior to the start of experiment D. During experiment C, t_0 occurred at 4 min (in all three experiment replicates) and a mean internal temperature of the closed mussel was 69 ± 5°C, while in experiment D t_0 was reached in 1 min with a mean internal temperature of the closed mussel being 44 ± 4°C.

The concentrations of viruses in mussels during the cooking experiments are shown in Fig. 1. Infectious FRNA bacteriophage were reduced by 1·19 $\log_{10}$ PFU g^{-1} DT and 0·36 $\log_{10}$ PFU g^{-1} DT in experiments A and B respectively. This reduction was not significant (P = 0·11 and P = 0·09), and at the conclusion of experiments A and B, infectious FRNA bacteriophage were detected at 5·85 ± 0·09 $\log_{10}$ PFU g^{-1} DT and 6·35 ± 0·36 $\log_{10}$ PFU g^{-1}DT respectively. Infectious FRNA bacteriophage were reduced to undetected levels after 8 min and 3 min from the beginning of experiment C and experiment D respectively (Table 2). Initial concentrations of infectious FRNA bacteriophage (7·05 ± 0·02 $\log_{10}$ PFU g^{-1} DT) were significantly reduced to 4·23 ± 0·3 PFU g^{-1} DT; in experiment C (P < 0·05) and to 4·79 ± 0·07 $\log_{10}$ PFU g^{-1} DT in experiment D (P < 0·01) at the time required for 50% of mussels to open. In experiment C, all viruses determined using RT-qPCR were reduced by >99% following a total cooking time of 8 min. In experiment D, concentrations of NoV GI were reduced from 1·93 ± 0·05 $\log_{10}$ genome copies g^{-1} DT to <LOD in 5 min total cooking time, while NoV GII were reduced from 5·51 ± 0·22 to 3·99 ± 0·27 $\log_{10}$ genome copies g^{-1} DT (96·44%) at the same time.

Thermal inactivation of viruses in mussel DT

Using RT-qPCR, initial concentrations of NoV GI, NoV GII and FRNA bacteriophage were 2·33, 5·44 and 7·43 $\log_{10}$ genome copies g^{-1} DT respectively. Similar concentrations were still detected at 200 s and no significant reduction was observed for each virus (1-way ANOVA; P > 0·37). However, infectious FRNA bacteriophage concentrations were reduced by 3·35 ± 0·03 $\log_{10}$ PFU g^{-1}

Table 2 Concentrations of viruses in mussels during cooking experiments C and D

	Cooking time		Log_{10} virus concentration per g ± SD (% reduction)			
	Total (min)	From 50% open mussles, min (t_x)	Infectious FRNA	FRNA RT-qPCR	NoV GI RT-qPCR	NoV GII RT-qPCR
Experiment C	4	0 (t_0)	4·23 ± 0·30 (99·85)	7·25 ± 0·31 (0·00)	1·79 ± 0·34 (71·33)	5·30 ± 0·39 (15·48)
	6	2 (t_1)	0·42 ± 0·17 (>99·99)	4·83 ± 0·90 (99·60)	0·74 ± 0·66 (97·40)	5·22 ± 0·13 (39·91)
	8	4 (t_2)	Undetected (>99·99)	4·79 ± 0·58 (99·63)	Undetected (99·41)	3·32 ± 0·21 (99·23)
	10	6 (t_3)	Undetected (>99·99)	4·40 ± 0·89 (99·85)	Undetected (99·41)	2·85 ± 0·41 (99·74)
Experiment D	1	0 (t_0)	4·79 ± 0·07 (99·45)	7·13 ± 0·14 (20·74)	1·93 ± 0·05 (59·78)	5·51 ± 0·22 (16·32)
	3	2 (t_1)	Undetected (>99·99)	4·92 ± 0·35 (99·50)	1·34 ± 0·28 (89·79)	5·25 ± 0·59 (35·47)
	5	4 (t_2)	Undetected (>99·99)	3·62 ± 0·03 (99·98)	Undetected (99·41)	3·99 ± 0·27 (96·44)
	7	6 (t_3)	Undetected (>99·99)	4·96 ± 0·14 (99·46)	Undetected (99·41)	3·66 ± 0·30 (98·34)

FRNA, FRNA bacteriophage; SD, standard deviation; NoV, norovirus.

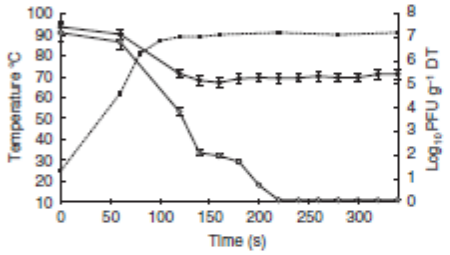

Figure 2 Reduction of infectious F-specific RNA (FRNA) bacteriophage in mussel hepatopancreas after incubation at 90°C. Shown are the mean ($n = 3$) internal temperature of the mussel HP (□) and log_{10} concentrations of infectious FRNA bacteriophage (○) and FRNA bacteriophage detected using RT-qPCR (●) during the heat-block experiments. Error bars represent the standard error of the mean.

DT from 7·19 ± 0·03 to 3·84 ± 0·01 log_{10} PFU g^{-1} DT (99·99% reduction) during the time required for the mussel DT to reach 90°C (100 s). Mean concentrations of infectious FRNA bacteriophage in mussel DT were reduced to undetectable levels within 220 s from the beginning of the experiment (Fig. 2). From regression analysis of C_t/C_0, the T_{90} value for infectious FRNA bacteriophage in mussel DT at 90°C was calculated to be 42 s.

Discussion

The risk of NoV illness from the consumption of contaminated shellfish when consumed raw or lightly cooked has been well documented (Lees 2000; Bellou *et al.* 2013). As current bacteriological standards are inadequate to indicate the risk of NoV illness, it seems likely that the recently standardized RT-qPCR method for NoV detection in foodstuffs (Anonymous 2013) will be used in a routine context. Monitoring of shellfisheries using the RT-qPCR assay may provide a suitable mitigation option for reducing the risk of NoV-contaminated shellfish from being placed onto the market and subsequent illness in consumers (EFSA 2012). However, limits based on NoV genome copies may not be entirely applicable to all shellfish species, particularly those that are intended to undergo cooking prior to consumption.

In 2011, EFSA recommended that post-harvest treatments for shellfish be shown to be effective and consistent in reducing virus concentrations prior to consumption (EFSA 2011). This was reflected in the Codex Alimentarius Commission guidelines 79-2012 (Anonymous 2012) which suggested that heat treatments of bivalve molluscs be further validated for their ability to inactivate viruses. While cooking is recommended to improve the sanitary quality of shellfish, a balance is often struck to retain the organoleptic quality of the shellfish (Richards *et al.* 2010) and domestic cooking procedures often use shell opening as an indication that the shellfish have been adequately cooked. We found that basing cooking processes on shell opening alone is insufficient to inactivate viruses and indicate safety.

The results from our experiments demonstrated that cooking mussels in boiling liquid (at a water temperature of >90°C) is more effective in reducing infectious virus concentrations than cooking in lightly simmering water (<76°C). We investigated inadequate cooking temperatures to simulate a worst-case scenario in a domestic setting and compared the reduction in viruses with those achieved using adequate cooking temperatures. In the inadequate cooking experiments using simmering water, the mean internal temperature of mussels ranged between 64 and 68°C at which point 50% had opened. However, the mussels still contained high concentrations of infectious FRNA bacteriophage despite all having opened at the conclusion of the experiment. Similarly, concentrations of NoV GI, NoV GII and FRNA bacteriophage

using the RT-qPCR assay were not significantly reduced when cooked in simmering water. However, placing mussels into boiling water for a period of at least 3 min ensured that the internal temperatures of the closed mussels reached 90°C and reduced infectious FRNA bacteriophage (a 7-log reduction) to undetectable levels. The reduction of infectious FRNA bacteriophage in mussels is consistent with that of Hewitt and Greening (2006) who found that infectious HAV was reduced to undetected levels (a 3·5 log reduction) in New Zealand Greenshell Mussels following 3 min in boiling water. This finding demonstrates that high cooking temperatures are necessary to completely inactivate viruses in mussels. Lighter cooking practices represent a potential risk of NoV transmission to consumers.

To further determine the thermal inactivation kinetics of infectious FRNA bacteriophage, mussel DT was placed in a pre-heated block set at 90°C and the time required for a 1-log reduction was calculated to be 42 s. Within the time taken for the mussel DT to reach 90°C, concentrations of infectious FRNA bacteriophage were significantly reduced (>1-log) and were reduced to undetected levels after 120 s at this temperature. Using spiked mussel DT placed into a heat block, Croci *et al.* (2012) found that infectious feline calicivirus was still detected after 15 min at 80°C. Sow *et al.* (2011) showed that murine NoV and HAV was reduced to undetected levels (a 5·4 log reduction) in DT from soft-shell clams that were heated to an internal temperature of 90°C for 3 min. As the results in this study are in agreement with previously published (2006) therefore seems that a temperature of >90°C provides the minimum temperature to ensure a significant reduction in the infectious viral load of mussels. In general, our results using infectious FRNA bacteriophage are within the ranges reported for other NoV surrogates and considering the ease of use of FRNA bacteriophage make them suitable indicators for further studies.

A significant discrepancy between the RT-qPCR and the plaque assay occurred during the thermal inactivation of FRNA bacteriophage in mussel DT. The RT-qPCR assay overestimated the concentrations of infectious FRNA bacteriophage and this finding is consistent with other studies (Hewitt and Greening 2006; Croci *et al.* 2012). Assuming that NoV behaves in a similar manner, this would have implications for assessing the sanitary quality of mussels post heat treatment using the standardised RT-qPCR assay. Alternative RT-qPCR methodologies involving long-range priming of the RT (determining genome integrity; Wolf *et al.* 2009) or using immunomagnetic capture PCR (determining capsid integrity; Dancho *et al.* 2012) may yield more informative results. Until such time as a consensus on the most appropriate molecular methodology to indicate NoV viability has been reached, further investigations using FRNA bacteriophage or other culturable NoV should be performed to determine the likely inactivation kinetics for NoV during shellfish processing.

The concentrations of viruses in the bioaccumulated mussels used in this study were greater than those likely to be present in mussels harvested from most commercial areas (Diez-Valcarce *et al.* 2012). As the RT-qPCR assay overestimated infectious FRNA bacteriophage concentrations in cooked mussels, this detection method is likely to overestimate the infectious NoV concentrations present in mussels and is therefore not suitable to assess NoV reduction during cooking practices. As FRNA bacteriophage are considered to represent a conservative indicator of NoV, the results presented in this study show that cooking mussels for a period of 3 min in boiling water (>90°C) is sufficient to fully reduce these high infectious virus concentrations to undetected levels. This regime therefore represents an effective mitigation strategy for preventing NoV illness in mussel consumers. We recommend that domestic consumers immerse mussels in boiling water (>90°C) for a minimum of 3 min to inactivate infectious viruses and reduce the risk of illness. This study also highlights that lightly cooking mussels (in water at ~70°C) is insufficient to fully inactivate enteric viruses and may pose a risk of illness to consumers.

Acknowledgements

The funding for this research was provided by Dŵr Cymru Welsh Water and by Bangor Mussel Producers and the European Social Fund via the KESS Scholarship programme.

Conflict of Interest

No conflict of interest declared.

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ADDENDUM TO CHAPTER 6

Materials and Methods

The human stool samples used in this experiment were positive for either NoV GI.4 or NoV GII.4. Genotype was determined according to sequencing of the capsid NS domain.

Results

Thermal inactivation of viruses in mussel DT

The paper suggests that similar qRT-PCR determined FRNA bacteriophage concentrations remained in mussel digestive gland after 200s incubation at 90 °C. In fact there was approximately a 2 $\log_{10}$ reduction in qRT-PCR determined titres during this period. However, this did not reflect the reduction in infectious FRNA bacteriophage revealed by plaque assay, which demonstrated a 3.35 $\log_{10}$ (99.99%) during only the time taken for the digestive gland to reach 90°C (100s) and a 6-log reduction after 200s, with infectious FRNA bacteriophage undetectable after 220s whilst PCR signal persisted with no further reduction. Therefore it stands that the qRT-PCR assay overestimated the concentrations of infectious FRNA bacteriophage to an increasing extent with time. Therefore the qRT-PCR assay is likely to overestimate concentrations of infectious NoV.

Discussion

The UK Ministry of Agriculture, Fisheries and Food recommended that the commercial cooking of bivalve molluscs achieve a minimum internal temperature of 90 °C maintained for 90s (Waterman 2001). This is adopted as an official requirement under European legislation. Our experiment conducted upon mussel digestive gland incubated at 90°C found that the T_{90} value for infectious FRNA bacteriophage at 90°C is 42s, suggesting that 90s at this temperature should provide a degree of protection to consumers via virus inactivation unless grossly contaminated. Experiments C & D conducted upon whole mussels cooked in water suggest that cooking regimes which do achieve this internal temperature offer a similar level of protection. Notwithstanding this, many domestic consumers use shell opening as a cooking guide and experiments A & B showed that mussels open at temperatures insufficient to inactivate enteric viruses, thereby posing a risk of illness.

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GENERAL CONCLUSION AND FURTHER WORK

The aim of this chapter is to summarise the information presented in this thesis with reference to the aims and objectives outlined in chapter 1.

Objective One

- Determine the appropriate sample size for *Mytilus edulis* when applying the ISO method to with regard to the variability between individuals.

Chapter two described an experiment which aimed to evaluate the inter-mussel variability in the accumulation of virus when exposed to contaminated water. The findings were in general agreement with previous work conducted on *Crassostrea gigas:* There was no evidence for the existence of "hot mussels" (i.e. mussels accumulating exceptionally high levels) which might cause upward distortion of virus levels determined for pooled samples. However, a small proportion of animals accumulated concentrations which were outliers of the distribution on the low end for NoV GI and GII and FRNA bacteriophage. By generating 5000 averages which theoretically represent pooled samples, it was shown that the pooling of ten animals provided a good model of the batch. Therefore, a minimum of ten mussels (as prescribed by the ISO method) should be used in surveillance, monitoring and research. Ten *Mytilus edulis* typically provide little in excess of the 2 ± 0.2 g recommend by the ISO method and therefore it is prudent to make more than ten animals. Further work should investigate a range of contamination levels and seek to confirm that a similar pattern is observed in environmentally contaminated animals where differential flow and topography across beds may lead to heterogeneous contamination.

Objective Two

- Evaluate the spatial pattern of NoV contamination around a coastal sewage discharge.

Chapter three described an experiment in which caged mussels were used to compare the spatial patterns of norovirus and faecal indicator organism contamination about an offshore effluent

discharge pipe, with the output of a hydrodynamic model. The results showed that NoV levels matched closely to the model predictions. A concentrated plume resulted in higher contamination of mussels which were predicted to be within its path than mussels which were nearer to the outfall but outside of its path. That a different pattern was observed for coliforms and *E. coli* in comparison to NoV demonstrates that they represent a poor indication of levels of sewage-derived viral pathogens. Hydrodynamic models may be a better risk-based approach for evaluating the potential for viral contamination.

Objective Three

- Assess the potential for offshore cultivation / relaying of *Mytilus edulis* to mitigate against viral and bacterial contamination

Chapter 4 describes an experiment in which mussels were experimentally relocated to a series of distances offshore. The experiment was complicated by the loss of equipment and an effect of distance could not be determined due to levels below the limit of quantification. However, levels of faecal indicator bacteria and norovirus reduced quite rapidly after relocation for 28 days. This effect was quite pronounced for norovirus GII (6540 gc/g - <LOQ). That levels remained low on each subsequent sampling suggests the suitability of the study site for production of mussels in line with EFSA advice given for oysters: That production in waters which are not faecally contaminated represents the most effective method for control of NoV risk. Further work would repeat the study through annual cycles to determine that good microbial quality is maintained and also investigate the equipment required to cultivate mussels in this location at commercial scale.

Objective Four

- Investigate the ability of a commercial depuration process applied to exported *Mytilus edulis* to improve their virological quality post-harvest

An investigation described in chapter five demonstrated that a commercial depuration at ambient winter temperatures was unable to contribute to the virological quality of commercially harvested mussels through reduction in norovirus concentrations. Further work should seek to determine whether increases to process temperature can improve norovirus elimination. However, recent research has suggested that whilst norovirus elimination in oysters is accelerated at higher temperatures, the effect may be smaller than necessary to eliminate norovirus within a commercially viable time period.

Objective Five

- Investigate the effectiveness of domestic cooking practices to reduce concentrations of infective virus in *Mytilus edulis.*

Chapter five describes an investigation which used FRNA bacteriophage as an indicator of viral infectivity during cooking of mussels. Infective concentrations of FRNA bacteriophage determined by plaque-assay reduced more rapidly during cooking than concentrations determined by RT-qPCR. The results showed that cooking "until the shells open" according to tradition and numerous recipes does not indicate that sufficient temperatures have been achieved to inactivate infective virus. However, immersing mussels in boiling water (>90 °C) for a period of three minutes was able to inactivate high infectious virus concentrations to undetectable levels. Therefore an advisory cooking instruction could reduce the risk of illness in mussel consumers. Further work should seek to determine first the consumer acceptance of mussels cooked to this specification. Regulatory limits for norovirus in bivalve molluscs have been proposed. However, the relatively few illnesses associated with commercially cooked shellfish suggest that cooking parameters adopted in legislation are effective. Mussels are usually cooked prior to consumption and this could justify relaxation of regulatory limits for products intended to be cooked prior to consumption and labelled as such. A survey of producers and consumers should seek to determine the acceptance of food advisory labels stating that the product is intended to be cooked and providing cooking recommendations.

APPENDIX I

Investigation of inter-mussel variability in norovirus concentrations in environmentally contaminated mussels

Introduction

The data presented in chapter two relates to artificially accumulated mussels. This provided important information relating to the variability in virus concentrations accumulated by individual mussels under controlled conditions. However, it can be argued that mussels naturally contaminated to a high level may be preferable.

Methods

Cages were constructed of stainless steel mesh (1" x 1") to hold 50 individual *Mytilus edulis* in discrete compartments. Mussels were collected from turbine 7 of Rhyl Flats Windfarm during low water slack 19/2/13. Mussels were deployed in cages on 19/2/13 and 22/2/13 at four sites in the Menai Strait at which they were expected to be exposed to varying levels of NoV contamination prior to intended collection. The sites chosen were Friar's road outfall, at which a cage was hung directly from the outfall marker buoy; Beaumaris castle CSO outfall, where an experimental mooring was deployed; at the mouth of the Afon Cadnant, where a cage was fastened to subtidal rocks; and at Britannia Bridge, where a cage was deployed in the channel retained by weighted rope attached to redundant ironwork at the base of a stanchion. This represented the closest possible deployment to the Treborth water treatment plant outfall which is in deep, fast flowing water West of the bridge. These cages were expected to represent a range of exposure levels.

For each cage, analysis was intended to proceed according to the method described in chapter two. The results for a pool of ten mussels would be compared with the range of results for thirty individuals and averages representing theoretical pools of ten.

Results

Unfortunately dangerous sea states prevented collection of cages until 22/04/14. The results for ten mussels randomly selected from each cage are shown in table one.

Site	NoV GI gc/g	NoV GII gc/g
Beaumaris	positive; <100 copies/g	positive; <100 copies/g
Britannia Bridge	positive; <100 copies/g	350 copies/g
Cadnant Mouth	144 copies/g	190 copies/g
Fryar's Road outfall	positive; <100 copies/g	positive; <100 copies/g

Table 1. Results for pooled smaples collected on 22/04/14.

These levels were considered insufficient to justify the cost associated with any analysis of individual animals. Norovirus of genogroup one was only detected at levels which could be quantified in pooled samples originating from the mouth of the of the Afon Cadnant. Norovirus of genogroup two was detected at quantifiable levels in samples originating from Britannia Bridge and the mouth of the Afon Cadnant. Norovirus of genogroup two was detected at 350 gc / g at Britannia Britannia Bridge but this level was considered relatively proximate to the LOQ. Therefore some individuals may have accumulated levels below the LOQ which would exclude them from quantitative analysis and any pattern in individual concentrations observed in individuals at this level may be hard to defend.

Conclusion

The highest levels of norovirus genogroup two were observed in a pooled sample originating from Britannia Bridge. Quantifiable levels of both norovirus genogroup one and two were observed in pooled samples at the mouth of the Afon Cadnant. It was decided that data derived from artificial bioaccumulation will provide important information.

APPENDIX II

Site-Specific and Industry directed research

Introduction: Funding body and project objectives

This PhD project was funded via the Knowledge Economy and Skills Scholarship (KESS) Programme. KESS scholarships attract European Social Fund (ESF) funding to build relationships between industries and academic institutions within the EU-designated convergence area of Wales. This project has contributed towards ongoing research collaboration between Bangor University (BU) and Bangor Mussels Producers (BMP), a consortium representing three local SMEs in North Wales, UK. The project has also received external supervision from Dr. James Lowther, representing the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) and has generated further links between BU, BMP and this executive agency of the Department for Environment, Food and Rural Affairs (DEFRA).

KESS participants spend four weeks per annum on internship with their industrial co-sponsor. The nature of the industry and of the research aims (including in both cases the seasonality) dictated that this be undertaken in smaller work packages and not block placements. It was necessary for the student to qualify for work on registered fishing vessels (Appendix IV) in order to carry out duties. The duties included performing the mandatory surveillance of by-catch during commercial movement of shellfish and several industry-directed surveys.

The following surveys addressed the immediate interests of the local shellfish industry. They also afforded the student an opportunity to acquire background data concerning the NoV situation in the local shellfishery and surrounding area. Interim reports were provided to the industrial partner.

Case Study: The Menai Strait (East) mussel (Mytilus edulis) fishery.

Introduction

Mussels (*Mytilus edulis*) were commercially harvested from Area 3 of the Menai Strait (East) Fishery on 3rd March 2011 and shipped to France via depuration in Holland. The information we received was that quality control checks in Holland were passed but subsequently the consignment (60,000 tonnes) was condemned due to shellfish vectored illness reported in France and traced to the production area. No information regarding the scale or aetiological agent responsible for illness was provided to us. The industrial partner requested assistance to determine whether there was an ongoing gross faecal contamination problem. At that time we were not equipped to assay for viral pathogens.

Materials and methods

The local shellfish producers requested seven sites across one commercially harvested mussel bed, located in the Menai Strait in North Wales (Fig. 1), be tested for faecal contamination of water / *in-situ* shellfish. Triplicate mussel and water samples were collected for laboratory analysis.

Mussel samples were processed to determine the total *E. coli* content as described in Chapter 3. Water samples were collected in sterile containers approximately 1 m below the water surface and processed as described in Chapter 4.

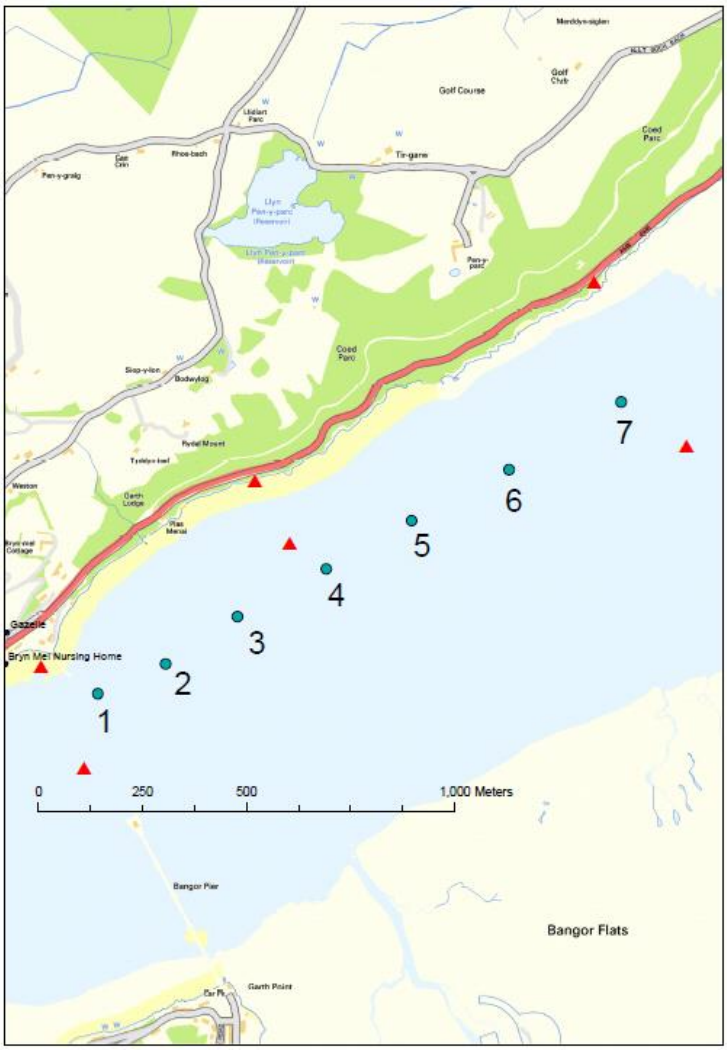


Fig.1 Location of sample sites within Menai Strait, North Wales (circles). Triangles mark location of outfalls.

Results

The results from water sampling are displayed in Figure 2. With the exception of site 2, all the sites demonstrated *E. coli* concentrations of less than 100 CFU / 100 ml. Site 2 demonstrated *E. coli* concentrations of over 800 CFU / 100 ml. Mussel sample results are displayed in Figure 3 and show that across all sites the *E. coli* concentration of the mussels was between 230 – 4,600 *E. coli* CFU /100 g which is in agreement with the long term classification held for the area (Class B).

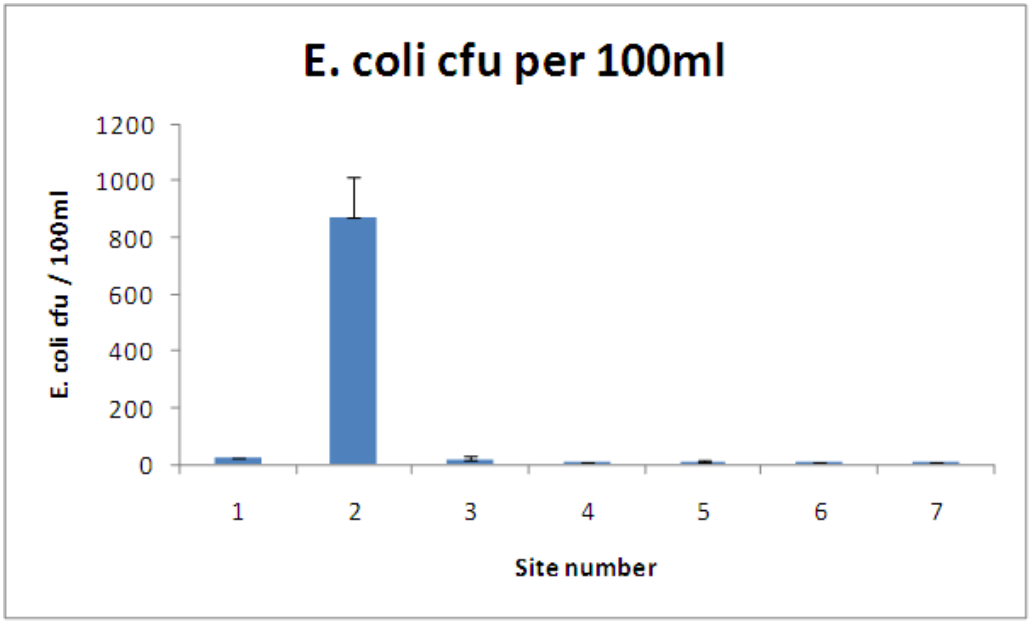


Fig. 2 Determined *E. coli* concentrations from water samples. Expressed as E. coli CFU / 100 ml. Error bars represent the SE and n = 3 in all cases.

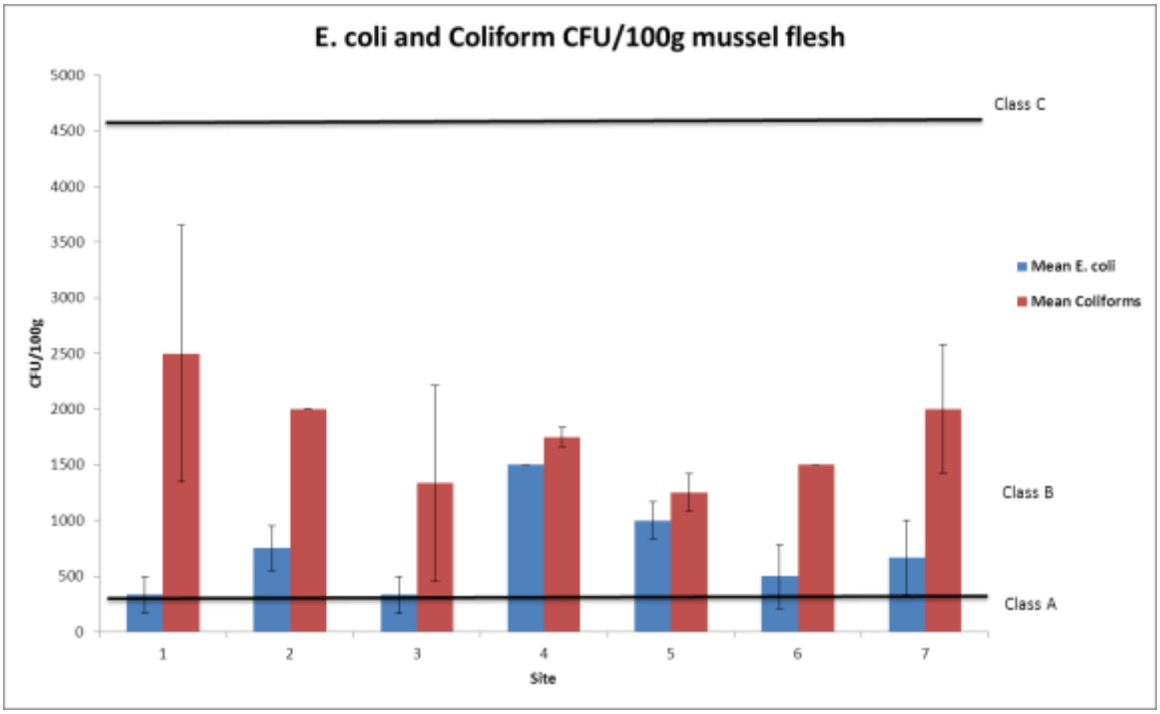


Fig. 3 Determined *E. coli* and total coliform concentrations from mussels, expressed as CFU / 100 g flesh and intervalvular fluid. Error bars represent the SE. n = 3 in all cases.

Discussion

The results from this study suggest that concentrations of Faecal Indicator Bacteria in mussels from Area 3 are at present in accordance with the Class B Long-Term classification. The unusually high *E. coli* concentration in water collected at site 2 could be explained by contamination during collection or laboratory processing and was not reflected in mussel samples. Therefore there was no evidence of an ongoing gross faecal contamination problem. However, it is possible that the mussels associated with illness in France were contaminated with human enteric virus either before or after harvest. It was suggested that the mussels may have been re-immersed after depuration. The data produced in this study does not help to determine the point of contamination. Loadings of Faecal Indicator Bacteria may have changed in the interim and a poor correlation between bacterial and viral concentrations in both water and bivalve molluscan shellfish has been repeatedly observed by researchers. Reasons for this poor correlation include the high environmental persistence of human enteric viral pathogens e.g. norovirus, the most common health risk associated with the consumption of faecally contaminated bivalve molluscan shellfish (BMS). Therefore, our data showing unexceptional *E. coli* levels in mussels does not determine that they were not or are not contaminated with human enteric viruses. Outbreaks of viral illness have previously been epidemiologically linked to shellfish grown in classified waters and demonstrating compliance with the regulatory *E. coli* standard post-depuration. Elimination of NoV from contaminated shellfish using traditional methods has been shown to be largely ineffective. Therefore, assuming that the shellfish harvested from this area were correctly implicated in the case(s) of shellfish vectored illness, we consider it equally conceivable that they were contaminated within their harvesting waters or upon re-immersion in contaminated waters. The latter is an illegal activity and has not been confirmed to us.

We recommend a regime for virus surveillance be developed for the local commercial beds and harvested batches because analysis of Faecal Indicator Bacteria may not represent the risk of viral contamination. In addition and subject to funding, an analysis of the sensitivity of the fishery to viral contamination, to identify sources and high risk periods will be of benefit to the industry.

Investigating the variability in NoV concentrations present in mussels from local beds relative to values for the designated monitoring point and suggested limits

Introduction

Local industry has an interest in understanding the spatial patterns of viral contamination within their commercially farmed shellfish beds. Infection with norovirus (NoV) is the most common health risk associated with the consumption of faecally contaminated bivalve molluscan shellfish (BMS) and was the target of this investigation. Introduction of virological monitoring to the official classification and control of shellfish harvesting areas has also been proposed (EFSA Panel on Biological Hazards 2012; CEFAS 2013). The EFSA report (2012) recommended that virus detection methods are now suitable for use in a legislative context. The method applied is now an ISO technical specification ISO TS 15216 and practical capability exists within the EU to introduce viral standards (CEFAS 2013). However, NoV contamination is expected to vary spatially within production areas according to environmental characteristics which may also differ considerably between production areas. There is currently no guidance on sampling regimes for NoV monitoring in bivalve molluscan shellfish (EFSA Panel on Biological Hazards 2012). The current sanitary measures used to reduce the risk associated with LBMs contaminated with faecally derived pathogens include classification and monitoring of harvest areas and food safety criteria for marketed products. All are currently based upon levels of the Faecal Indicator Bacteria (FIB) *E. coli*. The approach has been less effective in predicting risk from viral pathogens than it has against bacterial pathogens. The adoption of additional virus standards may provide greater protection to the consumer by controlling the risk of presence of specific pathogen at unacceptably high levels.

The current classification system for production areas is based upon levels of *E. coli* in shellfish tissue and its monitoring aims to determine general sanitary quality. The samples used to monitor *E. coli* levels are derived from a sample point determined by the sanitary survey. The sanitary survey aims to identify likely sources of contamination and the monitoring point is generally determined to reflect the worst-case scenario, i.e. the region of the bed likely to be most impacted upon by the identified sources general faecal contamination. A similar approach may be adopted for virological monitoring, but the sample would need to reflect the worst case human-specific faecal pollution and therefore may not coincide with identified *E. coli* monitoring points (CEFAS 2013). The representative monitoring site should be determined to provide the most effective protection to the consumer and to the industry against outbreaks of shellfish-vectored viral illness.

A full characterisation of the local shellfish production areas was beyond the scope and budget of this PhD. The objectives of this preliminary investigation were two-fold.

Objective one was to determine the range of NoV concentrations accumulated in *Mytilus edulis* across 6 systematically situated samples within each of three local production areas. In addition, we sampled the current CEFAS-designated monitoring point (*E. coli*) for each respective bed as a proof of principle. Concentrations of NoV on each bed were to be determined during one tide and the range compared to the concentration derived for samples taken from the respective official monitoring point.

Objective two aimed to look in greater detail at a single production area chosen by local industry. More intensive sampling was applied to improve our understanding of the magnitude of variability, rate of change over distance, and to help identify any patterns which may arise from factors including tidal height or source proximity.

The data will allow the magnitude of variability within each bed to be compared and for an appraisal of how representative the current CEFAS monitoring point would be, relative to the other samples taken from the same bed, if a single monitoring point were proposed. The three beds can also be compared in terms of degree of contamination during November 2011 and the information was relayed to industrial partners.

Methods

Experiment One

Mytilus edulis were collected on foot at low tide from Menai East (Area 4), Menai West (Barras 1) and Conwy (Morfa), on 23rd, 24th, 25th November 2011, respectively. Six samples were taken from each bed using GPS co-ordinates in a simple grid covering the bed but designed to avoid edge effects on these variably shaped beds. An additional sample was taken at the designated CEFAS monitoring point for each bed (SH59707490; SH47906510; SH76107970 respectively). Each sample comprised 20 *Mytilus edulis* animals. The digestive glands were excised within 4 h of collection, pooled, and 2 g aliquots frozen (-20 °C) until all equipment was sourced for adoption of the CEN method. Analysis proceeded according to the CEFAS SOP. A 2 g aliquot of glands was thawed for each sample, digested in Proteinase K and spiked with Mengo virus process control on 11/04/12. Nucleic Acid Extraction was undertaken on 12/04/12. The RNA extracts were stored at -80 °C until the method was verified under application at Bangor.

Samples were tested for NoV GI and NoV GII using the qRT-PCR method described by the CEFAS SOP in the week commencing 04/06/12.

We followed the same convention as Lowther et al. (2012) in scoring our data. Samples returning values which were below the Limit of Quantification (100 gc/g) were scored at 50 gc/g. Samples in

which NoV of a particular genogroup was not detected were scored with 20 gc/g for that genogroup, half the theoretical Limit of Detection.

Experiment Two

Mytilus edulis were collected from Menai East (Area 4) on 6/3/13 on foot at low tide. Samples were taken from the bed using pre-determined GPS coordinates for 18 sites in addition to the CEFAS monitoring point. These co-ordinates were applied in a grid to cover the entire bed, which is of a narrow, approximately rectangular shape. Each sample comprised 15 *Mytilus edulis* processed according to the CEFAS SOP. Data were scored as above.

Results

Round One – Menai West, Menai East and Conwy November 2011

The results of our analysis are shown in Table 1 and Table 2 for NoV GI and NoV GII respectively.

Table 1. Scored Data for NoV GI in six systematically sited samples and at the CEFAS monitoring point for each production area

	Production Area		
	Menai West (Barras)	Menai East (Area 4)	Conwy (Morfa)
1	20	50	20
2	50	50	20
3	50	50	20
4	50	226	121
5	50	199	20
6	50	20	20
CEFAS	50	50	50

Data are detectable NoV GI gc / g scored as described above. Mean is omitted due to frequency of <LOQ results.

Table 2. Scored Data for NoV GII in six systematically sited samples and at the CEFAS monitoring point for each production area

	Production Area		
	Menai West (Barras)	Menai East (Area 4)	Conwy (Morfa)
1	1082	1557	50
2	1002	2232	104
3	1416	2017	50
4	982	2284	183
5	822	3322	180
6	924	1023	50
CEFAS	1497	3017	50
$\bar{x}$	1103	2207	95

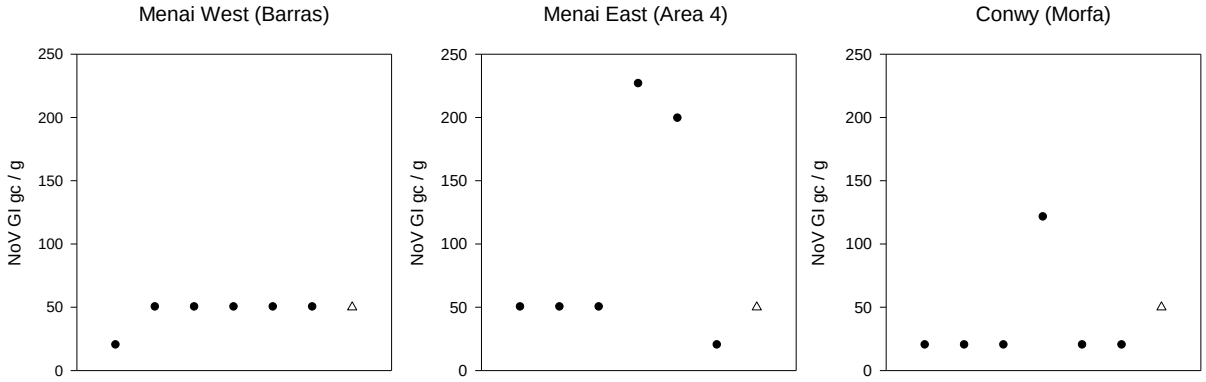
Data are detectable NoV GI gc / g scored as described above. Mean is allowed due to frequency of quantifiable results.

For Menai West (Barras), 6/7 samples including the CEFAS point shared the same quality: detectable NoV GI was present at concentrations which were below the Limit of Quantification (Fig. 1). No sample exceeded the result for NoV GI concentrations at the CEFAS monitoring point. At one site NoV GI was not detected. The CEFAS monitoring point also returned the highest NoV GII concentration (1497 gc / g) detected on the bed (Fig. 2), where all samples returned quantifiable results ($\bar{x}$=1103 gc /g. CV = 36%).

However, for Menai East, the CEFAS monitoring point did not reflect the highest concentrations of either NoV GI or NoV GII. Its <LOQ result for NoV GI was qualitatively similar to three experimental samples and exceeded levels at one site at which NoV GI was not detected. However, NoV GI was quantifiable in the region of 200 gc / g at two sites (Fig. 1). The result of 3017 NoV GII gc / g at the CEFAS *E. coli* monitoring point was close to the maximum observed (3322 gc /g) and exceeded all other sites. It was greater than the average concentration observed for the bed ($\bar{x}$=2207 gc / g CV=23%) and high relative to the range of data (1023 – 3322 gc / g) and towards the upper end of the distribution (Fig 2).

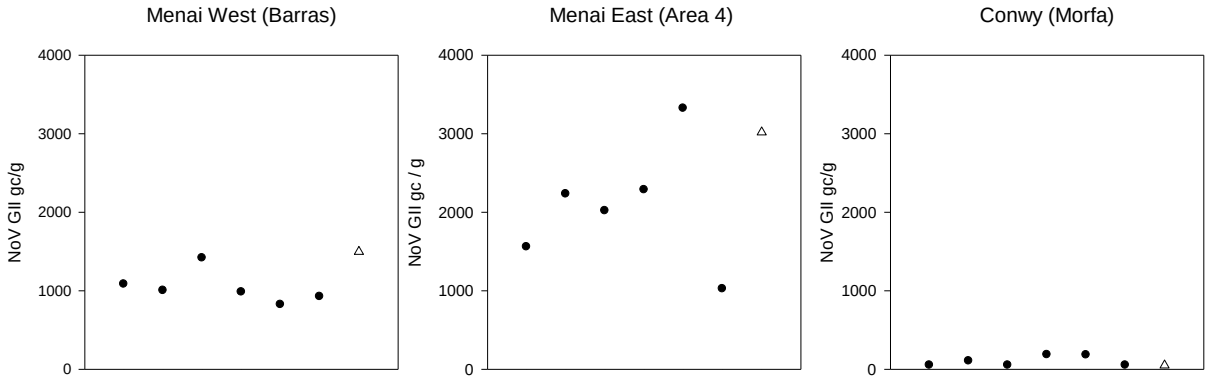
Levels of NoV (both GI and GII) on the Conwy (Morfa) production area were low relative to the LOQ and to the other areas studied on consecutive days. The CEFAS-designated monitoring point again did not reflect the highest concentrations on the bed. For NoV GI its <LOQ result was qualitatively greater than for 5 sites at which NoV GI was not detected (Fig. 1). But at one site, NoV GI was detected above the LOQ (121 gc / g). As a <LOQ result for the CEFAS monitoring point could reflect any concentration up to 100 gc / g, the difference in actual concentrations between this and the quantifiable result for sample 4 may be minimal. The NoV GII result (<LOQ) for the CEFAS monitoring point was qualitatively similar to three other sites but exceeded by three sites containing 104, 180 and 183 gc / g. Again, the difference between quantifiable and <LOQ results in this dataset may be minimal.

Fig. 1. Scored NoV GI concentrations in mussel samples.



Solid dots represent experimental samples 1-6. Hollow triangles represent the value derived for samples originating from the CEFAS-designated monitoring point. Samples in which NoV GI was not detected are scored 20 gc / g. Samples in which NoV GI was detected <LOQ (100 gc /g) are scored 50 gc / g.

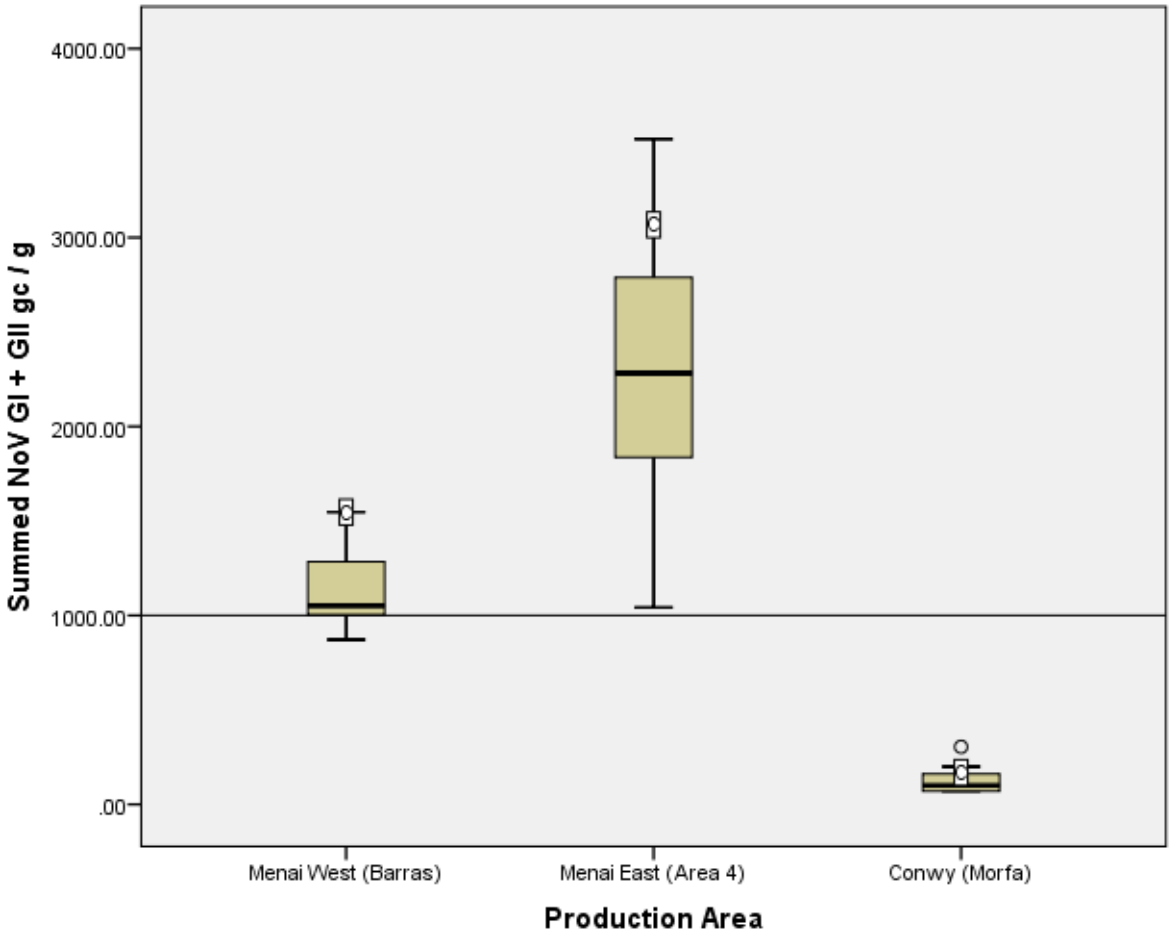
Fig 2. Scored NoV GII concentrations in mussel samples.



Solid dots represent experimental samples 1-6. Hollow triangles represent the value derived for samples originating from the CEFAS-designated monitoring point. Samples in which NoV GII was not detected are scored 20 gc / g. Samples in which NoV GII was detected <LOQ (100 gc /g) are scored 50 gc / g.

Concentrations of NoV GI and NoV GII (scored where appropriate) were summated according to the principle proposed by EFSA and CEFAS (2012; 2013) as this is an indicator of a risk which cannot be directly measured. The results are presented in Figure 3.

Fig. 3. Summated NoV GI and GII concentrations for each production area.



Boxplots show the distribution of summed GI + GII concentrations (NoV gc / g) in n=7 samples (6 + CEFAS-designated monitoring point). For each genogroup, raw data was scored before summation where appropriate. The y-axis reference line shows the upper limit (1000 gc / g) suggested by CEFAS (2013) for production areas. "C" Annotations have a y-axis position equal to the result (NoV summed GI + GII gc / g) for the currently designated monitoring point for *E. coli*.

In this view it is clear that the majority of samples (5/7) taken from Menai West (Barras) on 24/11/11 would exceed the upper limit (1000 NoV (GI+GII) gc /g) for production areas recommended by CEFAS (2013) and that the currently designated CEFAS monitoring point would reflect this fact. For Menai West, all samples taken from across the bed on 23/11/11 exceed this limit and the CEFAS monitoring point reflected this but did not contain the highest concentrations observed. For Morfa Conwy on 25/11/11, several samples exceeded the result returned for the CEFAS monitoring point, but interpretation is complicated by the frequency of results below the LOQ. Notwithstanding this, when results are proximate to the LOQ, it is clear that they are compliance with the 1000 gc / g upper limit suggested by CEFAS (2013).

When the production area was sampled in early March, levels tended to be below the LOQ for both NoV GI and NoV GII (Table 3 and Fig. 4).

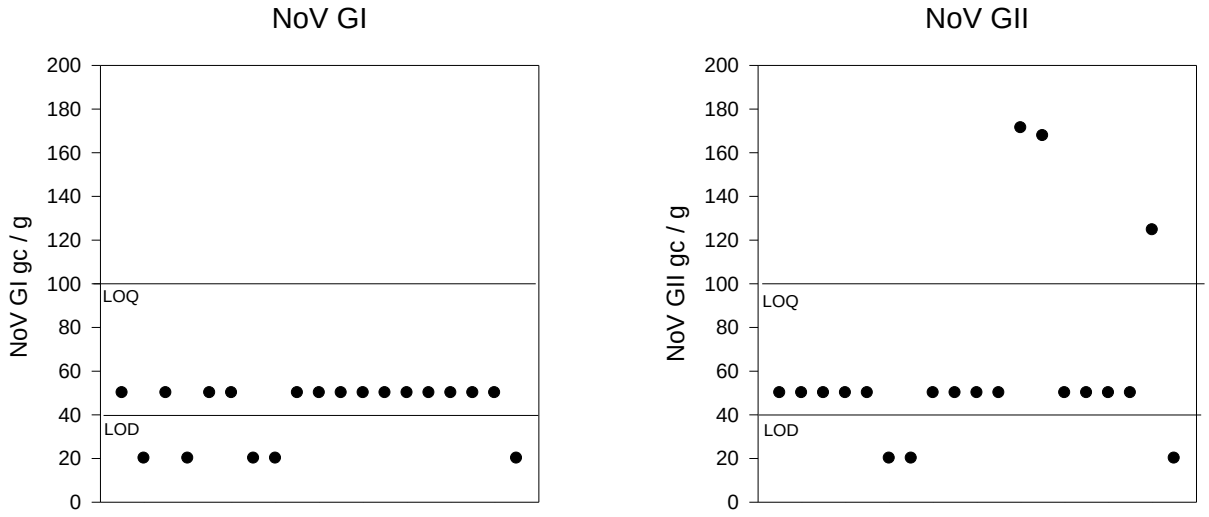
Table 3. Scored data for 19 samples.

	Menai West (Area 4)	
	NoV GI	NoV GII
1	50	50
2	20	50
3	50	50
4	20	50
5	50	50
6	50	20
7	20	20
8	20	50
9	50	50
10	50	50
11	50	50
12	50	171
13	50	168
14	50	50
15	50	50
16	50	50
17	50	50
18	50	125
19	20	20

Data are detectable NoV GI and NoV GII gc / g scored as described above.

Coverage of the bed with mussels was patchy and mussel samples could not always be collected at the pre-determined GPS co-ordinate. Therefore, new GPS co-ordinates were stored at the location from which each sample was taken. The CEFAS monitoring point was under water during this low tide. Five transects were started at the lowest point of the bed's profile accessible on foot at low tide. As far as possible this reactive sampling was undertaken to yield five transects of the intertidal area accessible on foot. Samples were taken as close to the predetermined GPS co-ordinates as possible. An additional sample was added to the transect at the widest part of the bed, in replacement of the inaccessible CEFAS monitoring point at the lower end of this transect.

Fig. 4. Score NoV GI and GII gc / g in mussel samples taken from Menai East (Area 4).



Solid dots show scored data for 19 samples. Reference lines represent the method Limit of Detection and Limit of Quantification. Samples in which NoV GII was not detected are scored 20 gc / g. Samples in which NoV GII was detected but at levels below the LOQ (100 gc /g) are scored at 50 gc / g.

All samples returned concentrations below the Limit of Quantification for NoV GI and in 5 samples, NoV GI was not detected. NoV GII was quantifiable at levels marginally above the LOQ in 3/19 samples. In all other it was <LOQ and in three cases was not detected.

For NoV GI, there was no clear spatial pattern to the qualities of non-detection / detection below the LOQ (Fig. 5). For NoV GII, two samples at a low elevation on the bed profile returned the two highest results. These sites were the nearest to the designated CEFAS monitoring point which was not accessible. However, the only other quantifiable result was returned from a sample originating high on the bed (Fig. 6).

Figure 5. Scored NoV GI gc / g on Menai East (Area 4) 6/3/13

Samples in which NoV GI was not detected are scored 20 gc / g (half the theoretical limit of detection. Samples in which NoV GI was detected, but at levels below the LOQ (100 gc / g) are scored at 50 gc / g.

Fig. 6. Scored NoV GII gc / g on Menai East (Area 4) 6/3/13.

Samples in which NoV GI was not detected are scored 20 gc / g (half the theoretical limit of detection. Samples in which NoV GI was detected, but at levels below the LOQ (100 gc / g) are scored at 50 gc / g.

Discussion

In objective one I set out to compare the range of NoV concentrations in six widely spaced samples taken from a production area, with the concentration determined for the CEFAS-designated *E. coli* monitoring point. Three beds were treated as replicates, to better generalise any findings and identify area-specific observations. The designated monitoring point is not determined to reflect human-

specific faecal contamination. However, at the time of sampling the result determined for the monitoring point performed well in reflecting the higher levels observed on the bed. For Menai West (Barras) and Menai East (Area 4), where GII levels tended to be quantifiable, the CEFAS point returned a concentration exceeding the average for the bed, either returning the highest concentration observed (Barras) or a position high in the distribution of data (Area 4). In situations where the result for the CEFAS monitoring point was <LOQ for NoV GI or GII, and exceeded by a quantifiable result elsewhere on the bed, the results were qualitatively different but the absolute difference in concentration may have been marginal.

The variability within the production areas appeared to be proportional to their respective mean levels, and a hypothesis that variability is proportional to contamination level is biologically defensible. When NoV inputs to the bed are low, variability across the bed is likely to be low. When NoV inputs are higher, a greater range of concentrations may be observed due to the factors such as shore height and exposure duration (Lobel 1987). However, these samples relate to the levels observed on one day only for each bed. Repeated measures on each bed taken across different contamination levels would demonstrate whether variability increases with degree of harvesting area pollution. Therefore it is plausible that Area 4 shows greater variability than Barras or Morfa due to factors which are irrespective of exposure level. Such factors may relate to environmental characteristics or to production processes. For example, in this data set an uncontrolled factor was apparent in Area 4. Although all mussels on Area 4 had been *in-situ* for >6 months and therefore should be equally representative of recent contamination, the area has been used for holding animals of different age / size classes by the producer. Mussel age / size may affect NoV uptake and this factor was uncontrolled. Repeated measurements through the NoV Season (Winter) would allow a better analysis of whether across-bed variability tends to be proportional to the mean. Conwy (Morfa) showed the lowest summed concentrations of NoV GI and GII and this information is interesting as concurrent investigations by another researcher (K. Clements) determined Conwy (Morfa) to be highly contaminated at this time with the Faecal Indicator Bacteria *E. coli,* relative to beds in the Menai Strait. A potential explanation for this observation may be that *E. coli* of animal origin, associated with livestock farming in the Conwy catchment contributes significantly to levels of the Faecal Indicator Bacteria on the bed. Menai East (Area 4) showed higher contamination levels than Menai West (Barras) or Conwy (Morfa) in November 2011. It is not clear from this investigation that Area 4 is typically more contaminated because this information relates to a single time point and NoV levels are expected to reflect prevalence in the local community which is also variable. Nevertheless, concentrations on Menai East (Area 4) clearly exceeded the upper limit for production areas (1000 gc / g) suggested by CEFAS (2013). The importance of this is not clear because the risk posed by bivalve molluscan shellfish contaminated with NoV may not be the same for all species and it is not clear how

the regulatory limit may be applied to various species. The health risk posed by different Live Bivalve Molluscs from enteric viruses may vary. According to epidemiological evidence, a greater risk is associated with species which are consumed raw (CEFAS 2013). *Mytilus edulis* are often cooked prior to consumption and thorough cooking can inactivate NoV (Flannery et al. 2014). However, *Mytilus edulis* may be exported to regions of Europe in which raw consumption is practiced. Further, in other regions traditional cooking methods based upon shell opening do not indicate sufficient cooking for NoV inactivation. Domestic consumers can be recommended to immerse mussels in boiling water (>90°C) for a minimum of 3 min to inactivate infectious viruses and reduce the risk of illness (Flannery et al. 2014). Whether *Mytilus edulis* could be marketed with an advice label indicating the risk of illness associated with inadequate cooking and with printed cooking guidelines is dependent upon the decision of risk managers and acceptance of producers and retailers. A social survey may be valuable in determining whether consumers would buy a product carrying such a label.

In objective two, we looked in some greater detail at Menai East (Area 4) in early March 2013. Levels of NoV were too low, and below or proximate to the Limit of Quantification for a thorough interpretation. For example, three sites returned quantifiable levels under 200 gc / g, making them qualitatively different to all others. However, there may be little real difference between concentrations <200 gc / g and concentrations <LOQ (100 gc / g). In addition, sampling at each site was not replicated. Therefore the significance of these differences is largely unknown. The interpretation of replicate data would remain complicated when levels are proximate to the LOQ. Therefore the pattern observed should not be over interpreted and it will be best to repeat the investigation when mussels are naturally contaminated to a high level. As mentioned under objective one, the determination of concentrations below the LOQ demonstrates clear compliance with the 1000 gc /g limit suggested by CEFAS (2013) hence the limitations of the method to quantify levels <100 gc / g is unlikely to complicate monitoring of production levels if this limit is adopted. Nevertheless, a proposed limit of 200 gc / g (summed GI + GII) for products placed on the market may be harder to implement because compliance would constitute a <LOQ result for each assay. Difficulties may be experienced where one assay returns a result in marginal excess of the LOQ and the other is unquantifiable as the sum would be unknown and may or may not be compliant. One option would be to stipulate that NoV must be <LOQ for both genogroups for products placed onto the market.

For limits applied to production areas, a greater problem may be the determination of a classification and monitoring system. If concentrations of NoV accumulated by *Mytilus edulis* in production areas should never exceed 1000 gc / g then Menai East (Area 4) may be considered unsuitable for production. Should it be decided that harvesting should not be allowed when levels exceed 1000 gc /

g then a longitudinal study is needed to determine the potential burden of closures. Reopening principles will also need to be established.

There are various approaches which may prove valuable in further work related to this investigation. There are two distinct directions for further work. The first direction seeks to address the key research questions surrounding the implementation of a robust monitoring scheme. The second aims to meet the needs of local industry to understand better the scale and extent of viral contamination, both spatial and temporal, affecting their production areas.

Development of a robust monitoring scheme

For the first objective it is important to determine the amount of sampling effort required for monitoring to reliably detect contamination at levels representing unacceptable risk. First that limit must be determined. In a discussion paper, CEFAS (2013) recommended that an absolute upper limit of <1000 gc/g for production areas would be appropriate considering the information from outbreaks, depuration studies and analysis of impact upon producers. PCR is known to detect infectious / non-infectious virus but is currently the only available method for determining virus concentrations in shellfish. PCR detectable concentrations represent an indication of risk and a dose response is apparent from epidemiological data (Lowther et al. 2010). A suggested end-product limit of <200 gc / g anticipates that depuration can be optimised. So assuming that the monitoring should reliably detect a level of contamination of 1000 gc / g on beds, there is a bottom up approach stage: Information regarding variability in contamination levels across beds is required. And a top down; For this variability and detection threshold, how should monitoring be undertaken? There appear to be two options. One approach is by desk study and visual survey. The current sanitary survey method applied to production areas assumes that a monitoring point can be determined to represent the worst-case faecal contamination on the bed and used to monitor compliance with *E. coli* standards. European Commission guidelines exist for conducting sanitary surveys and existing sanitary surveys should already identify any sources of human faecal contamination (EFSA Panel on Biological Hazards 2012). The information may be useful in assessing the likely extent of NoV contamination (Pommepuy and Le Guyader, 2008). A combination of limited monitoring and the sanitary survey could allow the relative risk of NoV contamination for harvesting areas to be characterised (EFSA Panel on Biological Hazards 2012). In addition, this information may assist in identifying a (possibly different) monitoring point to represent worst-case human-specific faecal contamination at which compliance with viral standards may be determined. This approach is supported by data in this thesis, which suggested that spatial patterns of NoV contamination about offshore outfalls can match well to tidally-driven effluent dispersal models and as such be predicted. However, shellfish beds may have complicated (and changing) hydrodynamics, topology and be exposed to a variety of human-faecal

inputs from wastewater effluent plumes, (untreated) combined sewer overflows and tributaries. The interaction of sources and their relative contributions may vary. The situation may also differ according to production technique, with broadcast (bottom culture) methods being exposed to different factors to those affecting (e.g. rope culture systems). The second option would be an empirical approach i.e. to apply a form of power analysis aimed to determine the sampling effort required for monitoring purposes. Some work has already been undertaken to estimate the probability of detecting contamination when samples are taken from batches of harvested shellfish (EFSA Panel on Biological Hazards 2012). NoV batch prevalence and concentration were shown to be factors upon which success of the testing regime to detect contamination strongly depend. However, the testing of every batch is likely to be prohibitively expensive and hence production area classification may reduce the necessity for batch screening. A simple approach to shed light upon a monitoring scheme for production area classifications would be to plot confidence intervals against number of samples taken to see where the reduction in CI levels out and how many samples are required to be confident that a bed is worthy of its classification for harvesting. To do so it is necessary to characterise as much of each bed as possible which requires stratification. Shellfish production areas are not uniform and topology and exposure will differ requiring consideration and it is important to recognise that if the beds are very variable this may be missed by under-sampling. Further information is also required to determine whether the variability is constant across contamination levels or changes. Essentially this means working towards some sort of power-simulation analysis where the probability of detecting unacceptable contamination can be explored for a particular monitoring scheme and variability. A different approach would be to try to quantify and characterise spatial variability. Nested systematic sampling was considered in this study but analysis costs for the samples required were beyond budget. Information from such studies would be valuable to the development of a robust monitoring scheme, but to investigate each production area longitudinally would be prohibitively expensive and the sanitary survey approach is most likely to be adopted. An empirical analysis of spatial variability in production areas would be extremely informative at this time but was estimated to be beyond the budget of this PhD.

Characterisation of local shellfish beds.

Local industry requires greater information regarding the characteristics of viral contamination in local production areas to inform mitigation options. The industry focussed angle would be to attempt to characterise the beds in terms of sensitivity to contamination. For example, Riou et al. (2007) investigated a coastal area subject to episodic faecal contamination to determine the impact of small tributaries leading into the area upon seawater and shellfish microbial quality. The study centred upon the development of a two-dimensional hydrodynamic model subject to decay rates appropriate to micro-organism behaviour and predicted the influence of faecal input on shellfish quality. Field

measurements of shellfish concentrations corroborated that when rainfall was less than 10 mm per day, water quality remained acceptable. This information helps to identify risk periods and also identified the areas affected. A similar study would be of benefit to local industry but was beyond the budget of the present PhD and would be subject to available funding.

Conclusion

Shellfish production areas are currently classified according to *E. coli* levels in shellfish tissue as this provides an indicator of faecal contamination and subsequently dictates the processing required post-harvest. This has provided protection to consumers against shellfish-vectored bacterial pathogens. However, *E. coli* has been shown to be a poor indicator of viral contamination and NoV infection is currently the most common health risk associated with consumption of faecally contaminated shellfish. Hence, recent developments in molecular detection methods provide an opportunity to develop surveillance strategies to control this risk. Proposed limits may be restrictive upon industry; the data in this study demonstrates non-compliance with suggested upper-limits. However, the risk of viral illness may not be the same for all bivalve shellfish, because some, e.g. *Mytilus edulis* are more typically cooked. Notwithstanding this, traditional cooking methods may not ensure viral inactivation and raw/light cooking may be practiced in regions to which shellfish are exported. Whether a cooking advice label is sufficient to justify less stringent regulations is a political decision, and producer/consumer acceptance is yet to be shown. Publication of a standard method (Anon 2013) and hydrodynamic model developments may assist industry in determining mitigation strategies.

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Snapshot comparison between Menai East Fishery and Rhyl Flats Windfarm

Introduction

Local industry has an interest in co-locating mussel production areas within the Rhyl Flats, Gwynt-y-Mor and N. Hoyle windfarms off the N. Wales Coast. It is hypothesised that these offshore locations may be less impacted by faecal contamination than the Menai East Fishery. Of particular interest are viral pathogens, which are considered to have high environmental persistence. Infection with norovirus (NoV) is the most common health risk associated with the consumption of faecally contaminated bivalve molluscan shellfish (BMS) and analytical methods for NoV in BMS are available. Therefore we investigated NoV concentrations in mussels originating from within the existing Menai East Production area, and in mussels originating from within Rhyl Flats Windfarm.

We are aware of several point sources located on the coast proximate to the windfarm, including (at N53°20.214 W3°34.152) the outfall of an activated sludge (no tertiary treatment) WTP serving a population equivalent of 77,953. The spatial pattern of NoV contamination in mussels caged about this outfall was investigated in Chapter 3. The experimentally derived levels of NoV GI and GII in mussels were similar, with NoV spread from the outfall showing a distinct plume which matched very closely to a tidally-driven effluent dispersal model. The model, and the agreement shown by our experimental data, suggested that the Rhyl Flats wind farm would not be exposed to high concentrations of sewage-derived pathogens originating from this outfall. Byssal attachment of wild *Mytilus edulis* to the turbine towers provides an opportunity to assess exposure of mussels in this situation to NoV.

An opportunity to access this location was afforded during the sampling of the same experimental sites referred to in Chapter 3 for sediment analysis, part of a follow-up study undertaken by T. Perkins (BU PhD student). J. Ben Winterbourn conducted this sampling with the assistance of BMP boats and skipper on behalf of T. Perkins. Access to the turbines is complicated by sea state and the timing of this sampling was largely opportunistic.

Methods

All samples were collected 14-11-12. Samples for "Menai East" were recovered from a single trawl of "The Horseshoe off Beaumaris" (Area 6; Menai Strait (East) Fishery Order Area) at a location N53°15.435 W4°05.104. Samples for Rhyl Flats Wind Farm were scraped from the intertidal section of turbine piling located at northernmost row, easternmost turbine N53°22.903 W3°36.600. Three replicate samples were processed for each site according to the CEFAS Standard Operating Procedure (SOP) for NoV analysis in BMS.

Results

All samples passed extraction and amplification efficiency, inhibition and positive controls according to CEFAS SOP.

SAMPLE	Genogroup 1 gc/g	Genogroup 2 gc/g
Rhyl Wind Farm 1	4.5 (<LOQ) i.e. Positive <100 gc/g	11.5 (<LOQ) i.e. Positive <100 gc/g
Rhyl Wind Farm 2	0.3 (<LOQ) i.e. Positive <100 gc/g	25.1 (<LOQ) i.e. Positive <100 gc/g
Rhyl Wind Farm 3	0.7 (<LOQ) i.e. Positive <100 gc/g	0 (<LOD) i.e. Not detected <40 gc/g
Rhyl WF Average	**1.8 (<LOQ)** Positive <100 gc/g	**12.2 (<LOQ)** Positive <100 gc/g
Horseshoe 1	111.4	314.3
Horseshoe 2	133.5	325.2
Horseshoe 3	111.1	133.1
Horseshoe Average	**118.7**	**257.6**

100 gc/g is the Limit of Quantification. Results for Rhyl Flats Wind Farm 14-11-12 should be described/interpreted as "detected; below LOQ" for both NoV GI and GII. These levels are qualitatively less than detected in samples originating from the Horseshoe on the same date.

Conclusion

The results presented here are consistent with the view that Rhyl Flats Wind Farm is less impacted by NoV contamination than area 6 of the Menai Strait (East) fishery. However, these results apply to a single date. It is possible that NoV infection was not prevalent in the community within this different geographic area and that in the event of an outbreak, more grossly contaminated receiving waters may extend to the wind farm. Notwithstanding this, an investigation into the spatial spread of NoV upon discharge of secondary treated sewage from the most significant proximate outfall (chapter 3) suggested that the plume matched very closely to a tidally-driven effluent dispersal model (within the extents of the experimental plot). The experimentally derived data did not extend to the windfarm but the position of the concentrated plume was in agreement with the model within the experimental plot. NoV demonstrates high environmental persistence. NoV was detectable in samples originating from the Wind Farm 14-11-12, at low levels which could not be quantified by the method. The wind farm may receive dilute contamination originating from coastal discharges.

Investigation into the effect of heavy rain upon NoV levels within the local fishery

Introduction

It has been widely noted that heavy rainfall leading to Combined Sewer Overflows (CSOs) can result in increased contamination of shellfisheries because untreated sewage may be heavily contaminated with enteric viruses (Maalouf et al. 2010). For the local fishery, ideally an analysis of the fishery's sensitivity to contamination would be undertaken similar to that conducted by Riou et al. (2007). Such an analysis is beyond the scope and budget of this PhD but is recommended for further work on behalf of the industrial partner. A period of heavy rainfall, resulting in localised flooding, followed the collection of samples from the Menai East Fishery 14/11/12 as collected for the previous investigation. The industrial partner provided further samples 26/11/12 for comparison after sustained heavy rainfall as part of a preliminary investigation into the sensitivity of the fishery to contamination after heavy rainfall.

Methods

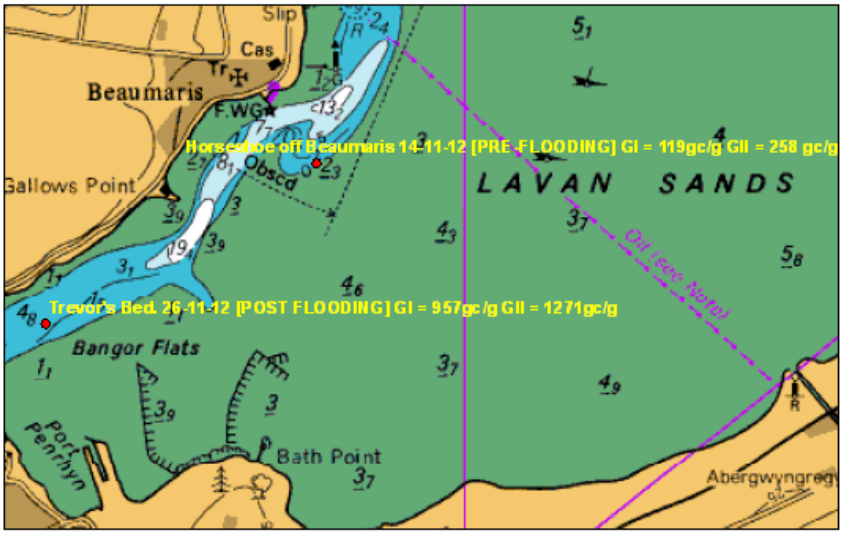
Samples for 14/11/12 were collected by J.B. Winterbourn after a short trawl within area 6 of the Menai Strait (East) Fishery at N53°15.435 W04°05.104. Samples for 26/11/12 were provided by BMP after a short trawl. The co-ordinate given was N53°14.725 W04°07.096. Three sample reps were processed for each site according to the CEFAS SOP.

Results

All samples passed extraction and amplification efficiency, inhibition and positive controls according to CEFAS SOP. Unfortunately, the samples cannot be considered repeated measures pre- and post-flood. Samples provided for 26/11/14 were collected during commercial fishing operations. The co-ordinates of these samples were subsequently determined to be within Area 3 of the Menai Strait (East) Fishery Order. Therefore results are tabulated for each area below and not as pre- and post-flood comparisons.

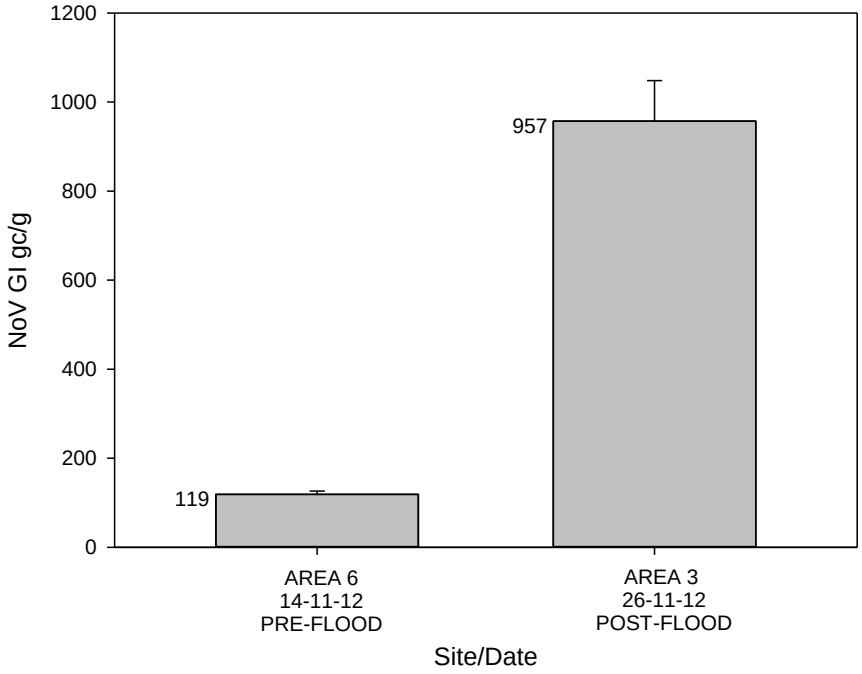
Sample	Genogroup I gc/g	Genogroup II gc/g
N53°15.435 W04°05.104 14-11-12 R1	111.4	314.3
N53°15.435 W04°05.104 14-11-12 R2	133.5	325.2
N53°15.435 W04°05.104 14-11-12 R3	111.1	133.1
Horseshoe (area 6) Average	**118.7**	**257.6**

SAMPLE	Genogroup I gc/g	Genogroup II gc/g
N53°14.725 W04°07.096 26-11-12 R1	1021	1289
N53°14.725 W04°07.096 26-11-12 R2	1073	1207
N53°14.725 W04°07.096 26-11-12 R3	777	1319
Area 3 Average	**957**	**1271**



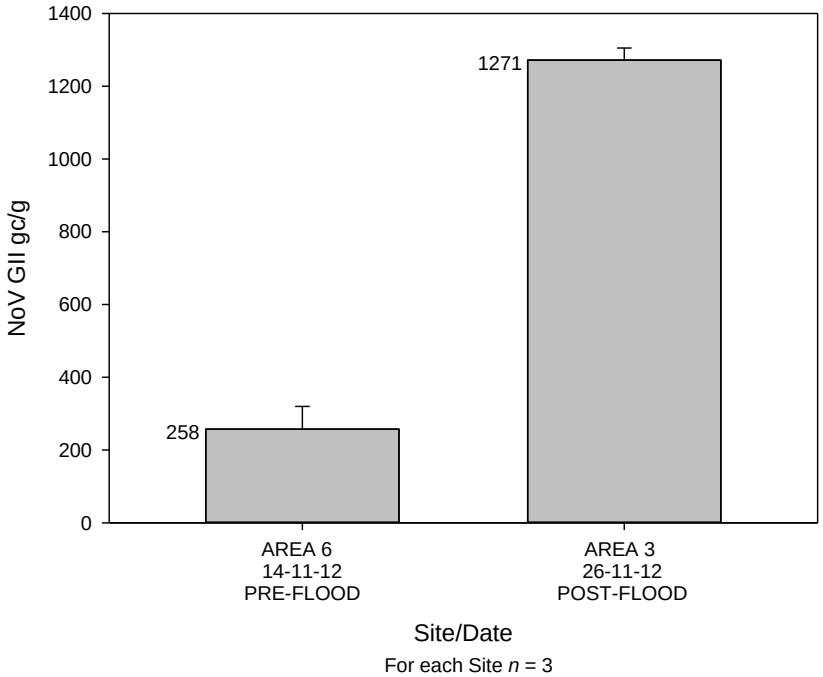
It can be stated that concentrations of GI and GII NoV were approximately 8- and 5-fold higher in samples collected on 26-11-12 than in samples collected 14-11-12 (fig 1 and 2).

Norovirus GI



For each site *n* =3

Norovirus GII



For each Site *n* = 3

Conclusion

We cannot confirm that higher levels observed 26/11/14 are the result of heavy rain alone. In this thesis, high spatial variability within production areas has been observed. Also demonstrated was close agreement between experimentally derived NoV levels in shellfish and tidally-driven effluent dispersal models. Exposure to NoV contamination via effluent plumes was shown to vary over smaller distances than the separation between the sample points in this study. There is good reason to hypothesise that levels in the Menai East Fishery have risen since the intense rainfall. But the caveat that the two sites investigated may be more or less exposed to concentrated effluent plumes must be sustained.

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Comparison between the Inland Sea (Holyhead) and two areas within Menai Strait (East) Fishery in January 2013

Introduction

Local industry has an interest in identifying new areas suitable for the production of shellfish for human consumption. The sheltered waters within the "Inland Sea", Holyhead, are attractive to the industry. Water quality and the potential for production of hygienic shellfish are key criteria in determining suitability. Infection with norovirus (NoV) is the most common health risk associated with the consumption of faecally contaminated bivalve molluscan shellfish (BMS). Viral contaminants have shown poor correlation with bacterial indicators. Therefore direct evaluation of exposure of shellfish to NoV within the potential production area constitutes a pragmatic component of investigation. This analysis, commissioned by the industrial partner, served to provide a snapshot comparison between two existing lays, and the potential area identified at the Inland Sea.

Methods

All samples were provided by Bangor Mussel Producers for analysis. Trial *Mytilus edulis* were harvested from the Inland Sea 17/01/13. Samples of farmed mussels, from Area 3 and Area 6 of the Menai Strait (East) Shellfishery, were collected 18/01/13 via short dredges. Mussels were collected to provide three replicate samples for each site to be processed using the CEFAS SOP.

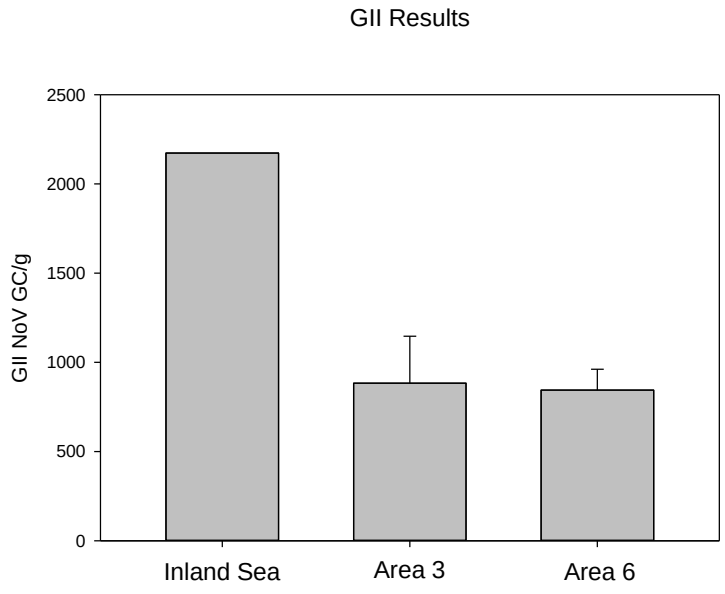
Results

Mussels originating from the Inland Sea had low meat content and small digestive glands. After dissection, insufficient material existed for replication. Digestive glands from three replicate samples were pooled to yield a single sample. Three replicate samples were processed for each Menai (East) Fishery site. All samples passed extraction and amplification efficiency and inhibition control. However, the GI assay suffered from failed negative control. Contamination was evident in 1 of 2 negative extraction controls (CT 39.02) and 2 of 2 no-template-controls (37.00;37.02) affecting the GI assay only.

SAMPLE	Genogroup 1 gc/g	Genogroup 2 gc/g
Inland Sea 17/1/13	>278	2173
Inland Sea Average	**N/A – No replicates**	**N/A – No replicates**
M.E. Area 6 R1 18/1/13	>252	1066
M.E. Area 6 R2 18/1/13	>356	798
M.E. Area 6 R3 18/1/12	>199	668
M.E. Area 6 Average	**>269**	**844**
M.E. Area 3 R1 18/1/13	>130	486
M.E. Area 3 R2 18/1/13	>107	783
M.E. Area 3 R3 18/1/13	>86	1381
M.E. Area 3 Average	**>108**	**883**

NoV GI results should not be interpreted. Presence of low-level contamination was evident in negative controls. It may be assumed that low level contamination would contribute to total genome copies detected to only small extent but it is not clear whether contamination affected samples systematically. Therefore comparison of sites according to NoV GI levels is inappropriate.

The sample from the Inland Sea returned a higher result for GII contamination than the mean of three replicates for either of the Menai East sites. Two Menai East sites did not show a significant difference ($P>0.05$).



Error Bars show Standard Error of the Mean for Trevor's Bed and Horseshoe. No error bars for inland sea due to absence of replicates.

Discussion

NoV GI results cannot be discussed. For all sites, it should be recognised that a single sample point cannot be used to characterise the production area, as spatial variability has not been properly characterised. NoV GII contamination in mussels originating from a short dredge in each of two Menai East sub-units was not significantly different. This observation provides some support to the view that heavy rain in November did result in elevated levels within the Menai East Fishery. It was discussed in the previous investigation that samples collected pre- and post-heavy rainfall originated from Area 6 and Area 3 respectively. Lack of significant difference observed between these sites on 18/01/13 provides weak evidence that the difference before and after heavy rain is probably not explained by generally greater contaminant exposure in Area 3, and rather by an impact upon the fishery after flooding and the operation of Combined Sewer Overflows in this region.

NoV GII contamination of mussels originating from the Inland Sea did appear to be higher than for either lay investigated in the Menai East Fishery. However, in the absence of replicates for the Inland Sea, the significance of this difference is unknown. It should also be noted that mussels collected from the Inland Sea appeared to be in poor physiological condition, with very low meat content and digestive gland mass. This in itself does not support the suitability of this growing area. In addition, the poor physiological condition of these animals may have affected the accumulation/elimination of NoV. Furthermore the nature of the sample was different to that processed for animals in better condition originating from the Menai East Fishery areas. Thirty animals were required to provide a single aliquot (2 g) suitable for testing.

In conclusion, unhealthy animals harvested from the Inland Sea appeared to contain higher NoV concentrations than healthy animals harvested the following day from the Menai East Fishery, suggesting that the Inland Sea does not provide advantageous mussel farming conditions.

Effect of Storage upon NoV concentrations in mussels stored between staggered deployment

Introduction

The local industry has an interest in whether norovirus titres can change in mussels during post-harvest storage. There is no clear reason why animals stored out of water should either eliminate or accumulate NoV. However, Neish (2013) and Ueki (2007) noted increasing NoV levels in BMS during a period following artificial exposure to contaminated water. This uptake lag may be attributed to the movement of NoV particles into the digestive gland after exposure to a concentrated spike and before the gland is removed for analysis. When faced with a requirement to stagger the deployment of mussel sentinels originating from the same batch, we considered it pragmatic to determine any change in detectable NoV concentrations between the immediately deployed animals and those which had to be stored for 3 d.

Mussels were collected from turbine 7 of Rhyl Flats Windfarm during low water slack 19/2/13 for relocation (caged) to sites within the Menai Strait. The purpose of this was to source mussels which were negative for norovirus (or minimally contaminated), prior to experimental re-location at sites where we expected them to be exposed to NoV. It was intended that variability in NoV accumulation between naturally-contaminated individuals would be investigated across a range of exposure levels as described in appendix I.

The collected mussels were prepared for experimental re-deployment during return to port in the Menai Strait but tidal constraints allowed only for deployment at Friar's Road outfall (Beaumaris) on the same day. The remaining mussels were stored (without being resubmerged) at Porth Penrhyn at ambient temperatures (day time temp ~5°C) until deployment of the remaining cages on 22/2/13. On this day a sentinel was deployed at 1) Beaumaris Castle Outfall 2) the mouth of the Cadnant (Menai Bridge), 3) at Britannia Bridge 4) at Hirael Bay Outfall.

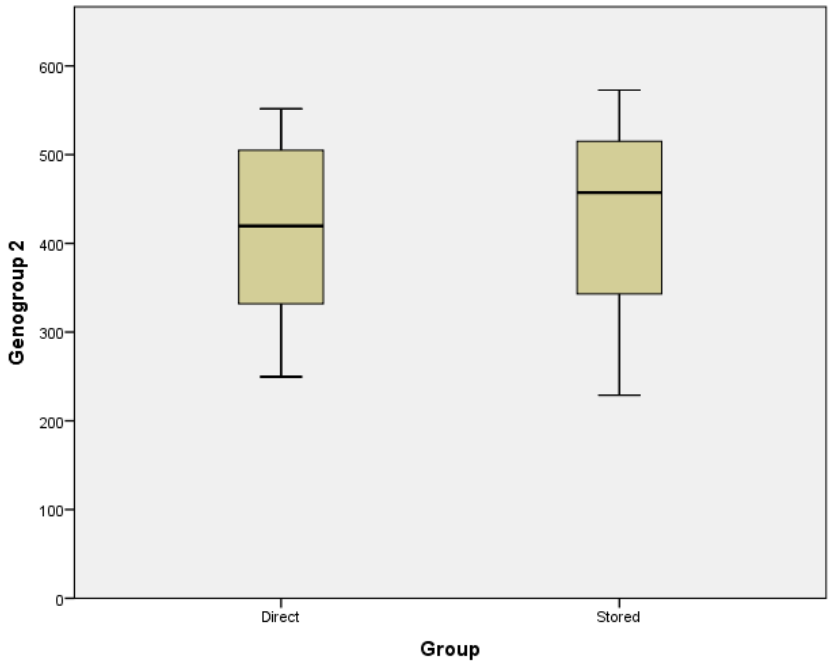
Methods

Nine replicate samples were taken from the harvested batch on day of collection and transferred immediately to the laboratory for processing within 4 h. After the necessity to store some of the mussels between deployments, a further three replicate samples were taken from the remaining batch and processed within 4 h of the secondary deployment to detect any change in detectable titre in the stored animals. All samples were processed according to the CEFAS SOP.

Results

All samples passed extraction, amplification, inhibition and positive/negative controls according to CEFAS SOP. Both batches returned results below the Limit of Quantification for NoV genogroup I and as such were not qualitatively different. All replicate samples returned valid results above the Limit of Quantification for NoV genogroup II. There was no significant difference in NoV GII titres between mussels analysed immediately after collection and mussels stored out of water at ambient (winter) temperatures for 3d.

	Direct (n=9)	Stored (n=3)
GI mean	16 (standard Error = 3.6) (<LOQ)	15 (standard error = 1.3) (<LOQ)
GII mean	414 (Standard Error = 38)	420 (Standard Error = 101)



Note different sample sizes. Direct n=9 Stored n=3

Discussion

This was not an experiment designed to test effect of short-term storage on NoV titres in mussels but was undertaken in response to a necessity to stagger deployment of mussel sentinels. It indicates that RT-qPCR detectable NoV concentrations in *Mytilus edulis* stored at ~5 °C for 3 d underwent no change. This justifies the later deployment of mussels from the same batch under similar baseline characterisation. Also, 100% of animals selected for analysis of the stored batch were alive and

responding to percussion. This suggests that the animals deployed on 22/2/13 remain suitable for use as sentinels. However, irrespective of storage, levels of GII NoV in mussels attached to pilings at Rhyl Flats WF have risen considerably since a previous sampling effort on 14-11-12, when they were below the Limit of Quantification. Levels for GI NoV remain below the LOQ. The contamination evident within this offshore windfarm demonstrates the persistent nature of NoV and ability of *Mytilus edulis* to efficiently concentrate NoV from the water column.

SAMPLE	Genogroup 1 gc/g	Genogroup 2 gc/g
Rhyl WF Average 14-11-12 (n=3)	**1.8 (<LOQ)**	**12.2 (<LOQ)**
Rhyl WF Average (T7) 19-02-13(n=9)	**16 (<LOQ)**	**414**

Levels of NoV GII detected in mussels from the windfarm in February 2013 exceed those detected in mussels originating from Area 6 of the Menai Strait (East) Fishery 14/11/12 (257.6 gc/g) but are less than those detected for Area 6 on 18/01/13 (844 gc/g).

APPENDIX III

Appendices for chapter three

Manuscript in short-communication format submitted to the proceedings of the 9th International Conference on Molluscan Shellfish Safety (ICMSS). Based on the work presented in Chapter 3.

This work was presented orally by J. Ben Winterbourn at the 9th ICMSS.

Evaluation of spatial contamination patterns for norovirus and faecal indicator bacteria near to a coastal sewage discharge using *Mytilus edulis* as biosentinels

James B. Winterbourn*[1], Katie Clements[2], James Lowther[4], Shelagh K. Malham[2], James E. McDonald[3] and Davey L. Jones[1]

[1]School of the Environment, Natural Resources & Geography, Bangor University, Bangor, Gwynedd, LL57 2UW, UK *afpc58@bangor.ac.uk, [2]School of Ocean Sciences, Bangor University, Bangor, Gwynedd, LL59 5AB, UK, [3]School of Biological Sciences, Bangor University, Bangor, Gwynedd, LL57 2UW, UK, [4]Cefas, The Nothe, Barrack Road, Weymouth, Dorset, DT4 8UB, UK

Abstract

Bivalve shellfish have the capacity to accumulate norovirus (NoV) from waters contaminated with human sewage. Consequently, shellfish represent a major vector for NoV entry into the human food chain, leading to gastrointestinal illness. Identification of areas suitable for the safe cultivation of shellfish requires an understanding of NoV behaviour upon discharge of sewage into coastal waters. This study exploited the potential of *Mytilus edulis* to accumulate NoV and employed the proposed international standard method for quantification of NoV within mussel digestive tissues. To evaluate the spatial and temporal spread of NoV from an offshore sewage discharge pipe, cages of mussels were suspended from moorings (n=13) deployed in a 1km grid array around the outfall. Caged mussels were retrieved after 30 days and NoV (GI and GII), coliforms and *E. coli* enumerated. The experimentally derived levels of NoV GI and GII in mussels were similar, with NoV spread from the outfall showing a distinct plume which matched very closely to a tidally-driven effluent dispersal model. A contrasting spatial pattern was observed for coliforms. These data demonstrate that coliform / *E. coli* concentrations do not accurately reflect viral dispersal in marine waters and contamination of shellfish by sewage-derived viral pathogens.

Keywords: food safety, mussels, human sewage, shellfish harvesting, viral gastroenteritis

Introduction

Contamination of bivalve shellfish with norovirus (NoV) from human faecal sources represents a well-established human health risk (Lees 2000). Bacteria including coliforms and enterococci have been used to estimate the level of faecal contamination of water and / or shellfish and may be referred to collectively as Faecal Indicator Bacteria (FIB). In Europe, *Escherichia coli* (*E. coli*), a coliform species commonly found in the lower intestine of warm-blooded organisms, is adopted as the traditional indicator of faecal (sewage) contamination in shellfish and used for risk assessment and management (Anon 2004). However, studies have indicated that *E. coli* provides a poor indicator of the risk of NoV contamination. Reasons for this poor correlation include the different environmental persistence of viruses and bacteria in marine water and differences in their seasonal discharge pattern (Fong and Lipp 2005).

Direct recovery and concentration of viral pathogens from coastal waters is problematic, often requiring large sample volumes and providing only a time-specific measure of contamination. However, bivalve shellfish have been shown to efficiently accumulate viruses (Asahina *et al.* 2009; De Donno *et al.* 2012; Nenonen *et al.* 2008) and sensitive quantitative methods which detect NoV genomes in molluscan shellfish using molecular techniques (PCR) now exist (Lees and CEN WG6 TAG4 2010; Anon 2013).

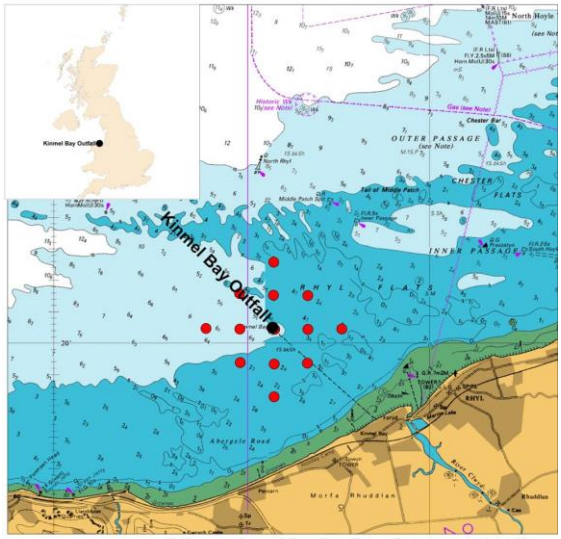
Materials and Methods

Site selection

The offshore sewage outfall pipe at Kinmel Bay, North Wales (53.336901N, 3.569200W (WGS84); Fig. 1), which serves a total population equivalent of 77,953, was selected for this study. The discharge is consented for up to 38,860 m^3 d^{-1} with a dry weather flow not

exceeding 15,941 $m^3 d^{-1}$. Sewage released from the outfall receives only secondary treatment (activated sludge). No tertiary treatment is applied. The outfall discharges into the coastal waters of Liverpool Bay at 4 km offshore, in 6.9 m of water at Lowest Astronomical Tide, to achieve compliance with EU bathing water quality standards. We hypothesized that these conditions could result in a significant release and persistence of potential human pathogens in marine waters.

Fig. 1. Outfall Location and sample sites



Sampling Regime and Shellfish Biosentinels

A diamond-shaped array of 13 independent sampling points was selected (Fig. 1) based on model simulations of sewage plume behaviour. The individual sample points were separated by 1 km in *x* and *y* dimensions. To minimise variability associated with growing conditions, *Mytilus edulis* were collected via a short trawl of broadcast-cultivated animals, from a commercial bed with an EU Class B classification. The animals were washed, size graded and 200 animals randomly selected for baseline enumeration of NoV and *E. coli* at time zero (T_0). Ten replicate samples of 10 animals were analysed for NoV and 10 replicate samples of 50 g shellfish flesh for coliforms and *E. coli.* Groups of 35 live animals of the same batch were then placed in net bags (300 x 300 mm). The net bags were placed in plastic cages and suspended at a sea depth of 1 m by attaching to a plough anchored Polyform A3 buoy. The cages were deployed on 12/03/12 and after 30 d the mesh bags containing shellfish were recovered. The samples were stored on ice before return to the laboratory for processing within 6 h.

Quantification of Norovirus in Mussels

NoV quantification in mussel digestive tissue was determined using quantitative reverse-transcription PCR (qRT-PCR) as described by (Lowther *et al.* 2012a). Modification was made to the formation of the positive extraction control, to the quencher used for the GII probe (TAMRA) and in addition, aliquots of chopped glands were frozen (-20°C) and thawed once prior to Proteinase K digestion rather than being digested fresh or after short-term (24hrs) refrigerated storage (4°C). The positive extraction controls consisted of homogenates prepared as per samples after the addition of 1 lenticule® disc of NoV Reference Material for each genogroup (HPA) to ten digestive glands. Average quantities enumerated from three aliquots of extracted RNA/sample give overall quantities in detectable genome copies/g digestive gland. For T_0 n=10. For *in situ* samples n=1 per site/month.

Quantification of *E. coli* and coliforms

Bacterial colony forming units (CFU) were enumerated from shellfish flesh by direct plating onto selective agar as described in Clements *et al.* (2013). For T_0 n=10. For *in situ* samples n=3 per site/month.

Statistical and geostatistical analysis

To ensure our data are comparable with UK survey data generated by the National Reference Laboratory (Lowther et al. 2012a), samples returning "not detected" results for a particular NoV genogroup were assigned a score of 20 copies/g for that genogroup (half the limit of detection (LOD)). Samples giving positive results below the limit of quantification (LOQ; 100 copies/g) were assigned a score of 50 copies/g. Statistical analysis was carried out using IBM SPSS Statistics 20 and Geostatistical analysis and presentation was carried out in

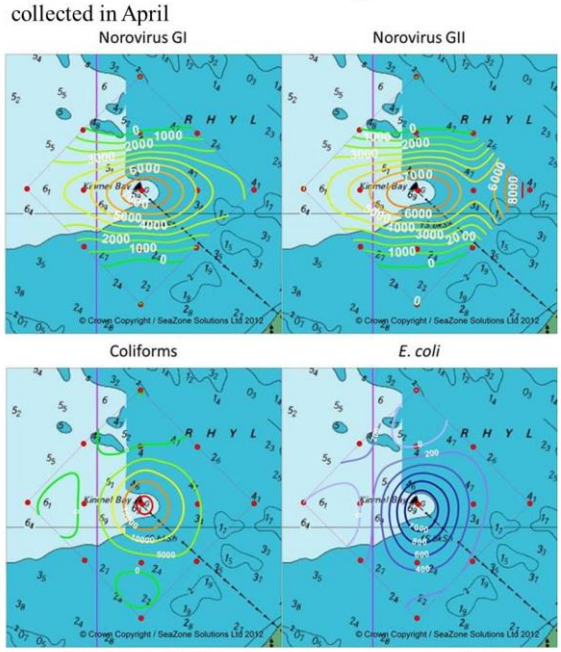
ArcMap 9.3.1 using the Spatial Analyst Extension.

RESULTS

Norovirus and Bacteria in Mussels

After 30 d, GI NoV levels had increased from a T_0 baseline value of 52.2 copies/g at all sites except two at which it was not detected and two at which levels remained <LOQ. For GII NoV, levels increased from a T_0 value of 3312 copies/g at four sites and decreased at all other sites. Similarly, *E. coli* contamination increased in mussels directly over the outfall from the T_0 value of 400 ± 163 to 1167 ±166 CFU/100g. The spatial patterns of NoV and coliforms / *E. coli* around the discharge point were however, very different with NoV showing much greater dispersion and symmetry about the outfall (Fig. 2).

Fig. 2. Norovirus and indicator organisms in mussels collected in April



For NoV GI and GII, contours represent scored data as detectable genome copies / g digestive gland. Not detectecd scores 20 gc / g. 1-100 (<LOQ) scores 50 gc / g. n= 1. For Coliforms and *E. coli* contours represent mean CFU / 100g shellfish flesh and intravalvular fluid. N=3.

Both GI and GII NoV results showed a pattern of contamination elongated to the East and West of the outfall. For NoV GI, levels decreased with distance in all directions from the outfall. But for NoV GII, highest levels (9958 c/g) were observed at the most Easterly sample point, 2 km to the East of the outfall. *E. coli* was detected at highest levels over the outfall but was not detected to the West of the outfall, being skewed East and towards the shore. Total coliforms were detected at highest levels over the outfall, were also skewed East and slightly towards shore, but were detected at all sites. On a site-by-site basis, there was a strong correlation between NoV GI and GII concentrations (r_s=.905; P<0.001). Total coliforms and *E. coli* also correlated (r_s=.747; P=.003). Correlation between total coliform and NoV GI concentrations was weakly significant (r_s=.601 P=.030), but correlation with GII was non-significant (r_s =.543 P=.055). *E. coli* did not correlate with either NoV GI (r_s=.296 P=.326) or GII (r_s=.220 P=.470).

DISCUSSION

The relatively high T_0 value for GII NoV allowed for clear differentiation between sites where levels in resituated mussels increased (up to 3-fold) and sites where they decreased to levels below the LOQ (approx. 66-fold decrease; 3311 to half LOQ), suggesting that the pattern observed is representative of contamination *in situ.* Furthermore, spatial contamination patterns for GI and GII NoV were correlated, except for a disparately high GII result at the easternmost site. Further work seeks to integrate model data for the nearby Clwyd River (Fig. 1), into which sewage is also discharged, possibly resulting in an additional impact of greater magnitude at Eastern sites and containing different GI/GII composition.

The most contaminated sites by either NoV genogroup all occupy the East-West transect through the centre point of the array, over the outfall, and concentrations declined steeply with distance both to the North and South. This is in visual agreement with hydrodynamic model predictions for the sewage discharge plume (data not presented). However, agreement between model predictions and measured *E. coli* and coliform concentrations was less apparent. Furthermore, whilst *E. coli* correlated with total coliforms and NoV GI

correlated strongly with GII, the only statistically significant correlation between the FIB selected for enumeration and NoV was coliforms with NoV GI and this association was not strong.

Indeed NoV GI and GII were detected in mussels at very high levels at sites at which *E. coli* was not detected, notably to the West of the outfall. We are aware that the tidal current was flowing to the East at the time of sampling and therefore animals to the West are likely to have been less recently exposed to the effluent plume. This is consistent with evidence that FIB are an indicator of recent faecal contamination but norovirus can persist longer in shellfish tissue. The water is deeper to the West of the outfall and a differential effect of water depth upon NoV/FIB behaviour is also plausible given potential association with particles and related sedimentation / resuspension phenomena. But importantly, all sentinels were suspended at 1 m below the surface rather than on the seabed.

Given that current regulations in Europe are based on concentrations of *E. coli* in shellfish flesh, mussels containing these levels of NoV could legitimately be sold for consumption following minimal treatment - potentially exposing consumers to an unacceptable risk of illness. It is possible that the method applied detected some inactivated NoV and may overestimate the amount of infectious virus present. However there is recent evidence that amount of genome detected is generally proportional to risk (Lowther *et al.* 2012b).

Conversely, FIB were detected at sites at which NoV was not detected, with the distribution of FIB being somewhat more skewed towards the shore. We hypothesise that secondary non-point sources, which may be of animal origin, affect this pattern. Therefore, this study suggests that FIB indicate the presence of faecal contamination but may not accurately reflect persistent contamination by viral pathogens associated with human-sewage effluent.

Much of the research concerning accumulation / elimination dynamics in shellfish has focussed upon oysters which are associated with more outbreaks than other species, possibly as a result of traditional raw consumption. However, with potential in Europe for virological standards applicable to all bivalve molluscan shellfish, similar data relating to *Mytilus edulis* (and other bivalves sold for consumption) is urgently required.

ACKNOWLEDGEMENTS

This study was funded by Bangor Mussel Producers, Welsh Water and the European Social Fund via the KESS Scholarships programme. We thank Ian Harris for help with the GIS.

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Possible observation of a seasonality in factors affecting extraction efficiency

The offshore experiments described in chapters three and four ran approximately simultaneously during the summer of 2012. In chapter two only the spatial pattern of contamination observed in April was described. This is because NoV concentrations in caged declined in summer presumably due to a reduced community prevalence of infection and environmental factors including water temperature (Fig 1).

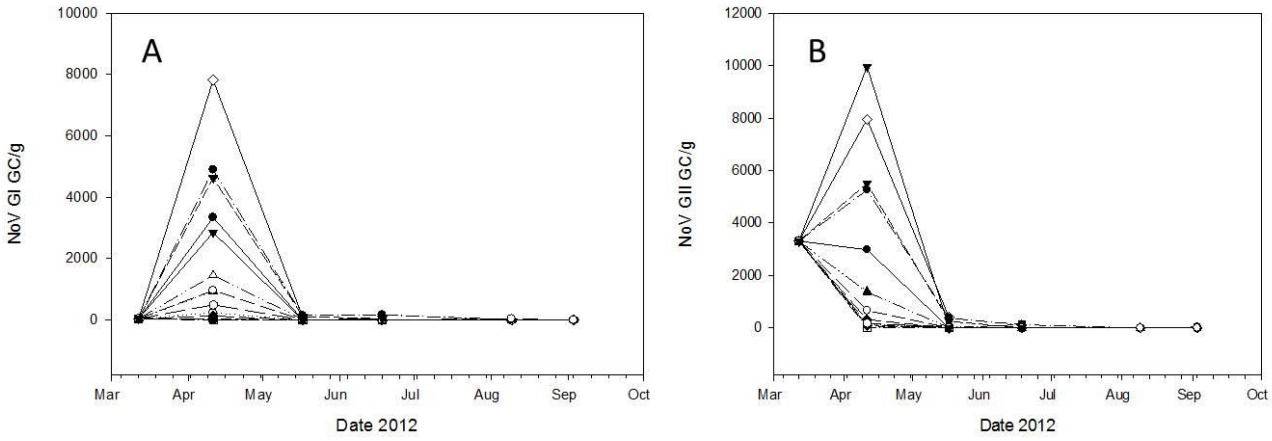

Fig 1. Results of longitudinal plume investigation. NoV GI gc/g in caged mussels are shown in panel A. NoV GII gc/g in caged mussels are shown in panel B. Note the relatively high baseline value for GII was reduced after relocation of mussels to some sites and greatly increased at others.

Therfore the spatial pattern was most distingt in the month of April. In addition the loss of experimental moorings reduced the resolution of data in subsequent months. Reinstating lost moorings to continue the experiment through the following Winter was beyond the budget of this PhD.

However, when exploring the data for the longitudinal experiments an interesting trend was observed: Sample extraction efficiencies determined using mengovirus as an extraction control appeared to decline in summer (Fig. 2). All samples demonstrated extraction efficiencies considered acceptable by ISO TS-15216 (>1%). Extraction efficiencies for samples originating from the transect ranged from 1.19-84.53%. Extraction efficiencies for samples originating from the plume investigation ranged 1.02-77.24%. In both cases there appeared to be a seasonal trend and extraction efficiencies were similar at each experimental site for a given month.

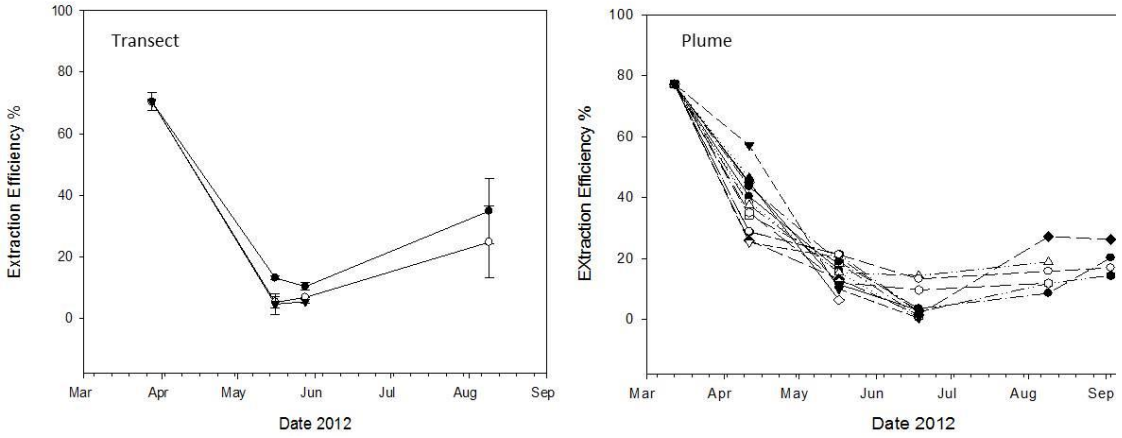

Fig. 2. Extraction efficiencies by date for transect and plume experiments. Transect plot shows mean and standard error for eacg site. Plume plot shows value for each site. Extraction efficiencies were highest at the start of each experiment, were low in May and June and appeared to rise towards the end of the experiment.

Very low extraction efficiency in summer could theoretically result in underestimation of norovirus titre. However, normalisation of data by extraction efficiency is not recommended under the ISO TS 15216 protocol for bivalve molluscan shellfish. The use of mengo virus extraction control provides only an estimate of extraction efficiency and guards against false negatives occurring due to failed extraction. Therefore results should normally be reported uncorrected.

Extraction efficiencies were lowest in May and June and an anecdotal observation was of simultaneous high seawater turbidity during associated with algal bloom affecting this water body. Tissue composition including lipid content is also known to vary in bivalves according to reproductive cycle. An emulsion was observed to form during proteinase K digestion of samples originating during this period. This emulsion interfered with the formation of a clear supernatant during centrifugation.

It is not clear whether this effect was related to a geographically confined environmental factor (e.g. algal bloom) or a physiological factor affecting extraction from *Mytilus edulis* during summer. Seasonal variation in extraction efficiency requires further study.

Spatial and temporal patterns of pathogenic bacteria within sediments surrounding a coastal sewage discharge.

N.b This abstract introduces a continuation of the study described in chapter 3. The sampling of caged mussels deployed around the outfall as described in chapter three was commenced by JBW in March 2013. TLP adopted the same sampling regime and experimental sites to contribute to our understanding of microbial behaviour in areas surrounding coastal outfalls. JBW devised the initial experiment using caged mussels to evaluate spatial patterns of norovirus and faecal indicator bacteria contamination. JBW undertook the fieldwork, collecting sediment samples alongside mussel and water samples. Sediment sampling continued after recovery of the remaining experimental moorings. TLP undertook bacterial analysis for sediments and is preparing the manuscript.

T.L. Perkins, J. B. Winterbourn, S. K. Malham, D. L. Jones and J. E. McDonald

The discharge of treated sewage into coastal waters is a common global practice which represents a significant point source for pathogenic bacteria entering the aquatic environment. Recent studies have suggested that coastal sediments may act as a significant reservoir for pathogenic bacteria. Subsequently these bacteria may be re-suspended back into the water column and adversely impact shellfish growing waters, bathing waters and human health. The objective of this study was to investigate the spatial and temporal patterns of pathogenic bacteria within coastal sediments, in close proximity to a long submarine offshore domestic sewage outfall. Sediment samples were collected monthly from a 1km grid array surrounding a sewage outfall. Cultureable *E. coli*, total coliforms, *Vibrio* and *Enterococcus* spp. were enumerated. This study revealed a spatial pattern of culturable bacterial dispersal within sediments showing a strong correlation with a tidally driven effluent dispersal model. Thus it suggests that discharged bacteria may not always disperse out to coastal waters but can settle into the surrounding sediments, which under certain conditions may be resuspended back into the water column and transported. The findings from this study also highlighted alternative areas with elevated bacterial concentrations, suggesting that whilst the sewage outfall may be the principle source of bacterial contamination within the study region, alternative point and diffuse sources may also represent significant bacterial inputs. In addition, a high degree of temporal variation was observed across all bacterial groups and sites. These findings show the potential for sediments to act as a reservoir for sewage-derived bacteria.

APPENDIX IV

Training Undertaken

Laboratory training in application of CEN/ISO method for quantification of NoV in Bivalve Molluscan Shellfish	01/10/11	Centre for Environment, Fisheries and Aquaculture Science, Weymouth.
First Aid Basic Sea Survival Health and Safety Basic Firefighting and Prevention	09/02/13 10/02/13 11/02/13 13/02/13	Maritime and Coastguard Agency / Sea Fish Industry Authority
Rhodamine WT Dye Tracing	22/03/13	New South Wales Food Authority / US Food and Drug Administration
Graduate School	23/05/13	Knowledge Economy Skills Scholarships
QuantStudio™6 Flex real-time PCR training course	25/11/13	Life Technologies™

APPENDIX V

Participation and Industrial Engagement during this PhD

Nov 2013 – Participant in Public Health Wales investigation into shellfish-vectored outbreak of viral illness.

Lead: **Dr. Christine Whiteside**- Consultant in Communicable Disease Control, Health Protection Team, Public Health Wales, Chris.whiteside@wales.nhs.uk

Agencies involved: Public Health Wales, Centre for Environment Fisheries and Aquaculture Science, Conwy Council Environmental Health, Natural Resources Wales, Dŵr Cymru Welsh Water, Food Standards Agency, European Food Safety Authority, 4x Ltd. Companies.

European Fisheries Fund Stakeholder Meeting. Assisted in hosting meeting with local industry, Environmental Health Officers, Dŵr Cymru Welsh Water, Anglian Water, FSA, CEFAS and SeaFish Industry Authority. Bangor

Shellfish Association of Great Britain. Delegate at Mollusc Committee and Norovirus focus meetings. London

Local Shellfish Action Group. Active in stakeholder group comprising industry, regulators, Water and Public Health Authorities. Colwyn Bay.