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Bacterial reservoirs in shellfish and shellfish harvesting waters

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Bacterial reservoirs in shellfish and shellfish harvesting waters

Katie Clements

September 2013



PRIFYSGOL
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A thesis submitted to Bangor University in candidature
for the degree

Philosophiae Doctor

School of Ocean Sciences

Bangor University, Menai Bridge, Anglesey, LL59 5AB

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Summary

Shellfish have been recognised as an important human food source since roman times and are now routinely consumed by inhabitants across five continents. However, shellfish are also well known vectors for human illness as they are capable of bio-accumulating pathogenic micro-organisms from the wider environment within somatic tissues and hence, are capable of transferring these pathogens into the human food chain. Current European efforts to safeguard consumers include the routine bacteriological monitoring of shellfish tissues using *E. coli* as a proxy for potential pathogenic micro-organisms. The aims of this thesis were firstly, to identify and quantify the bacterial reservoirs present in commercial shellfish harvesting areas. Secondly, to determine the relative contribution of these reservoirs under different mitigation techniques, and thirdly, to examine the relationship between the bacterial and viral reservoirs present within shellfish tissues.

A single commercial mussel (*Mytilus edulis*) bed was intensively surveyed to identify both spatial and temporal changes in the bacterial reservoir present within mussel tissues and to examine the relationship between the bacterial reservoir present within the mussel tissues and concentrations of bacteria present in the underlying sediment. This study concluded that the underlying sediments represented a greater bacterial reservoir than within the mussel tissues, however no spatial relationship between the two reservoirs was evident. In addition, we investigated the potential of epizoic organisms to act as a bacterial reservoir. The findings from this study demonstrated that epizoic barnacles contained more than 80% of the total coliform bacteria present and, as such, represent a previously unidentified, but significant bacterial reservoir in shellfish harvesting areas.

Based on previous findings, the effect of standard mitigation techniques i.e. depuration and offshore relaying on the previously identified bacterial reservoirs were investigated. A standard 48 hour depuration treatment was shown to be effective in the reduction of indicator bacteria from shellfish tissues, but ineffective in reducing the bacterial content of epizoic organisms to beneath acceptable levels. Offshore relaying was shown to be an effective measure to reduce both bacterial and viral concentrations in shellfish tissues, however, these concentrations demonstrated no relationship with the bacterial content of the surrounding waters.

Finally the relationship between the bacterial and viral content of shellfish tissues was examined and compared to modelled *E. coli* concentrations in the water surrounding a sewage outfall. No relationship between the bacterial and viral content of the shellfish was observed. This finding supports previous studies suggesting that bacterial indicators are poor surrogates for viral contamination of shellfish. However, interestingly the determined concentrations of norovirus within shellfish tissues were more closely correlated with the modelled predictions than the determined *E. coli* concentrations from the shellfish tissues.

In conclusion, the bacterial reservoir within shellfish flesh may be over-shadowed by larger bacterial reservoirs present within the wider shellfish harvesting area. The interaction between these environmental bacterial reservoirs and the bacterial reservoirs within shellfish flesh remains largely unknown, and represents an area for further study, especially with regard to the impact of alternative environmental bacterial reservoirs on the bacterial content of shellfish destined for human consumption.

Contents

Declaration and Consent	2
Summary	6
List of Figures.....	11
List of Tables.....	14
Dedication	15
Acknowledgements	16
Abbreviations	17
CHAPTER 1.....	20
Introduction.....	20
1.1 General information on shellfish	21
1.2 Global shellfish production.....	22
1.3 Nutritional benefits of shellfish consumption	24
1.4 Shellfish as vectors for human illness.....	26
1.5 Factors affecting pathogen accumulation in shellfish.....	32
1.6 Methods for determining the source of contamination in shellfish	34
1.7 Legislation governing shellfish production	38
1.8 Effectiveness of current legislation for safeguarding shellfish consumers	40
1.9 Mitigation strategies for reducing microbial contamination in shellfish.....	41
1.10 The Conwy mussel fishery.....	47
1.11 Objectives and outline of this thesis	48
1.12 Literature cited	52
CHAPTER 2.....	65
Spatial and temporal heterogeneity of faecal indicator bacteria across an intertidal shellfish bed: Implications for routine bacterial monitoring	65
Abstract	66
2.1 Introduction.....	67
2.2 Materials and methods.....	70
2.3 Results	74
2.4 Discussion.....	83
2.5 Acknowledgements.....	88
2.6 Literature cited	88
CHAPTER 3.....	92
Epizotic barnacles act as pathogen reservoirs in shellfish beds	92

Abstract	93
3.1 Introduction.....	94
3.2 Materials and methods.....	96
3.3 Results	98
3.4 Discussion.....	102
3.5 Acknowledgements.....	104
3.6 Literature cited	105
CHAPTER 4.....	109
Differential depuration dynamics of bivalve shellfish and epizoic barnacles: Implications for current depuration practices.....	109
Abstract	110
4.1 Introduction.....	111
4.2 Materials and methods.....	113
4.3 Results	116
4.4 Discussion.....	122
4.5 Acknowledgements.....	125
4.6 Literature cited	126
CHAPTER 5.....	129
Assessment of the potential for offshore shellfish cultivation to mitigate against bacterial and viral contamination.....	129
Abstract	130
5.1 Introduction.....	131
5.2 Materials and methods.....	134
5.3 Results	139
5.4 Discussion.....	148
5.5 Acknowledgements.....	152
5.6 Literature cited	153
CHAPTER 6.....	158
Use of <i>Mytilus edulis</i> biosentinels to evaluate spatial contamination patterns of norovirus and faecal indicator organisms in close proximity to a coastal sewage discharge.....	158
Abstract	159
6.1 Introduction.....	160
6.2 Materials and methods.....	162
6.3 Results	167
6.4 Discussion.....	173
6.5 Acknowledgements.....	176

6.6 Literature cited	177
CHAPTER 7.....	184
General Discussion.....	184
7.1 Discussion.....	185
7.2 Identification and quantification of bacterial reservoirs in commercial shellfish harvesting areas within North Wales, UK.....	186
7.3 Determination of the relative contribution of previously identified bacterial reservoirs in shellfish tissues under different mitigation strategies	193
7.4 Examination of the relationship between bacterial and viral reservoirs within shellfish tissues	196
7.5 Final conclusions and future management recommendations	197
7.6 Literature Cited	199
APPENDIX I.....	203
Spatial variation of waterborne <i>Escherichia coli</i> – Implications for routine water quality monitoring	203
APPENDIX II	208
Using PCR-DGGE to track the flux of <i>Escherichia coli</i> communities through an estuarine nature reserve	208
APPENDIX III.....	223
Epizootic barnacles act as pathogen reservoirs on commercial shellfish beds: Implications for the shellfish industry.....	223
APPENDIX IV.....	228
Evaluation of spatial contamination patterns for norovirus and faecal indicator bacteria near to a coastal sewage discharge using <i>Mytilus edulis</i> as biosentinels.....	228
APPENDIX V	233
Sediment composition influences spatial variation in the abundance of human pathogen indicator bacteria in the Conwy Estuary, North Wales	233
APPENDIX VI.....	235
Fate of norovirus during commercial and laboratory simulated depuration of naturally contaminated mussels	235
APPENDIX VII	237
Evaluation of the use of brilliance® selective media for the enumeration of <i>E. coli</i> and total coliforms from food and environmental samples.	237
APPENDIX VIII.....	240
Industry directed research	240
APPENDIX IX.....	246
Other investigations undertaken	246
APPENDIX X.....	251

Photographs depicting the experimental methodologies..... 251

List of Figures

Fig.1.1. Total UK shellfish landings divided by shellfish type (thousand tonnes) in 2011.

Fig.1.2. Fat content of various shellfish types compared with fish, chicken and beef.

Fig.1.3. Omega-3 (n-3) fatty acid content of different human food sources.

Fig.1.4. Number of reported outbreaks in the UK resulting from the consumption of contaminated shellfish by decade (1970-2000).

Fig.1.5. Reported disease outbreaks from different shellfish types 1969-2000.

Fig.1.6. *E. coli*, faecal coliform bacteria and *enterococci* MPN counts in estuarine sediments under base and high flow conditions.

Fig.1.7. Routine microbiological sampling results for the Conwy mussel beds 2008-2013.

Fig.2.1. Map showing the study sampling location. Inset diagram shows the location and approximate positions of the five transects across the shellfish bed.

Fig.2.2. Contour plots showing the distribution of bacteria across a mussel bed. *E. coli* distribution in mussels (a) and sediments (b), coliform distribution in mussels (c) and sediment (d), distribution of *Vibrio spp.* (e) and marine heterotrophic bacteria in mussels (f).

Fig.2.3. Bacterial concentrations of a) *E. coli* and b) coliforms within mussel flesh, over differing temporal scales, demonstrating the effect of tidal state and seasonality.

Fig.2.4. Bacterial concentrations of a) *Vibrio spp.* and b) marine heterotrophs within mussel flesh, over differing temporal scales, demonstrating the effect of tidal state and seasonality.

Fig. 3.1. Total coliform population observed in mussels and barnacles across three commercial shellfish beds.

Fig.3.2. *Vibrio spp.* and total marine heterotroph (TVC) population size observed in both mussels and barnacles across three commercial shellfish beds.

Fig.4.1. Total coliform population observed in a) mussels and b) barnacles from three commercial shellfish beds held over a 72 hour time period in a depuration facility.

Fig.4.2. Presumptive *Vibrio spp.* population observed in both a) mussels and b) barnacles from three commercial shellfish beds held over a 72 hour period in a depuration facility.

Fig. 4.3. Total viable count (TVC) of the marine heterotroph population observed in both a) mussels and b) barnacles from three commercial shellfish beds held over a 72 hour period in a depuration facility.

Fig. 5.1. 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).

Fig. 5.2. Concentrations of (a) *E. coli* and (b) total coliforms observed in offshore relayed mussels from differing distance offshore over 124 days. For comparison (c) total coliform concentration observed in seawater at differing offshore distances over 124 days.

Fig. 5.3. Results for (a) norovirus GI and (b) norovirus GII detected in replicate mussel samples from different offshore distances for each time point.

Fig. 5.4. Mussel shell length as observed in offshore relayed mussels from differing distance offshore over 124 days.

Fig. 5.5. Mussel flesh weight measured pre-deployment on 28th March 2012 and post-deployment on 28th May 2012 over differing distance offshore.

Fig. 5.6. Percentage mortality observed in offshore relayed mussels from differing distance offshore over 124 days.

Fig. 6.1. Map showing the outfall location and sample sites.

Fig. 6.2. Concentrations of Norovirus GI and GII and indicator bacteria determined from caged mussels after one month *in situ*.

Fig. 6.3. Concentrations of Norovirus GI, GII, *E.coli* and total coliform bacteria determined from mussel flesh samples after one month *in situ*. Data are expressed per site, across two transects a) west - east and b) north - south.

List of Tables

Table 1.1. Quantity and value of shellfish types cultured in the UK in 2010.

Table 1.2. Percentage of reported, shellfish vectored, disease and deaths attributed to different shellfish species 1984 - 1994.

Table 1.3. Summary of human pathogens transmitted through shellfish consumption.

Table 1.4. Microbial classification of shellfish harvesting waters.

Table 1.5. Published values for elimination rates of potential human pathogens.

Table 2.1. Correlation coefficient (r) matrix demonstrating the relationships between bacterial concentrations, nutrients and physico-chemical parameters over a commercial mussel bed.

Table 3.1. Species composition of barnacles on the surface of mussels obtained from three commercial shellfish beds.

Table 3.2. Comparison of ecological parameters across three commercial mussel (*M. edulis*) beds.

Table 3.3. Proportion of the coliform reservoir associated with mussels and their epizoic barnacles in three commercial shellfisheries.

Table 4.1. Comparison of the elimination rate of total coliform bacteria for mussels and barnacles from three commercial shellfisheries, over a 48 hour depuration procedure.

Table 4.2. Ecological evaluation and bacteriological comparison of three commercial mussel (*M. edulis*) beds. In all cases $n = 15$, bracketed numbers represent the Standard Error.

Table 5.1. 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.

Table 6.1. Baseline concentrations of norovirus GI, GII, *E. coli* and total coliforms.

Table 6.2. Correlation co-efficient matrix demonstrating the relationship between determined norovirus GI, GII, *E. coli*, total coliforms and the predicted (modeled) *E. coli* concentrations.

Dedication

This PhD is dedicated to my parents.

Without your support this work would not have been possible.

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The production of this thesis would not have been possible without the help and assistance of many different people and I would like to take this opportunity to thank everyone who has assisted me in this endeavour. Firstly, I would like to thank my funding body, KESS, and in particular, Penny for her tireless efforts in dealing with my queries and problems. I would also like to thank Bangor Mussel Producers and Deepdock Ltd, in particular James Wilson for providing valuable local advice and equipment, not to mention the match funding for this project.

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Abbreviations

%	Percent
µL	Micro litre
°C	Degrees Centigrade
CAMS	Centre for Applied Marine Sciences
CEFAS	Centre for Environment Fisheries and Aquaculture Science
CFU	Colony Forming Units
cm	Centimetre
d or d ¹	Day or per day
dl	Discharge Limit
DNA	Deoxyribonucleic Acid
EC	Electrical Conductivity
EC	Electrical Conductivity
EFSA	European Food Safety Authority
ESF	European Social Fund
EU	European Union
<i>Ex situ</i>	Off site
FIB	Faecal Indicator Bacteria
g	Gram
gc /g	Genome copies per gram
GI	Genogroup 1
GII	Genogroup 2
h or h ¹	Hour or per hour
HBGA	Histo-Blood Group Antigen
HPP	High Pressure Processing
<i>In situ</i>	In position

KESS	Knowledge Economy and Skills Scholarship
kg	Kilogram
km	Kilometre
L or l	Litre
LAMP	Loop Mediated Isothermal Amplification
LCRI	Low Carbon Research Institute
LH-PCR	Length heterogeneity Polymerase Chain Reaction
LOD	Limit of Detection
m	Meters
m or m² or m³	Meters, Meters squared, Cubic meters
min	Minute
mL or ml	Millilitre
mm	Millimetre
mm	Millimetre
MPN	Most Probable Number
MST	Microbial Source Tracking
NH₄⁺	Ammonium
NO₂⁻	Nitrite
NO₃⁻	Nitrate
NoV	Norovirus
NRL	National Reference Laboratory
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PO₄³⁻	Phosphate
qPCR	Quantitative Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction

Rep-PCR	Repetitive Element Polymerase Chain Reaction
rev /min¹	Revolutions per minute
RNA	Ribonucleic Acid
s or s⁻¹	Second or per second
SE	Standard Error
spp.	Species
T₀	Time at point “0”
TN	Total Nitrogen
TOC	Total Organic Carbon
T-RFLP	Total Restriction Fragment Length Polymorphism
TVC	Total Viable Count
UK	United Kingdom
US	United States
UV	Ultra Violet
VBNC	Viable But Non-Culturable
w/v	Weight to Volume ratio
WWTP	Waste Water Treatment Plant

CHAPTER 1

Introduction

1.1 General information on shellfish

Shellfish: noun

(plural) -fish, -fishes

“any aquatic invertebrate having a shell or shell-like carapace, esp such an animal used as human food. Examples are crustaceans such as crabs and lobsters and molluscs such as oysters”. Collins English Dictionary (2011).

Globally there are approximately 8,500 species of shellfish which are classified according to their shell form (Potasman et al. 2002). Shellfish are not represented by a single phylum, rather the classification system for shellfish encompasses three separate phyla; Mollusca, Crustacea and Echinodermata (Hayward and Ryland 1995). Molluscan shellfish are characterised by the number of shell valves; bivalve shellfish (oysters, mussels and clams) have two shell valves hinged by an elastic ligament, univalve shellfish (limpets, whelks and winkles) have a single shell valve and cephalopods (octopus, squid and cuttlefish) have no shell valves. Crustacean shellfish are characterised by having an external skeleton and include prawns, barnacles and crayfish. Echinoderm shellfish include starfish, sea urchins and sea cucumbers. For the purpose of this thesis the term “shellfish” refers to molluscan bivalve shellfish unless otherwise stated.

Bivalve shellfish can be found in a range of aquatic habitats, with species found in both freshwater (European freshwater pearl mussel; *Margaritifera margaritifera*) and saltwater ecosystems (common European mussel; *Mytilus edulis*). Bivalve shellfish can be found both in the tropics (Baker et al. 2007) and in the higher Northern and Southern latitudes (Hilbish et al. 2000). The majority of bivalve molluscan shellfish have a benthic – pelagic life cycle broadly consisting of a pelagic larval stage and a sessile benthic adult stage (Roughgarden et al. 1988, Possingham and Roughgarden 1990, Ackerman et al. 1994). The pelagic larval stage enables effective colonisation of both new and existing shellfish beds via natural processes such as water currents and tidal movements (Stancyk and Feller 1986, Alexander and Roughgarden 1996, PiNeDa et al. 2007), and anthropogenic activities such as transportation via ballast water in ships (Mackie 1991).

Bivalve shellfish larvae typically settle close to their con-specifics (Grünbaum 2011) and can form dense ‘beds’ of adult bivalve shellfish. These ‘shellfish beds’ can occur naturally or be artificially created for commercial species (Quayle and Newkirk 1989). Shellfish beds have important ecological implications, as dense aggregations of shellfish are capable of engineering

the natural ecosystem by affecting localised hydrodynamic processes (Stevens et al. 2008), altering the nutrient flux (Forrest et al. 2009) and outcompeting native organisms (Aldhous 2009). Nevertheless, bivalve shellfish are a crucial component of the diet of many species including crabs (Elner 1981), fish (French III 1993), birds (Carter 1968) and humans (Teplitski et al. 2009).

Bivalve shellfish are filter feeders, consuming a mixed diet of phytoplankton and detritus (Lesser et al. 2010) and are commonly found in areas of sheltered water where the nutrient levels are high (Potasman et al. 2002). The digestive physiology varies within the class (Riisgård and Larsen 2000) and for the majority of species the effect of environmental parameters (water velocity, food concentration, temperature and salinity) on feeding rate have yet to be accurately determined (Newell et al. 2001). The precise mechanism of filtration feeding in molluscan shellfish is controversial (Bayne 1998, Dolmer 2000). Jørgensen (1996) argued that shellfish feeding is autonomous, whilst conversely others have argued that certain species are able to feed selectively and are able to regulate their food uptake depending on prevailing environmental conditions (Shumway et al. 1985, Lucas et al. 1987, Wong and Cheung 1999). Despite this uncertainty, many studies have shown that shellfish are capable of bio-accumulating potentially pathogenic micro-organisms from their surrounding environment, which can then be transferred to humans via the consumption of contaminated individuals (Wittman and Flick 1995, Potasman et al. 2002, Teplitski et al. 2009).

1.2 Global shellfish production

Shellfish have been recognised as an important food source since Roman times and are now consumed routinely by inhabitants on 5 continents (Potasman et al. 2002). It is estimated that, on average, each inhabitant of the planet consumes 16 kg of seafood per annum (Teplitski et al. 2009). Over the last 30 years, shellfish production and harvesting has increased dramatically (Potasman et al. 2002). This trend has been attributed to increased awareness of the health benefits of shellfish consumption (Woolmer 2010).

1.2.1 Shellfish capture fisheries

Driven by consumer demand, 19.9 million metric tonnes of “wild caught” shellfish were landed globally in 2010, with an estimated global value of £9.23 billion. Molluscan shellfish species represented 71% of this total (Anon 2012^a). In the UK in 2011; 600 thousand tonnes of ‘seafish’ were landed from UK registered vessels at a total estimated value of £828 million.

Of the total UK ‘seafish’ landings, 151.3 thousand tonnes were shellfish (Elliott et al. 2012). In 2011, shellfish formed the majority of all landings into England, Wales and Northern Ireland (56, 14.7 and 13.2 thousand tonnes respectively (Elliott et al. 2012)). Shellfish landing data from 2011 (UK) shows that scallops represented the highest quantity of shellfish landed, followed by nephrops and crabs (Fig.1.1). Export data shows that 56% of the UK bivalve shellfish harvest is exported, mainly to Europe where there is a high market demand for processed shellfish (Lake and Utting, 2007).

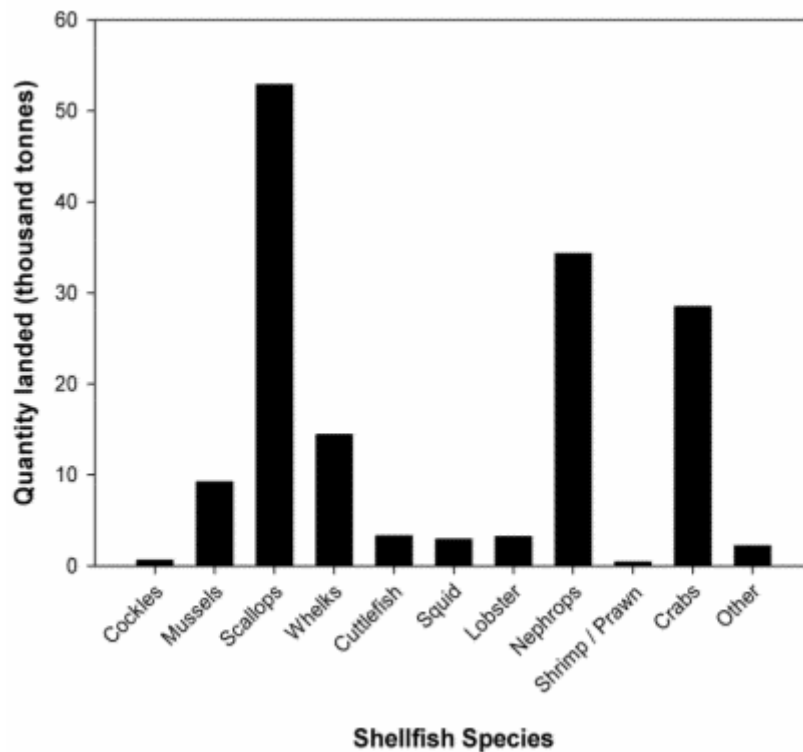


Fig. 1.1. Total UK shellfish landings divided by shellfish type (thousand tonnes) in 2011. Compiled from Elliott et al. (2012).

1.2.2 Shellfish aquaculture

Aquaculture is the fastest growing food supply sector in the world (Anon 2012^b). In Europe, molluscan shellfish production in 2010 was 0.63 million metric tonnes which equates to approximately 4.5% of the global total, at a value of 1.21 billion US dollars (Anon 2012^b). In Europe, total aquaculture production equating to 1.3 million tonnes or 3.2 billion euros represents a quarter of all European production (including fish, molluscs and crustaceans) (Anon 2012^b). In Europe, mussels are the main shellfish group that are cultivated, producing a total of 477,000 tonnes in 2010 (representative of all species). Pacific oysters were the second

major shellfish group cultivated in Europe in 2010, with a total production of 105,000 tonnes (Anon 2012^b).

Table 1.1. Quantity and value of shellfish types cultured in the UK in 2010 (Anon 2012^b).

<i>Tonnes</i>	Scotland	England	Wales	Northern Ireland	UK Total
Pacific oyster	241.00	646.00	3.00	260.00	1,150.00
Native (flat) oyster	28.00	88.50	0.05	-	116.55
Scallops	7.60	-	-	1.00	8.60
Queens	7.30	-	-	-	7.30
Mussels	7,199.00	3,233.00	8,960.00	10,820.00	30,212.00
Clams	-	26.50	-	-	26.50
Cockles	-	7.30	-	-	7.30
Estimated value (£ million)	8.30	3.40	6.10	7.70	25.50

Shellfish production in the UK (2010) was valued at approximately £25 million from a total production of approximately 31,500 tonnes (Table 1.1). In accordance with the production statistics for Europe, the major shellfish group produced in all UK countries, in 2010, were mussels, followed by pacific oysters (Table 1.1).

1.3 Nutritional benefits of shellfish consumption

Shellfish are becoming more widely recognised as a nutritious food source, evidenced by increasing consumption rates and value (Glude 1983, Oliveira et al. 2011). Shellfish are currently promoted in developed countries as the ‘healthy eating’ alternative to consuming meat and poultry. The attributes (minimal processing and free from additives) of shellfish as a food source also appeal to consumers in these nations (Acebron and Dopico 1999, Murchie et al. 2005). However, in developing countries the importance of shellfish as a human food source may be under-estimated, as in many countries shellfish represent up to 50% of the total animal protein consumed (Smith et al. 2010).

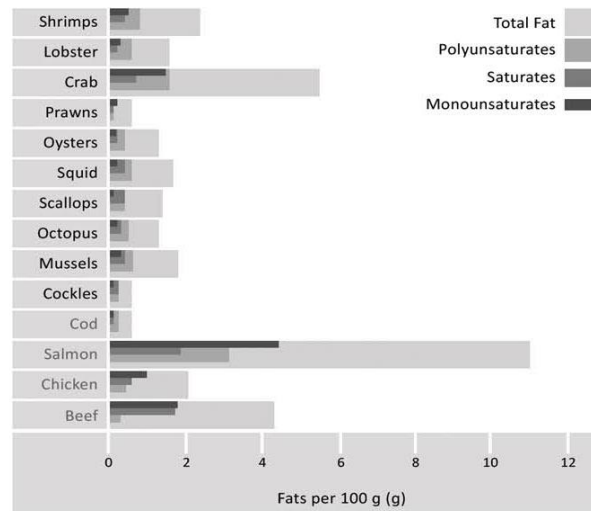


Fig. 1.2. Fat content of various shellfish types compared with fish, chicken and beef (Woolmer 2010).

Shellfish contain less saturated fat per 100 g than chicken, beef, and salmon (Fig.1.2) which is significant as saturated fats have been linked with cardiovascular disease and high cholesterol (Woolmer 2010). Shellfish have high levels of polyunsaturated fats (Fig. 1.2) which are high in levels of n-3 fatty acids, also known as omega-3 (Fig. 1.3), which are known to promote wellbeing when consumed by humans (Woolmer 2010). Long chain n-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DPA) cannot be synthesised within the human body and are exclusively obtained from the diet; it is in this respect that shellfish are considered to be an important component of the human diet (Arts et al. 2001).

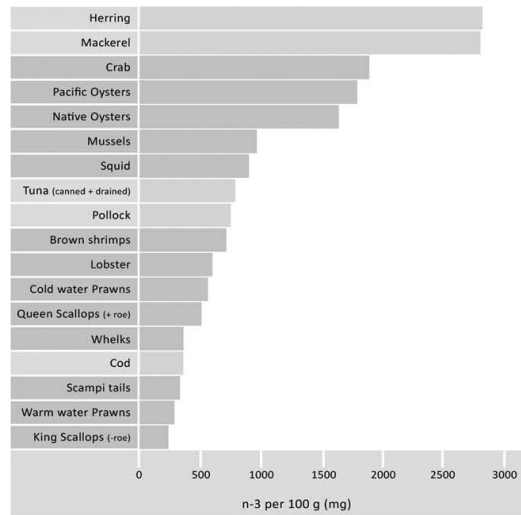


Fig. 1.3. Omega-3 (n-3) fatty acid content of different human food sources (Woolmer 2010).

The benefits of increased n-3 fatty acid consumption vary from protection against high cholesterol and cardiovascular disease, including reducing the risk of fatal myocardial infarctions by up to 20% by consuming 200 g or more shellfish per week (Yuan et al. 2001), to helping promote a healthy weight loss diet (Børresen 2008). Alternative benefits of consuming n-3 fatty acids also include a lower risk of colorectal, breast and prostate cancer (Woolmer, 2010) with increased protection against inflammatory bowel disease (Børresen 2008). In addition, increasing consumption of n-3 fatty acids also has positive effects on a number of other disorders such as schizophrenia, attention deficient hyperactivity disorder (ADHD), eczema, diabetes, Alzheimer’s disease, dementia and Parkinson’s disease (Woolmer 2010). As well as preventing disease, shellfish are also an important source of vitamins such as Thiamin B1, Riboflavin B2, Niacin B3, B6, B12 and vitamin E, and are a rich source of iron, selenium, copper, phosphorus, zinc and iron, therefore contributing to a healthy and balanced diet (Woolmer 2010).

1.4 Shellfish as vectors for human illness

Bivalve shellfish are filter feeders capable of bio-accumulating pathogenic micro-organisms that are present either within their surrounding environment *in situ* (Metcalf et al. 1979, Larkin and Hunt 1982, Burkhardt and Calci 2000, Lees 2000, Nappier et al. 2008) or after exposure to pathogenic micro-organisms post harvesting via secondary contamination (Sagoo et al. 2007). Subsequent consumption of contaminated shellfish, with minimal post

retail treatment (i.e. cooking) by humans, may vector pathogenic organisms into the human food chain and cause illness amongst the human population. Therefore, despite the nutritional benefits of consuming shellfish, the number of cases per annum of disease and death vectored by consumption of contaminated shellfish is significant enough to cause concern among the general public (Wittman and Flick 1995). Coupled with an increase in the recognition of an inter-relationship between human health and the oceans, research efforts are now being directed towards investigating the linkages between public health and the oceans (Fleming et al. 2006) with an aim to preserving the long term sustainability of the shellfish industry.

1.4.1 Outbreaks of human illness associated with shellfish consumption.

It is estimated that over 2 billion people worldwide rely on seafood as a major source of protein in their diet (Fleming et al. 2006). However, research shows that 1 in 4 Americans suffer a food-borne illness each year, totalling over 76 million cases, with 5000 deaths per annum as a direct result of contracting a food-borne illness (Tauxe 2002). These figures are thought to significantly under-estimate the true numbers of cases due to severe under-reporting, as national statistics rely on the affected individual self-reporting to their local GP. Studies have shown that despite the consumption of bivalves across 5 continents, only 12 countries have reported any significant shellfish-related disease outbreaks. Particularly notable is the lack of any reports of outbreaks from Africa, demonstrating that the published figures for global shellfish related outbreaks are severely underestimating the number of cases per annum (Potasman et al. 2002). A case study by Ang (1998) highlights the under-reporting of symptoms in the UK. In February 1997 an outbreak of viral gastroenteritis caused by the consumption of contaminated oysters was discovered. In this case, none of the individuals affected saw their GP, and the outbreak was only discovered as some of the affected diners were part of a birthday party and knew one another. Following this discovery an enquiry was launched and many more affected individuals were discovered who were not part of the birthday party. Studies such as Fleisher and Kay (2006) have also shown that there is a “risk perception bias” concerning the reporting of symptoms. For example, those individuals who perceive themselves to be more at risk are more likely to report their symptoms. The number of reported outbreaks has increased per decade (Fig.1.4); however this could be due largely to an increase in consumer awareness. Tauxe (2002) argues that the rationale behind the increase in the number of reported outbreaks is due to anthropogenic involvement. For instance, altering the ecology of an area, or improvements in processing technology can directly connect pathogens into the food chain (e.g. the shipping of shellfish with low level contamination may

increase their pathogen load during long-distance transport, making the source of contamination harder to identify).

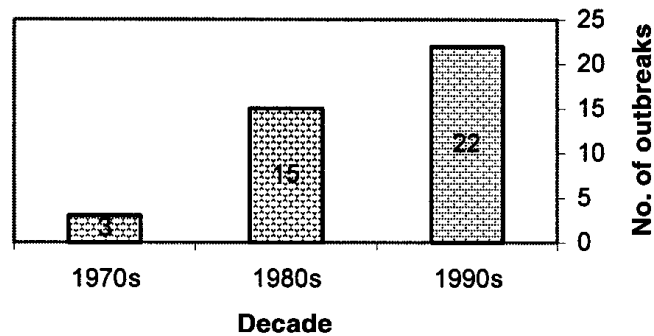


Fig. 1.4. Number of reported outbreaks in the UK resulting from the consumption of contaminated shellfish by decade (1970-2000) (Potasman et al. 2002).

The first reported case of disease linked with shellfish consumption occurred in 1816 (Potasman et al. 2002). Since then the pathogens responsible for food-borne infections have remained in flux; over time well established pathogens have been removed or eliminated and new pathogens have emerged (Tauxe 2002). For example, outbreaks of Typhoid fever induced from consuming contaminated shellfish were prevalent in the US until the 1950's, however no further outbreaks have been documented since 1994 (Rippey 1994). This can be contrasted to newly emerging pathogens such as *Escherichia coli* O157:H7 which was first identified as a pathogen in 1982, and continues to be identified as the agent responsible for disease outbreaks worldwide (Tauxe 2002). In 2004, one of the first outbreaks of Hepatitis E was recorded from the consumption of contaminated shellfish in India (Swain et al. 2010). It is reported that since 1977 a new food-borne pathogen is discovered at the rate of one pathogen every two years, but as more knowledge is gained about the pathogen, it is brought under control and eventually eradicated (Tauxe 2002).

1.4.2 Shellfish types responsible for vectoring human illness

The shellfish industry is entirely driven by the needs of the consumer and at present the consumer preference is for raw or lightly cooked shellfish, although definitive statistics on this aspect are lacking (Wittman and Flick 1995, Potasman et al. 2002). This poses a problem for the shellfish industry as it requires more extensive efforts to ensure a safe supply of shellfish supplied to the consumer. Essentially the safety issues focus on the quality of the shellfish harvesting waters and the conditions under which the shellfish are harvested, processed and distributed (Wittman and Flick 1995).

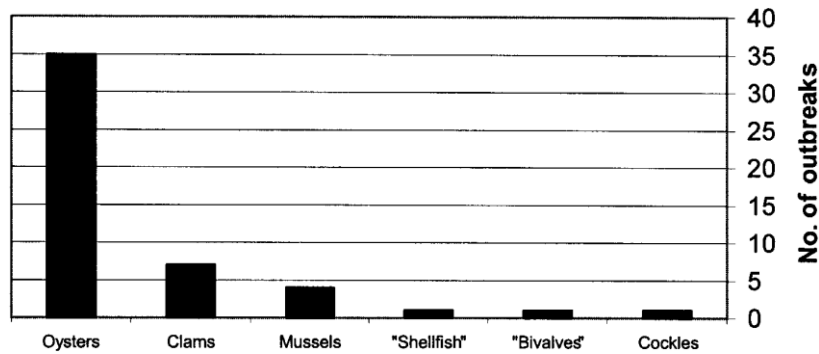


Fig. 1.5. Reported disease outbreaks from different shellfish types 1969-2000 (Potasman et al. 2002).

Shellfish species that are traditionally served raw or lightly cooked are more often cited as the vector for outbreaks of disease when compared to shellfish species that are more often served cooked or pickled (Fig. 1.5; Table 1.2). Oyster species such as *Crassostrea gigas* are regarded as a delicacy and are traditionally served raw, in shell, whereas shellfish such as cockles (*Cerastoderma edule*) and mussels (*Mytilus edulis*) are traditionally served either cooked or pickled. The process of cooking or pickling increases the likelihood that any micro-organisms responsible for food-borne illness will be either be inactivated or eliminated from the shellfish flesh. It is also postulated that other commercial shellfish species such as scallops are less likely to contain potentially harmful micro-organisms as they are motile species and possess the ability to swim away from unfavourable locations. It should be noted, however, that there is very little published data to support this theory.

The majority of disease outbreaks have been linked with the consumption of oysters (Fig. 1.5; Table. 1.2), with the first recorded outbreak in 1816 (Potasman et al. 2002). The largest recorded outbreak occurred in Shanghai in 1988 and was linked to the consumption of clams which resulted in 290,000 individuals contracting Hepatitis A, and 47 deaths (Potasman et al. 2002). However, it is estimated that 99% of all deaths and 20% of disease cases linked with the consumption of shellfish are a result of contamination by naturally occurring bacteria (Wittman and Flick 1995). Recent research has focused on viral agents, such as norovirus, as the primary pathogen responsible for human infections (Butt et al. 2004), however illnesses requiring hospitalisation and fatalities are most frequently associated with bacterial pathogens (Oliveira et al. 2011). The symptoms of diseases contracted through shellfish consumption vary in severity from mild gastrointestinal problems to death. In the majority of fatal cases, the individuals belonged to a pre-defined 'at risk' group. These groups include immunocompromised individuals, children, elderly individuals or third trimester fetuses (Wittman

and Flick 1995). It is for this reason that individuals within these groups are advised to refrain from eating raw or lightly cooked shellfish.

Table 1.2. Percentage of reported, shellfish vectored, disease and deaths attributed to different shellfish species 1984 - 1994. Compiled from (Wittman and Flick 1995).

Type of shellfish	Disease (% of total)	Deaths (% of total)
Oysters	49	97
Clams	38	2
Mixed	12	1
Mussels	2	0

1.4.3. Pathogenic micro-organisms responsible for human illness

Many of the pathogenic organisms responsible for public health outbreaks through the consumption of contaminated shellfish have been isolated and extensively studied (Table 1.3). However, variation in public health outbreaks over both temporal and spatial scales does exist. Therefore it is important to distinguish the naturally present microbial flora from the microbes shed from both humans and animals throughout individual catchments. This can be achieved to some extent through baseline monitoring of individual catchments (Colwell 1978).

Table 1.3. Summary of human pathogens transmitted through shellfish consumption.

Pathogen	Organism type	Source	Disease caused in humans	Citation
<i>Vibrionaceae vulnificus</i>	Bacteria	Naturally occurring	Gastrointestinal disease and septicæmia	(Pommepuy and Le Guyader 1998)
<i>Vibrionaceae cholerae</i>	Bacteria	Naturally occurring	Cholera	(Pommepuy and Le Guyader 1998)
<i>Vibrionaceae parahaemolyticus</i>	Bacteria	Naturally occurring	Acute gastroenteritis	(Pommepuy and Le Guyader 1998)
<i>Aeromonas spp.</i>	Bacteria	Naturally occurring	“Traveller’s diarrhoea”	(Pommepuy and Le Guyader 1998)
<i>Escherichia coli spp.</i>	Bacteria	Human and livestock faeces	Gastroenteritis	(Pommepuy and Le Guyader 1998)
<i>E. coli</i> O157:H7	Bacteria	Human and livestock faeces	Acute gastroenteritis	(Chadwick et al. 2008)
<i>Salmonella enterica</i>	Bacteria	Human and livestock faeces	Gastroenteritis (Typhoid fever depending on serotype).	(Pommepuy and Le Guyader 1998)
<i>Listeria monocytogenes</i>	Bacteria	Human and livestock faeces	Listeriosis	(Pommepuy and Le Guyader 1998)
<i>Campylobacter jejuni</i>	Bacteria	Human and livestock faeces	Gastroenteritis	(Pommepuy and Le Guyader 1998)
Norwalk like virus	Virus	Human faeces	Viral Gastroenteritis	(Karamoko et al. 2005)
Human enteric virus	Virus	Human faeces	Viral Gastroenteritis	(Karamoko et al. 2005)
Small round structured virus	Virus	Human faeces	Gastroenteritis	(Ang 1998)
Hepatitis A virus	Virus	Human faeces	Hepatitis A	(Crocì 2003)
Non B enteral hepatitis	Virus	Human faeces	Hepatitis E	(Swain et al. 2010)
<i>Giardia intestinalis</i>	Protozoa	Human and livestock faeces	Giardiasis	(Geurden et al. 2010)
<i>Cryptosporidium parvum</i>	Protozoa	Human and livestock faeces	Cryptosporidiosis	(da Fonseca et al. 2006)
Adenoviruses	Virus	Human, dog, pig and reptile faeces	Gastroenteritis	(Hundesä et al. 2010)
<i>Enterococcus faecalis</i>	Bacteria	Human and livestock faeces	Gastroenteritis	(Roslev et al. 2009)

1.5 Factors affecting pathogen accumulation in shellfish

The microbial communities present in shellfish harvesting waters are not globally ubiquitous; variation can occur over both temporal and spatial scales and can be caused by both anthropogenic factors such as sewage discharges and changes in land use, as well as natural factors such as soil type, topography, local climate and vegetation cover (Baker 2003).

1.5.1. Point and diffuse sources of microbial pollution

The sources of microbial pathogens can be classified either as ‘point’ or ‘diffuse’ sources of pollution. Point sources of pollution include sewage discharges and discharges from industries; these occur at a known geographical point and can be relatively easily monitored to quantify temporal microbial changes in the water quality (Kay et al. 2008c). Recent research advances are now focusing on the importance of non-point or diffuse sources of pollution within river catchments. These include faecal inputs from agricultural animals, surface run-off from urban areas, and groundwater leaching (Chadwick et al. 2008, Dowd et al. 2008, Kocasoy et al. 2008). These, non-point sources of pollution are notoriously difficult to determine and even more difficult to quantify. Conflict between different stakeholder and user groups compounds the problem of identifying the non-point pollution sources and impedes any remediation measures (Meays et al. 2004).

Typical remediation measures for improving water quality on a larger catchment scale have focused mainly on point sources of pollution, such as repairing faulty sewers and monitoring discharges from sewage treatment works and industry. In most cases, however, this has resulted in no significant improvement of the shellfish waters of the UK (Kay et al. 2008b). Presently there is a need for a fully integrated catchment management program, incorporating both point and diffuse sources of pollution (Stapleton et al. 2007), to appropriately ascertain the dominant sources of pollution and allocate resources accordingly. Prior to any management decisions being carried out each catchment must be thoroughly studied and information collected on climatic, aquatic, and topographic variables as well as on the microbial dynamics of the catchment (Stapleton et al. 2007). Ultimately, this will enable mathematical models to predict pathogen flux throughout the catchment (Kay et al. 2005).

1.5.2. Precipitation and land use

Pathogen flux within an individual catchment is not constant over a temporal scale. Precipitation can lead to a significant change in the quantity of many pathogens in shellfish

waters as a result of run-off from both urban and rural land types. Figure 1.6 shows the MPN (Most Probable Number) counts of *E. coli*, faecal coliforms and *enterococci* in an estuary in New Orleans USA. As can be seen under dry or ‘base flow’ conditions at both sites the bacterial counts were low. However, following a rainfall event or at ‘high flow’ the bacterial counts spiked at over 100 times the concentration observed during base flow conditions. The latter was ascribed mainly to urban run-off, and sewage discharges, washing microbial contaminants into the estuary. Riou et al. (2007) noted that there was a significant “risk period” to shellfish consumers for a minimum of three days following a high flow (+10 mm rainfall) event. Kay et al. (2005) attempted to integrate rainfall and land use data into a model for the Ribble catchment, UK. They concluded that whilst the model was useful in predicting microbial flux throughout the catchment, further research into the effects of differential land use and the impacts of increased precipitation is needed in order to improve future models.

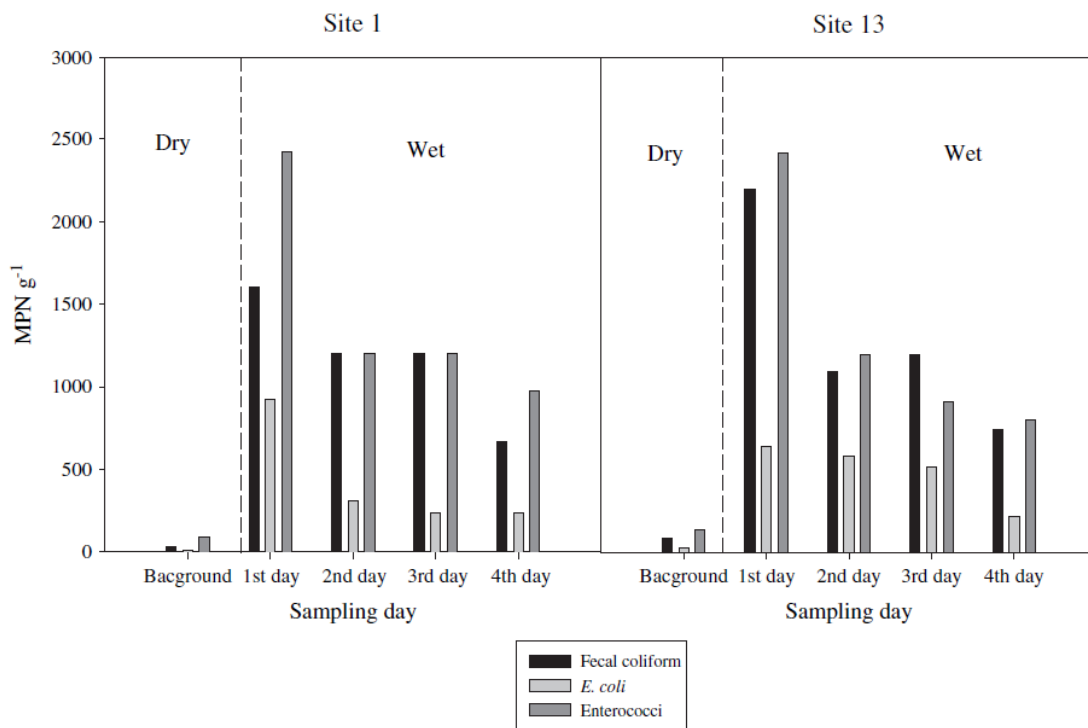


Fig. 1.6. *E. coli*, faecal coliform bacteria and *enterococci* MPN counts in estuarine sediments under base and high flow conditions (Jeng et al. 2005).

1.5.3 Seasonality of pathogens

Many pathogenic organisms demonstrate strong seasonality which contributes to temporal variability within shellfish and seasonal patterns of illness reporting among consumers (Rippey 1994). Seasonality may be linked to water temperature and salinity (Hernroth et al.

2002, del Refugio Castañeda Chávez et al. 2005), or localised weather patterns (Lipp et al. 2001). Further research is required into the behaviour of the pathogens under differing environmental conditions such as changes in temperature and salinity (Hernroth et al. 2002).

1.5.4 Environmental interactions between *in situ* pathogen reservoirs

Shellfish and the surrounding waters are well known reservoirs for pathogenic micro-organisms. However, little research has been conducted on alternative *in situ* reservoirs and the microbial flux between them. Wilkinson et al. (2006) noted that bacterial flux from re-suspended sediments in muddy estuaries was a significant diffuse pollution source, whilst (Characklis et al. 2005) noted that research concerning sediment flocs and settlement of bacteria was lacking. Recent research has demonstrated the role of epizoic barnacles as a pathogen reservoir on shellfish beds (Clements et al. 2013). Further work is required to determine other potential pathogen reservoirs and the microbial flux between different reservoirs both *in* and *ex situ*.

1.5.5 Nutrient loading

Increased nutrient loading may occur from both point and diffuse sources throughout coastal catchments (Mallin et al. 2000) and may alter the indigenous microbial community present in shellfish harvesting waters (Lessard and Beck 1990). Different bacterial species are known to be better able to utilise certain inorganic nutrients within the marine environment (Kirchman 1994) and shifting nutrient ratios may impact on the result of microbial competition (Azam et al. 1983). In addition, bacterial cells are known to enter a dormant state (Viable but Non-Culturable or VBNC) whereby, they are present in the environment but cannot be directly cultivated (Oliver 2000). Nutrient starvation has been cited as one of the stressors that can induce VBNC in bacterial cells (Trevors 2011). Therefore nutrient loading has the potential to resuscitate bacterial cells present in the environment in a state of VBNC and further work is needed to determine the effects of different nutrient parameters on the concentrations of pathogenic micro-organisms present in shellfish and shellfish harvesting areas.

1.6 Methods for determining the source of contamination in shellfish

Monitoring the water quality of shellfish harvesting waters, by point sampling and the enumeration of faecal indicator bacteria such as *E. coli* and other faecal coliform bacteria, provides only an indication of the level of faecal contamination present at a selected

geographical location and time point. This method, however, fails to identify the sources of the contamination (Field and Samadpour 2007). At present, many techniques are being developed and tested to track the sources of faecal contamination through river catchments (Meays et al. 2004). The process of tracing the origins of contaminants is termed Microbial Source Tracking (MST) and the ultimate goal of MST is to devise a technique that can be universally applied to all river catchments to identify sources of contamination and quantify their relative significance (Porter 2008).

Increased recognition of the significance of diffuse sources of faecal contamination within river catchments has created a shift from the monitoring of point sources of pollution, to a more holistic, catchment-based management approach, incorporating both point and diffuse sources of pollution and highlighting the need for the development of a valid and reliable MST method (Stapleton et al. 2007). The basic principle of MST is to determine a characteristic of faeces which enables it to be identified in a water body and assigned to a specific host, then assuming that the proportions of that marker remain the same the relative contribution of the source can be inferred (Field and Samadpour 2007). Information gleaned from MST results can then be applied in management strategies. For example, accurate identification of sources of contamination can be used to prioritise remediation efforts to avoid wasting resources (Santo Domingo et al. 2007).

MST methods can be broadly separated into two categories, molecular methods and non-molecular methods. The non-molecular methods do not allow for a fully quantitative assessment of a pollution source, therefore, there has been a shift towards molecular MST methods in recent studies (Porter 2008). MST is a rapidly developing field with new methods being continually developed and tested and older methods becoming progressively discarded in favour of new and emerging techniques (Field and Samadpour 2007).

MST methods can be categorised as either culture-based or culture-independent and as either library-dependant or library-independent. Culture-based techniques are more time consuming compared to culture-independent methods, and are limited to using only microbes that can be easily cultured. A detailed knowledge of microbial communities must also be employed as the community structure may alter during culturing and this must be accounted for in the subsequent analysis (Field and Samadpour 2007). Culture-independent methods are significantly quicker than culture-based methods and they can sample whole populations with little or no culture bias. However, the markers used have not been correlated with indicator bacteria and this is essential to match the results to European legislation (Field and Samadpour 2007). Library-dependent methods require a reference library to be created first before

comparing environmental samples to the referenced samples. Creating the reference library is time consuming and expensive compared to cheaper and quicker culture independent methods (Field and Samadpour 2007).

1.6.1 Culture-based, library-dependent methods

Examples of culture-based library-dependent methods of MST are antibiotic resistance and DNA fingerprinting techniques such as Denaturing Gradient Gel Electrophoresis (DGGE), Repetitive element palindromic PCR (Rep-PCR), ribotyping and pulsed-field gel electrophoresis (PFGE). Esseili et al. (2008) was able to utilise DGGE community fingerprinting techniques to target three genes and to identify with 85-86% accuracy the origins of *E. coli* from 150 host samples including, pigs, horses, cows and raw human sewage. This study concluded that DGGE analysis could be a useful tool in the tracking of bacteria through catchments. A drawback is that this method is not quantitative and only provides an indication of bacterial source to species level which is not solely sufficient, however, DGGE community fingerprinting could be used in conjunction with other techniques. Ribotyping as a method of tracking bacteria has been applied to over 80 case studies worldwide over 12 years (Meays et al. 2004). However, even after such an extended development period the success rate of distinguishing between eight different host species (human, chicken, pig, dog, turkey, goose, cow and horse) ranged between 46 and 96% depending on the types of enzymes used and the protocol employed (Carson et al. 2001). Antibiotic resistance studies test *E. coli* isolates against panels of antibiotics to discriminate only between human and animal faecal pollution. However, many antibiotics share the same resistance mechanisms providing unreliable results and, in addition; antibiotic resistance is not geographically or temporally stable (Meays et al. 2004).

1.6.2 Culture-based, library-independent methods

Culture-based, library-independent MST methods include monitoring the ratio of faecal streptococci to faecal coliforms, however, this is a wholly unreliable method as the ratio changes over time and differs under different environmental conditions such as temperature (Field and Samadpour 2007). The genotyping of F⁺ RNA coliphages has enabled the distinction between human and animal faecal contamination, however, the genotype distribution was shown to be significantly different to the European expected distribution (Field and Samadpour 2007).

1.6.3 Culture independent, library-dependent methods

Culture-independent, library-dependent methods include bacterial community fingerprinting, utilising targeted sampling data and analysing bacterial composition based on overlapping peaks by comparison to a reference library. This method could not distinguish naturally occurring environmental bacteria from target species, nor could it identify specific faecal sources when the samples were mixed (Field and Samadpour 2007).

1.6.4 Culture-independent, library-independent methods

Culture-independent, library-independent methods include chemical source tracking utilising compounds such as caffeine to distinguish human contamination from non-human sources (Field and Samadpour 2007) and utilising fluorometry to track optical brighteners in water (from laundry detergents). This method was utilised by (Hartel et al. 2007) to distinguish human and non-human sources of contamination, through targeted sampling. Although fluorometry is a relatively inexpensive technique, the results gained in this study were inconclusive due to background organic matter also fluorescing and the relatively quick degradation of the optical brighteners in the environment. Molecular based techniques such as LH-PCR (Length heterogeneity polymerase chain reaction) and T-RFLP (terminal restriction fragment length polymorphism) are also culture-independent, library-independent methods, but they require expert technical knowledge and expensive equipment, thus restricting their use (Meays et al. 2004). The use of bacterioidales 16S rRNA was only able to distinguish human from non-human sources of faecal contamination (Dick et al. 2005) however; in this study the results obtained showed no correlation with the predetermined database. It was also postulated that enteric bacteria may co-evolve with their hosts and this may be a significant factor for MST.

1.6.5 Limitations of Microbial Source Tracking

The most promising technique to be considered for MST was a culture-independent, library-dependent method. Hosts shed blood and intestinal cells in faeces and by using real time PCR and quantifying the amplicons during the early phase of the PCR reaction, this allowed the quantification of the PCR products and provided a quantitative assessment of the sources of contamination. This technique became known as quantitative PCR or qPCR (Gilbride et al. 2006). Focused research devised primers to target specific hosts and improve the specificity of the assay. However, Stapleton et al. (2009) found no correlation between bacterial qPCR analysis and faecal indicator bacteria, thus the results could not be corroborated with legislation. It was also discovered in the same study that the qPCR signal was lost following

UV treatment of water, thus this technique could not be used downstream of any sewage treatment plant that utilised UV treatment. Porter (2008) also noted that bird markers were occasionally present in human samples and this was attributed to poultry being consumed as part of the human diet.

New methods of MST are constantly emerging (Santo Domingo et al. 2007) and the development of new methods continues to drive research in this area. (Velusamy et al. 2010) investigated the use of biosensors as a quantitative method of MST that was both rapid and was capable of being used *in situ* in the environment to quantify pathogen flux. (Yano et al. 2007) developed a novel method of MST known as Loop Mediated Isothermal Amplification (LAMP) which has a much lower detection limit than conventional PCR and a more rapid amplification time. The results can be visually monitored through monitoring the turbidity within the sample; therefore specialist equipment is not required.

Significant drawbacks to the concept of MST are the lack of performance standards on which to assess the accuracy of any results and the lack of any formal standard operating procedures for any techniques (Santo Domingo et al. 2007). Current MST methods also focus on correlations with faecal indicator bacteria in order to comply with current legislation, however if MST is to be used to protect human health, MST needs to be specifically correlated with pathogens, therefore there is an urgent need for specific pathogen source tracking (Field and Samadpour 2007). Presently, MST should be regarded as a 'new' science requiring further focused research for validation prior to its application (Stapleton et al. 2009).

1.7 Legislation governing shellfish production

The Shellfish Waters Directive 79/923/EEC (Anon 1979) was adopted on the 30th October 1979. This directive set the benchmark for all subsequent legislation concerning shellfish as it provided the first set of microbial and sampling guidelines for shellfish harvesting waters. This directive cited only one microbial guideline (less than 300 faecal coliform units per 100 grams of mussel flesh or 100 ml of inter-valvular fluid) and cited that a 5 tube, 3 dilution most probable number (MPN) method should be used for the processing and enumerating of quarterly shellfish samples. This directive was superseded by the Shellfish Waters Directive 2006/113/EC (Anon 2006). However, the microbial indices and sampling strategy remained unchanged. These directives relate entirely to the waters where shellfish are located and/or grown, and they provide only indirect protection for shellfish consumers. Protection for the consumers of shellfish products was provided by the Shellfish Hygiene Directive 91/492/EEC

(Anon 1991) which set out microbial standards for shellfish intended for human consumption. This directive imposed a classification system for shellfish harvesting areas, and clear microbial limits for shellfish at their point of sale. This directive was superseded by European legislation implemented in 2006; however, the microbial standards and sampling methodology were not amended. Currently, shellfish harvesting areas and shellfish intended for human consumption are governed by European Directives EC 852/2004 (EU 2004c) which governs the hygiene of food stuffs. Directive EC 853/2004 (EU 2004b) which lays down specific rules for foods of animal origin, including procedures for harvesting, depurating and relaying bivalve shellfish, and Directive EC 854/2004 (EU 2004a) which specifies the requirements and classification of shellfish harvesting areas and the subsequent requirements for the sale of live bivalve shellfish (Table 1.4). The ‘competent authority’ tasked with completing the necessary monitoring of the shellfish beds in England and Wales is the Food Standards Agency. This monitoring is accomplished via monthly samples, of which (at least) 90% of shellfish must meet the legislative requirements. Formally the directive cites no specific sampling strategy, however, it does stipulate that a representative sampling strategy must be utilised.

Table 1.4. Microbial classification of shellfish harvesting waters. Adapted from EC 854/2004 (EU 2004a).

Classification	Microbial standard	Information
A	<230 <i>E. coli</i> 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 <i>E. coli</i> 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 treatment in an approved purification centre or after relaying so as to meet the standards for Class A.
C	<46,000 <i>E. coli</i> 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 <i>E. coli</i> per 100 g flesh and intervalvular liquid. Determined by 5 tube, 3 dilution MPN.	No formal restrictions are placed on harvesting.

The most recent piece of legislation concerning water quality and shellfish is Directive 2000/60/EC of the European Parliament and Council of 23rd October 2000 which establishes a framework for community action in the field of water policy (Anon 2000). This directive is more simply known as the “Water Framework Directive”. This directive was conceived in 2000 and became law in 2003. The overriding aim of the Water Framework Directive was to tie in all aspects of water quality legislation and streamline legislation for all water sources up to one nautical mile offshore. Rather than using the traditional political or administrative boundaries, the Water Framework Directive focuses on river basins as a whole, shifting to a much more integrated management style and an individual catchment based approach (Stapleton et al. 2008). The Water Framework Directive divides the UK into 11 separate river basin catchments and requires that the competent authority devises an individual management plan for each river basin district, thus shifting from ascertaining and resolving point source pollution problems to a much more holistic approach (Kay et al. 2008a, Stapleton et al. 2008). The Water Framework Directive sets long term goals for each river basin district and requires that each catchment achieves “a good ecological standard” based on chemical, physical and biological parameters by 2015 (Anon 2000).

1.8 Effectiveness of current legislation for safeguarding shellfish consumers

The current microbial standards used in all European legislation rely solely on the use of bacterial indicators such as *E. coli* and other faecal indicator bacteria. However, numerous studies have shown that bacterial indicators (such as *E. coli*) show no correlation with viruses, and that whilst good water quality can be seen with regard to bacterial indicators, this may not be the case with viruses and this can pose a concern to human health (Burkhardt and Calci 2000, Romalde et al. 2002, Santo Domingo et al. 2007). (Romalde et al. 2002) cites EU legislation as “inadequate” for assessing viral contamination of shellfish, however, molecular methods for virus detection are still awaiting field validation before they can be incorporated into European law. (Chigbu et al. 2005) and (Elmanama et al. 2006) comment on the seasonal variations of indicator bacteria, the former working in Mississippi, USA and the latter working in Gaza. These studies both noted that concentrations of indicator bacteria increase dramatically following heavy rainfall through run-off, sewage discharges and groundwater leaching. Therefore in order to comply with EC 854/2004 (EU 2004a) these factors must be accounted for to ensure a representative sampling strategy.

1.9 Mitigation strategies for reducing microbial contamination in shellfish

Remediation measures implemented on a catchment scale often show no direct impact on the microbial contamination of the shellfish harvesting waters (Kay et al. 2008c). To improve the microbial quality of shellfish there are various mitigation options that could be utilised both pre and post-harvest, to prevent the risk of shellfish vectored illness. Some mitigation measures are stipulated by law, such as depuration and offshore relaying (EU 2004a) under certain circumstances, however they may still be utilised, even if not required by law, to provide a better quality product. Other mitigation measures to improve the microbial quality of shellfish focus on methods at the point of retail and beyond, for example, investigating ways in which to treat marketed shellfish products to reduce the risk to the consumer, for example high pressure processing and irradiation (Mallett et al. 1991, Murchie et al. 2005). A further alternative is to treat the shellfish product prior to ingestion i.e. cooking, canning or pickling.

1.9.1 Offshore cultivation / relaying

Shellfish are frequently grown in shallow near-shore coastal waters that are vulnerable to contamination by raw or partially treated human sewage (Lessard and Beck 1990). One option to improve the microbial quality of the shellfish, prior to harvesting, is to relocate them to waters which are less faecally contaminated (EFSA Panel on Biological Hazards 2012).

Offshore relaying is the practice of relocating adult shellfish cultivated in near shore environments to areas which are less faecally contaminated (typically in offshore areas), allowing them to ‘purge’ themselves of their microbial load. This is a requirement of European law for all shellfish that are classified as Class ‘C’ (Containing 4,600 – 46,000 *E. coli* per 100 g flesh; Table 1.4). Despite the suggestion of offshore relaying as a method to achieve compliance with EU legislation, it is not widely practiced as a routine harvesting measure due to the low market value of the product and high economic costs associated with relaying shellfish into offshore locations (Diagne et al. 2004, Adams et al. 2011).

Offshore cultivation of shellfish is routinely practised in many countries worldwide (Motes and DePaola 1996, Chalermwat et al. 2003, Spencer 2008) to establish new shellfish beds in offshore locations with a primary aim of increasing shellfish productivity. There are a variety of shellfish aquaculture techniques that are used to create offshore ‘shellfish farms’ such as rope culture (Dare and Davies 1975) long lines (Strohmeier et al. 2005) and shellfish racks (Dealteris et al. 2004). The precise method used is determined predominantly by the local

resources available and the shellfish species to be cultured. Offshore cultivation of shellfish in areas which are not subjected to high levels of microbial contamination not only increases shellfish productivity but also, as a secondary effect, produces shellfish of superior microbial quality (Buck et al. 2008).

1.9.2 Depuration

Depuration is a technique used post-harvest, but pre-retail to mitigate against the risk of microbial contamination of shellfish. It is a requirement of European law that all shellfish harvested from class 'B' areas are subjected to depuration treatment (Table 1.4). In England and Wales all bivalve shellfish are self-purged or "depurated" in closed recirculation systems, with UV water treatment. All commercial depuration systems must be approved by 'Seafish' and the conditions of approval for each system and species is determined by The Centre for Environment, Fisheries and Aquaculture Sciences or CEFAS (Lee, 2008). Currently in the UK there are five approved commercial depuration systems which have been validated by stringent bacteriological testing and are now regarded as 'proven' designs which require less stringent on-going bacteriological testing (Lee 2008). Despite this, "zero risk" to public health is not achievable due to high variability of naturally occurring pathogens (Kay et al. 2004).

E. coli can be successfully depurated from different species of shellfish (in order to comply with legislation requirements) but other potential human pathogens cannot be successfully removed during the same time period and using the same methods (Power and Collins 1989, Muniain-Mujika et al. 2002, Nappier et al. 2008, Barile et al. 2009, Nappier et al. 2010). (Pommeuy et al.) concurred that shellfish that comply with EU standards could still be implicated in disease outbreaks due to differential elimination of pathogens. Therefore it can be concluded that the current microbial standards (using *E. coli* as an indicator species) are inadequate to protect public health.

Studies conducted to determine the elimination rate of *E. coli* and draw comparisons with other observed human pathogens (Table. 1.5) are limited in their usefulness as they are often performed under experimental conditions, with varying environmental parameters, units, and methodologies which may not correspond to commercial systems and makes comparisons between studies difficult (Lee and Younger 2002). The initial concentrations of pathogens have been shown to affect the elimination rate (McLeod et al. 2009). Other environmental parameters such as temperature and salinity and different treatment variables such as using attached or unattached mussels (Rajagopal et al. 2005) have also been shown to affect the

elimination rate of pathogens. The actual shellfish tissues sampled can also show differential rates of elimination due to different physiological mechanisms (Power and Collins 1989, Wang et al. 2008).

Table 1.5. Published values for elimination rates of potential human pathogens.

Depurated species	Pathogen	Pathogen elimination time (hours)		<i>E. coli</i> elimination time (hours)	Depuration system	Reference
<i>Mytilus galloprovincialis</i>	<i>Vibrio parahaemolyticus</i>	36-48hrs		6-12	Commercial recirculation system, treated with UV.	(Barile et al. 2009)
<i>Mytilus galloprovincialis</i>	<i>Vibrio cholerae non - 01</i>	168		72	Fresh seawater	(Marino et al. 2005)
<i>Mytilus edulis</i> <i>Crassostrea gigas</i>	Male specific F+ Bacteriophage	47.3 (mussels) (oysters)	60.8	6.5 (90%)	Commercial recirculation system, treated with UV.	(Doré and Lees 1995)
<i>Crassostrea ariakensis</i>	<i>Cryptosporidium parvum</i>	29 days (still present)		n/a	Fresh artificial seawater	(Nappier et al. 2010)
<i>Crassostrea gigas</i>	Hepatitis A virus	23 (still present)		n/a	Laboratory trials	(McLeod et al. 2009)
<i>Crassostrea gigas</i>	<i>Poliovirus</i>	23 (still present)		n/a	Laboratory trials	(McLeod et al. 2009)
<i>Crassostrea gigas</i>	<i>Norovirus</i>	23 (still present)		n/a	Laboratory trials	(McLeod et al. 2009)
<i>Crassostrea gigas</i>	<i>Cryptosporidium parvum</i>	24 (still present)		n/a	Commercial recirculation system, treated with UV.	(Sunnotel et al. 2007)
<i>Mytilus galloprovincialis</i>	<i>Hepatitis A virus</i>	24 (still present)		24	Commercial recirculation system	(Franco et al. 1990)
<i>Mytilus galloprovincialis</i>	<i>Poliovirus 1</i>	24 (still present)		24	Commercial recirculation system	(Franco et al. 1990)
<i>Scrobicularia plana</i>	<i>Cryptosporidium parvum</i>	24 (depending on initial concentrations)		n/a	Laboratory trials representing commercial systems with UV treatment.	(da Fonseca et al. 2006)
<i>Mytilus edulis</i>	<i>Cryptosporidium parvum</i>	24 +		n/a	Laboratory trials representing commercial systems with UV treatment.	(da Fonseca et al. 2006)

It has been shown that current depuration legislation and techniques are ineffective in eliminating many potential pathogens as the methodology was designed for the removal of bacteria and does not take into account ‘new’ and emerging pathogens such as viruses and protozoan parasites (da Fonseca et al. 2006). Recent research has focused on determining an appropriate time period to eliminate emerging pathogens from differing shellfish species as opposed to researching *E. coli* elimination times (Table 1.5). Previously published data cannot be used to design new microbiological safety standards as there is no consistent methodology in use (Table 1.5). In addition, scientific studies typically focus on isolated pathogenic species to determine the elimination rate, however, few studies have been published that examine the interaction of different pathogenic species within a single shellfish population (McLeod et al. 2009). This is important commercially, as current legislation stipulates there should be no mixing of species in the depuration facility, but shellfish of the same species, from different regions may be depurated together (Robertson 2007). This has implications for public health as shellfish from different regions may harbour different pathogens.

(Muniain-Mujika et al. 2002) suggested that using the current depuration methods, a depuration time of 5 days would improve food safety. (Teplitski et al. 2009) highlighted the need to understand the function of commensal relationships between naturally occurring bacteria such as *Vibrio spp.* and shellfish so that depuration methods can be improved.

Conventional depuration systems have shown to be effective at eliminating *E. coli* from shellfish to a standard that complies with current legislation requirements. However, conventional systems are unable to successfully depurate ‘new’ and emerging pathogens and current legislation is inadequate in this respect, to protect public health. Further research needs to be conducted on the elimination rates of different pathogens from shellfish species to determine elimination rates and any possible interactions between pathogenic species. Researchers must devise a standard research methodology that can be applied to commercial depuration systems and the potential application of new microbial safety standards must be investigated, in order to reduce the risk from new and emerging pathogens.

1.9.3 High Pressure Processing, Irradiation, Cooking

Shellfish are traditionally consumed either raw or lightly cooked (Potasman et al. 2002), and despite the ‘risk’ to the consumer this remains the standard practice in both restaurants and domestic homes, for selected shellfish species such as oysters. Potential mitigation measures to reduce the risk to the consumer of shellfish vectored illness is to treat the final shellfish product either pre or post-retail.

Methods for treating shellfish pre-retail include High Pressure Processing and irradiation. High Pressure Processing (HPP) is a non-thermal approach which preserves the raw qualities of the shellfish. (Berlin et al. 1999) demonstrated that HPP was effective at inactivating all strains of *Vibrio spp.* HPP works by breaking down the detrimental enzymes, leaving the stronger covalent bonds intact, which preserve the flavour and taste of the shellfish. Irradiation of food is also another option reducing the microbial content of shellfish. Irradiation involves treating the shellfish pre-retail with doses of ionising radiation which can eliminate potential microbial pathogens whilst preserving both the taste and nutritional qualities, with the added benefit of extending the shelf life of the product. (Harewood et al. 1994, Schreiber et al. 1994, Farkas 1998). (Farkas 1998) demonstrated that pathogenic bacteria such as *Salmonella spp.* and *Staphylococcus aureus* could be inactivated by irradiation, as could *Campylobacter*, *Listeria monocytogenes* and *E. coli*. The authors also claim that irradiation is effective even when the food source is frozen, demonstrating its suitability for a wide range of shellfish products.

Despite the clear advantages of these procedures in reducing the microbial content of shellfish, these techniques are not widely applied. This is due in part to legislative requirements on food stuffs treated with radiation, consumer preference for minimally treated shellfish products, and the opposition of consumers to irradiated food products based on psychological perceptions and a lack of public knowledge (Farkas 1998), in addition to the ineffectiveness of techniques such as irradiation in the elimination of viral pathogens (Harewood et al. 1994).

Methods to reduce the 'risk' of shellfish vectored illness post-retail include treatments such as cooking, canning or pickling. These methods not only reduce the microbial content of shellfish, but also serve to increase the shelf life of the shellfish product. Many shellfish species are routinely cooked (mussels, clams and prawns) prior to consumption and others are routinely either pickled or canned (cockles, winkles and whelks). Despite this, there is still a large consumer demand for minimally processed shellfish, particularly for high value species such as oysters and clams, as cooking alters not only the taste and texture of the shellfish, it also impacts on the nutritional quality. In addition, treatments such as cooking have also been shown to be ineffective in entirely removing the risk of shellfish vectored illness to consumers (McDonnell et al. 1997).

1.10 The Conwy mussel fishery

Historically, a fishery for wild mussels (*Mytilus edulis*) has existed in Conwy since 1835 with landings being recorded from 1916 onwards after the installation of a purification plant (Edwards 1987). The mussels take between 3-5 years to reach a marketable size of 45 mm and are harvested using traditional methods of either hand picking on intertidal beds or raking on sublittoral beds. Mussel farmers, more commonly known as “musselmen” are required to hold licences issued by Conwy Borough Council only to “bona fide” fishermen. Post World War Two no more than 50 licences had been issued per annum. At its peak in 1939 the Conwy mussel fishery produced 950 tonnes per annum, however, an average annual mussel harvest was between 200-600 tonnes per annum which was mostly sold onto local inland markets such as Birmingham and Sheffield (Edwards 1987).

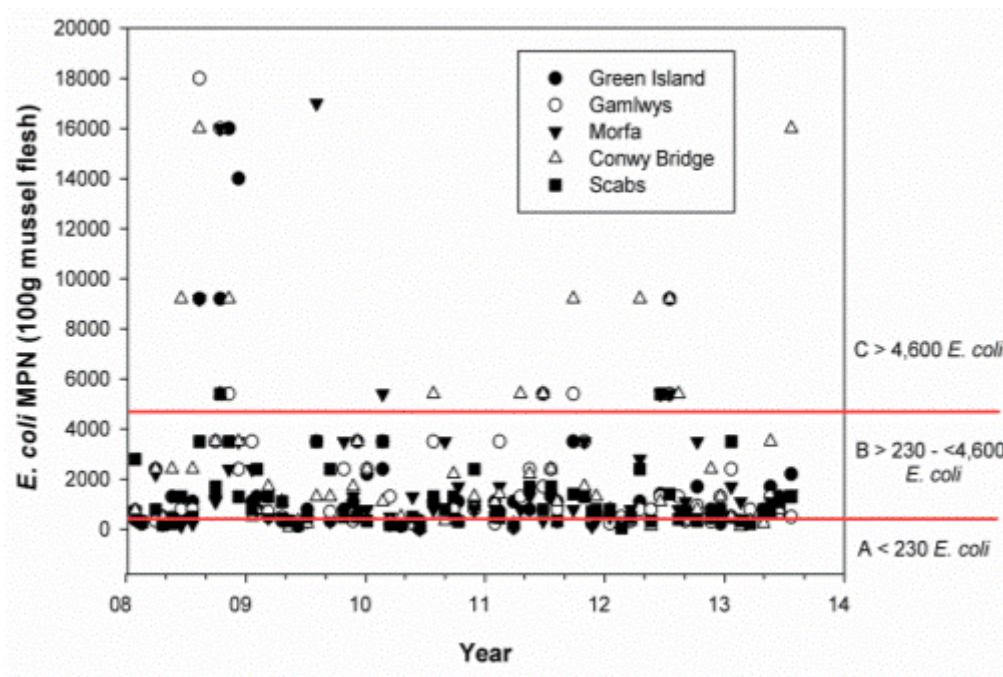


Fig. 1.7. Routine microbiological sampling results for the Conwy mussel beds 2008-2013. Values are expressed as *E. coli* MPN (Most Probable Number) per 100 grams of mussel flesh and inter-valvular fluid. Classifications (A-C) according to European legislation (EU 2004a) are represented by solid lines. Data publicly available from CEFAS (Centre for Environment Fisheries and Aquaculture Sciences) (CEFAS 2010).

In 2001, 75% of all UK mussels produced originated from Wales. In North Wales, one tonne of seed mussel produced 1 tonne of marketable mussels (+45 mm) after 2 - 2.5 years (Saurel et al. 2004). In 2004, the average price for one tonne of mussels was £160 per tonne (Lake and Utting 2007). Average Conwy mussel harvests of 300 tonnes per annum generated an estimated income of £270,000 locally, in addition to providing employment for the mussel fisherman, the owners and operators of the purification plants and the local sellers. However; in 2009 the Food Standards Agency partially downgraded the Conwy mussel beds from a Grade 'B' to a Grade 'C' based on poor microbiological results (Fig.1.7), which decreased the annual yield from 300 to 100 tonnes and caused huge economic losses to the region (Trevor Jones - Conwy Mussels. *Pers. Comm*). This combined with increased boat, transportation and cleaning costs have significantly lowered the profit margin for the mussel fishermen. Increased competition and lower prices have forced many fishermen to leave the fishery (Edwards 1987). This has had a significant impact on the local economy as the productivity of the region declined as well as the region experiencing social changes with job losses and a loss of "cultural heritage" (Trevor Jones - Conwy Mussels. *Pers. Comm*).

Much of the work for this thesis takes place within the Conwy production and harvesting area as the local shellfish producers are keen to understand the rationale behind the poor microbiological results experienced in 2009 (this thesis was part funded by the local shellfish industry) with a view to preventing a similar event in the future and to secure the future economic prosperity of the Conwy mussel fishery.

1.11 Objectives and outline of this thesis

Bacterial reservoirs present within shellfish tissues can pose a significant risk to human health. Current research efforts to safeguard both human health and the future economic prosperity of the shellfish industry largely focus on the identification of the sources of shellfish contaminants and the identification of their potential transport pathways through coastal catchments, with a view to optimising remediation efforts and subsequently reducing the risk to shellfish consumers. Current research into contaminants within shellfish flesh focuses largely on methodological development for the detection and quantification of viruses and marine bio toxins, as these are currently perceived to be a greater risk to human health. However, the bacterial content of shellfish is currently utilised within European legislation as an indicator of overall shellfish contamination levels and pathogenic bacterial strains are still commonly

contained within shellfish flesh and still represent a risk to human health. Whilst the bacterial content of shellfish had been previously researched; the behaviour and interaction of bacterial reservoirs present both within shellfish tissues and within the wider shellfishery environment represents an area that is currently under-researched.

This thesis focuses on the identification of environmental bacterial reservoirs in shellfish and shellfish harvesting areas, both *in situ* and during different post-harvest treatment regimes. This thesis provides an overview of both the spatial and temporal variation of bacterial reservoirs present within shellfish tissues and attempts to assess the relative significance of this reservoir with respect to alternative environmental bacterial reservoirs both *in situ* and under different mitigation strategies. The development of a new methodology for the quantification of norovirus within shellfish tissues also enabled a direct comparison of the viral and bacterial content of shellfish tissues to examine differences in spatial contamination patterns and to assess whether the current use of bacterial indicators in European legislation is sufficient to safeguard shellfish consumers against shellfish vectored viral illness.

Therefore, the key objectives of this thesis are as follows:

- **Identification and quantification of bacterial reservoirs in commercial shellfish harvesting areas within North Wales, UK.**
- **Determination of the relative contribution of previously identified bacterial reservoirs in shellfish tissues under different mitigation strategies.**
- **Examination of the relationship between bacterial and viral reservoirs within shellfish tissues.**

This thesis is presented as a series of manuscripts prepared for publication in peer reviewed scientific journals. The key objectives of this thesis link all the individual chapters together and are discussed in detail below.

Identification and quantification of bacterial reservoirs in shellfish harvesting areas.

- *To investigate the spatial and temporal changes in the bacterial reservoirs contained within shellfish tissues.*

A single commercial mussel (*Mytilus edulis*) bed was extensively surveyed to assess for spatial and temporal changes in the concentrations of faecal indicator bacteria present within the shellfish tissues. Results are presented in Chapter 2.

- *To investigate the relationship between the bacterial reservoirs present in the shellfish flesh and in the underlying sediments.*

The concentrations of faecal indicator bacteria within the underlying sediment were determined in conjunction with the faecal indicator bacterial concentrations from the shellfish tissues over a single *Mytilus edulis* bed. Results are presented in Chapter 2.

- *To investigate the relationship between the bacterial reservoirs present in the shellfish tissues and in the surrounding water.*

Mussel (*Mytilus edulis*) and water samples were collected simultaneously from two separate geographical regions over varying temporal and spatial scales to determine the respective concentrations of faecal indicator bacteria. Results are presented in Chapter 5.

- *To investigate the potential for epizoic barnacles to act as a bacterial reservoir.*

Samples of mussels (*Mytilus edulis*) complete with attached (epizoic) barnacles were collected from three separate intertidal mussel beds, the respective concentrations of faecal indicator bacteria were determined. Results are presented in Chapter 3.

- *To investigate the relationship between different bacterial species present within shellfish tissues.*

The composition of the bacterial reservoir within shellfish tissues was examined both *in situ* (on an intertidal mussel bed) and *ex situ* (during the depuration process) to examine the relationship between faecal indicator bacteria and naturally occurring bacterial species such as *Vibrio* spp. and marine heterotrophs. Results are presented in Chapters 2, 3 and 4.

- *To investigate the relationship between selected nutrient and physico-chemical parameters and concentrations of faecal indicator bacteria in both shellfish tissues and sediments.*

Selected nutrient parameters (total organic carbon, total nitrogen, nitrate, nitrite, ammonium and phosphorous) in addition to salinity and pH were determined from sediment samples to examine the relationship between key nutrient parameters and concentrations of faecal indicator bacteria in both sediments and shellfish tissues over a single mussel (*Mytilus edulis*) bed. Results are presented in Chapter 2.

Determination of the relative contribution of previously identified bacterial reservoirs in shellfish tissues under different mitigation strategies.

- *To investigate the effects of depuration on the concentrations of different bacterial species (*E. coli*, total coliforms, vibrio spp. and marine heterotrophs) within shellfish tissues.*

Mussel (*Mytilus edulis*) samples were taken from three separate intertidal mussel beds and subjected to a 72 hour depuration procedure in a scaled down commercial depuration facility. The concentrations of the selected bacterial species were determined at pre-determined time points. Results are discussed in Chapter 4.

- *To investigate the effects of depuration on the concentrations of different bacterial species (*E. coli*, total coliforms, vibrio spp. and marine heterotrophs) within epizoic barnacles.*

Barnacle samples (attached to mussels) were taken from three separate intertidal mussel beds and subjected to a 72 hour depuration procedure in a scaled down commercial depuration facility. The concentrations of the selected bacterial species were determined at pre-determined time points. Results are discussed in Chapter 4.

- *To investigate the effects of offshore relaying on the bacterial and viral concentrations of shellfish tissues.*

Caged mussel (*Mytilus edulis*) samples were experimentally deployed at varying offshore distances for a total of 124 days. At pre-selected time intervals the bacterial (faecal indicator bacteria) and viral (norovirus) concentrations of the shellfish tissues was determined. Results are presented in Chapter 5.

Examination of the relationship between bacterial and viral reservoirs within shellfish tissues.

- *To investigate the spatial contamination patterns of both faecal indicator bacteria and norovirus within shellfish tissues.*

Caged mussels (*Mytilus edulis*) were experimentally deployed at pre-determined locations surrounding a sewage outfall for approximately one month to investigate the differential uptake / elimination kinetics of both bacteria and virus particles within shellfish tissues. Results are presented in Chapter 6.

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CHAPTER 2

Spatial and temporal heterogeneity of faecal indicator bacteria across an intertidal shellfish bed: Implications for routine bacterial monitoring

KATIE CLEMENTS¹, RICHARD S. QUILLIAM^{2,3}, LUIS GIMÉNEZ¹, DAVID L. JONES³, JAMES WILSON⁴ AND SHELAGH K. MALHAM⁵

¹*School of Ocean Sciences, Bangor University, Menai Bridge, Anglesey, LL59 5AB, UK.*

²*Biological & Environmental Sciences, University of Stirling, Stirling, FK9 4LA. UK.* ³*School of*

Environment, Natural Resources and Geography, Bangor University, Bangor, Gwynedd, LL57

2UW, UK. ⁴*Deepdock Ltd, Bwthyn Y Mor, Llanfaethlu, Holyhead, Anglesey, LL65 4HD,*

UK. ⁵*Centre for Applied Marine Sciences, Bangor University, Menai Bridge, Anglesey, LL59*

5AB, UK.

*Corresponding author. Email: ktclements@hotmail.co.uk; ospc02@bangor.ac.uk

Running head: Spatial and temporal heterogeneity of faecal indicator bacteria on shellfish beds.

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Abstract

Routine bacterial monitoring of shellfish beds using indicator species is a common global practice designed to prevent contaminated shellfish products from entering the human food chain. However, current procedures which focus on the quantification of faecal indicator bacteria (FIB) as a proxy for microbial pollution lack transparency and are often not representative of contamination levels in shellfish harvesting areas. The objective of this study was to critically assess the accuracy of current monitoring strategies by quantifying the spatial and temporal concentrations of FIB within a single intertidal commercially harvested shellfish bed. Spatial and temporal dynamics (including the effects of tidal state and seasonality) of FIB were quantified in mussel flesh and sediment samples from a single, intertidal mussel (*Mytilus edulis*) bed. Our results confirmed that FIB concentrations across a shellfish bed were heterogeneous over larger spatial and temporal scales, and had no relationship with concentrations of autochthonous bacteria or the physico-chemical parameters of the sediment. These results have important implications for both public health and the economic prosperity of the shellfish industry, and demonstrate that routine monitoring is subject to both high spatial and temporal fluctuations. We conclude that FIB monitoring may not accurately represent levels of microbial contamination within shellfish harvesting areas and that more robust microbiological testing procedures are needed.

KEY WORDS: Environmental reservoirs, Sediment, *Vibrio spp.*, Pathogen, Seasonality.

2.1 Introduction

The global demand for seafood products has risen dramatically over the previous three decades (Potasman et al. 2002) with an average human consuming 16 kg of seafood per annum (Teplitski et al. 2009). The demand for shellfish products is expected to increase as they become more widely recognised as a relatively cheap and nutritious food source (Gjedrem et al. 2012). However, the increase in shellfish consumption is accompanied by an increase in shellfish-vectoring illness in humans (Potasman et al. 2002). Thus, the challenge for the shellfish industry is to supply a good quality product that is safe for human consumption (Lee and Younger 2002).

Shellfish are commonly cultivated in sheltered waters which are vulnerable to microbial contamination from both point-source pollution, e.g. sewage outflow, and diffuse pollution, e.g. agricultural runoff (Oliveira et al. 2011). Contamination of shellfish in harvesting waters by bacterial pathogens such as *Escherichia coli* O157 (Riou et al. 2007) and viral pathogens such as Norovirus (Lees 2000) is common, especially during storm events when sewage treatment facilities exceed capacity, leading to the release of un-treated sewage into the sea (Lessard & Beck 1990). This sewage release not only increases the microbiological loading into shellfish harvesting waters, but also raises nutrient levels, which in turn can alter indigenous microbial communities. Bivalve shellfish are capable of bio-accumulating pathogenic micro-organisms from the surrounding water and concentrating them within somatic tissues at increased concentrations relative to the surrounding water (Teplitski et al. 2009). As some shellfish are traditionally consumed either raw or lightly cooked, they are capable of vectoring potentially pathogenic micro-organisms into the human food chain (Potasman et al. 2002) and several reported outbreaks of gastroenteritis in the human population have been attributed to shellfish consumption (e.g. Norovirus and oysters; Ang 1998; Lee & Younger 2002; Rippey 1994). These outbreaks reduce consumer trust and challenge the promotion of shellfish as a “safe” food source.

To safeguard against contaminated shellfish products entering the human food chain, many countries have stringent legislation in place to regulate all aspects of the shellfish industry, i.e. from classifying shellfish harvesting areas to controlling post-harvest treatment and processing. In the European Union, shellfish quality assurance is currently governed under EC/854/2004 (EU 2004a) and EC/853/2004 (EU 2004b) where the hygiene status of shellfish is monitored via the use of faecal indicator species such as *E. coli* and coliforms as a proxy for pathogenic microorganisms, e.g. *E. coli* O157, that are too costly to screen individually

(Quilliam et al. 2011). The presence of *E. coli* is widely accepted as being an important indicator for faecal contamination, although there remains uncertainty about its relevance as an indicator for viral contamination (e.g. hepatitis A, norovirus) or naturally occurring pathogenic bacterial strains such as *Vibrio parahaemolyticus* and *V. vulnificus* (Muniain-Majika et al. 2002; Marino et al. 2005; Romalde et al. 2002).

In the United Kingdom, commercially harvested shellfish beds are initially assessed for potential sources of contamination before routine monitoring points are established. The shellfish bed is assigned a classification grade under EC/854/2004 (EU2004a) based upon *E. coli* concentrations within shellfish flesh. Subsequently, the classification grade assigned to an individual shellfish bed impacts, not only consumers, but also the shellfish industry, as it dictates the level of post-harvest treatment required for shellfish products at each classification grading. Routine bacterial monitoring is conducted either monthly or bi-monthly by the local authority and the shellfish samples are analysed by the National Reference Laboratory (NRL) at the Centre for Environment Fisheries and Aquaculture Science (CEFAS) using a standardised national protocol ISO/TS 16649-3:2005. The results determine the classification of a shellfish harvesting area, which could promote either a change in management practice or a temporary closure of the harvesting area. Therefore the classification grading assigned to each shellfish harvesting area has substantial economic implications for both the local and wider shellfish industry.

Previous research has shown that environmental factors such as seasonality, tidal state and rainfall events may alter concentrations of *E. coli* detected within shellfish tissues (Cook 2007; Stapleton et al. 2007; Riou et al. 2007; Kay et al. 2008) and hence affect the classification grading assigned to a harvesting area. In comparison, however, the spatial variation of *E. coli* within single mussel beds and the implications of this potential heterogeneity on shellfish quality monitoring have received scant attention.

The overarching aim of this study was to critically assess the concentrations of faecal indicator bacteria (FIB) within mussel tissues across a single, commercially harvested mussel (*Mytilus edulis*) bed over both spatial and temporal scales. In order to do this, we have quantified mussel flesh FIB concentrations in longitudinal and transverse transects across a mussel bed during different seasons and tidal states. In addition, to assess the suitability of *E. coli* as an indicator of microbial quality, we simultaneously quantified the concentration of naturally occurring bacteria such as *Vibrio spp.* within mussel tissues. Finally, we examined whether the sediment underneath the mussel bed was providing a dynamic reservoir for FIB, and whether this was regulated by nutrient levels or physico-chemical parameters.

“Previous research has highlighted possible major shortcomings of current research methods used for the assessment of mussel beds in the UK. These shortcomings have major implications for mussel producers and consumers as well as regulating bodies. This study investigates the pivotal role of a thorough spatial sampling regime for future updates/improvements of the current UK assessment system for biological safety of shellfish beds.”

2.2 Materials and methods

2.2.1 Sampling location and large-scale transects

Sampling was conducted on a commercial intertidal mussel (*Mytilus edulis*) bed at Conwy Morfa (53.298015N, 3.854535W) in North Wales, UK, which is currently classified according to EC 854/2004 (EU 2004a) as ‘class B’ containing 230 - 4,600 *E. coli* 100 g⁻¹ of mussel flesh. This mussel bed was surveyed and mapped using GPS and was estimated to be approximately 231 m in length (north-south) and 140 m in length (east-west). Five vertical transects running east-west were used to survey the entire mussel bed from the mean low water mark (MLW) to the upper limit of marketable-size mussels (140 m from MLW). All transects were evenly spaced 57.5 m apart from one another and samples were taken across all transects at 10 m intervals beginning from MLW (0 m) to the upper limit at 140 m (Fig. 2.1). One transect was surveyed per day, over five consecutive days one hour either side of low water. Only mussels of marketable size (> 45 cm) were included in the sampling strategy, areas of smaller ‘seed mussels’ were excluded. At each sample point three replicate mussels, and four sediment samples (0 - 5 cm depth) were collected, stored at 4 °C and processed within 12 h of collection.

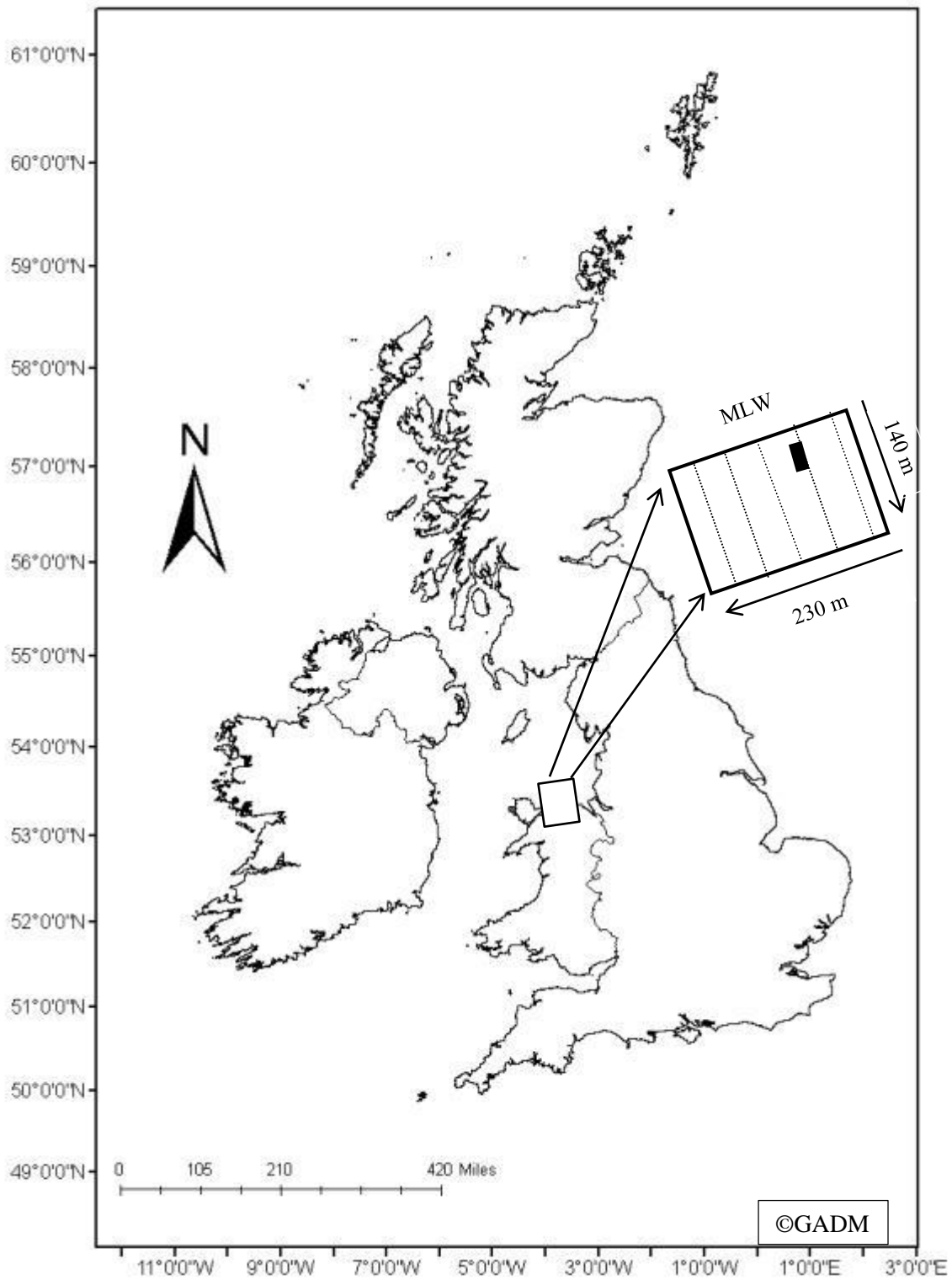


Fig. 2.1. Map showing the study sampling location. Inset diagram shows the location and approximate positions of the five transects across the shellfish bed. The approximate intensive sampling location is shown in the black highlighted area. Inset panel not to scale.

2.2.2 Intensive spatial sampling

An adaptive cluster sampling strategy was utilised to provide a measure of variability over spatial scales smaller than the 10 m intervals described above. Sampling took place at low water, one month after the initial sampling on 16th June 2011. An area of mussel bed 10 m in length and previously determined to have increased *E. coli* concentrations was selected for analysis. At 0.5 m intervals over the 10 m selected area, three replicate mussel samples were collected (Fig. 2.1). For comparison, triplicate mussel samples were collected at both MLW (0 m) and at 140 m, the upper limit on the mussel bed for marketable sized mussels. Mussel samples were stored at 4 °C and processed within 12 h of collection.

2.2.3 Temporal monitoring

To provide a measure of temporal variability mussel samples (in triplicate) were collected quarterly from the same location as the intensive spatial samples (Fig.1) including the comparative sites at both MLW and at 140 m. Over the selected 10 m area, mussel samples were collected at 2 m intervals and transported as described above. Samples were collected in June (summer), September (autumn), December (winter) and March (spring) 2011 - 2012. Samples were collected one hour either side of low water on both spring and neap tides to allow for a comparison of different tidal states. Spring tide sampling was duplicated to allow for comparison over a single tidal cycle. Samples were collected at low water in the morning (AM tide) and evening (PM tide) approximately 12 hours later.

2.2.4 Quantification of FIB and Vibrio spp. in mussel tissue.

Only live mussels were selected for analysis. Sample preparation and subsequent FIB quantification from mussel tissue followed an adapted methodology from Clements et al. (2013). Mussel samples were washed with sterile seawater to remove any residual sediment and debris and all encrusting organisms were removed, before rinsing in 100% methanol to surface sterilise the shell. Individual mussels were opened aseptically and the flesh and extra - cellular fluid were combined in a sterile container (each replicate of 50 g was obtained from approximately ten individual animals). Samples were homogenised for 60 seconds at 10,000 rev min⁻¹ using a Bamix® blender (Seal Rock Enterprises Ltd., Bishop's Stortford, UK). From the resulting homogenate, 200 µl was plated onto Brilliance® selective agar (#CM1046; Oxoid Ltd, Basingstoke, UK) to determine *E. coli* and total coliform counts. In addition, 10 µl of the homogenate was added to plates containing TCBS cholera agar (#CM0330; Oxoid, UK) and to marine agar (#1059; Conda Lab, Madrid, Spain) to determine total *Vibrio spp.* and total

marine heterotroph counts as a measure of the total viable counts (TVC) respectively. All plates were inverted and incubated at 37°C (Brilliance agar) and 25°C (TCBS and marine agar) and bacterial Colony Forming Units (CFU) were enumerated after a 24 h incubation period.

2.2.5 FIB and physico-chemical status of sediments

Fresh sediment (5 g) was added to 10 ml of sterile seawater and the samples shaken for 15 min at 225 rev min⁻¹, vortexed four times in 5 second bursts and subsequently allowed to settle for 5 minutes. Serial dilutions were made using sterile seawater and enumerated for *E. coli* and coliform bacteria as described above using Brilliance® selective agar. Bacterial CFU counts were expressed as CFU g⁻¹ dry weight. Available ammonium (NH₄⁺), nitrate (NO₃⁻) and nitrite (NO₂⁻) in distilled water extracts were determined using the colorimetric salicylate-hypochlorite procedure of Mulvaney (1996) as cited in Sparks et al. (1996) and the vanadate procedure of Miranda et al. (2001) respectively. Phosphate (PO₄³⁻) was determined colorimetrically using the molybdate blue procedure of Murphy and Riley (1962). Total dissolved organic carbon (TOC) and total dissolved Nitrogen (TN) were determined using a TCN-V analyser (Shimadzu Corp., Kyoto, Japan). Electrical conductivity (EC) and pH were measured using standard electrodes, in distilled water, in a 1: 5 w/v ratio.

2.2.6 Statistical analysis

Data was analysed using PASW Statistics v18 (IBM Corp., Armonk, NY). Normality was assessed using a one sample Kolmogorov-Smirnov test ($P \geq 0.05$). Correlations between data sets were made by Spearman Rank Correlation Coefficient (Significance level; $P \leq 0.05$). Comparisons between related samples were made using the Wilcoxon signed ranks test (significance level; $P \leq 0.05$). Differences in the intensive spatial data set (significance level; $P \leq 0.05$) were examined using an independent samples Kruskal Wallis test with a fixed factor of distance. Temporal data were analysed by one-way analysis of variance (ANOVA) and least significant difference (LSD) *Post Hoc* test.

2.3 Results

2.3.1 Spatial monitoring: determining bacterial distributions

E. coli and total coliform CFU were significantly higher in sediment samples than in the corresponding mussel flesh samples ($P < 0.01$; Fig. 2.2 a-d). The distribution of *E. coli* and total coliforms (in both mussel flesh and sediment) showed a patchy distribution across the whole shellfish bed (Fig. 2.2 a-d), and although there were 'hotspots' these were not spatially consistent across the mussel bed. Numbers of *E. coli* in mussel flesh were highest at a location at the edge of the mussel bed (Fig. 2.2a), whereas total coliform 'hotspots' were located at the lower end of the mussel bed, nearest to the MLW (Fig. 2.2c). In contrast, mussels in the upper half of the bed (furthest distance from MLW) contained lower concentrations of total coliforms. The concentrations of both *E. coli* and total coliforms within the sediment increased towards the upper limit of the shellfish bed (furthest from MLW) (Fig. 2.2 b,d) which was the opposite of the total coliform concentrations within the mussel flesh (Fig. 2.2c).

Presumptive *Vibrio spp.* and total marine heterotrophic bacteria contained within the mussel flesh also showed a 'patchy' distribution over the shellfish bed (Fig. 2.2 e-f). However the distribution of both *Vibrio spp.* and total marine heterotrophs were significantly different from the distribution of both *E. coli* and total coliforms over the same shellfish bed ($P < 0.01$ in all pair-wise comparisons).

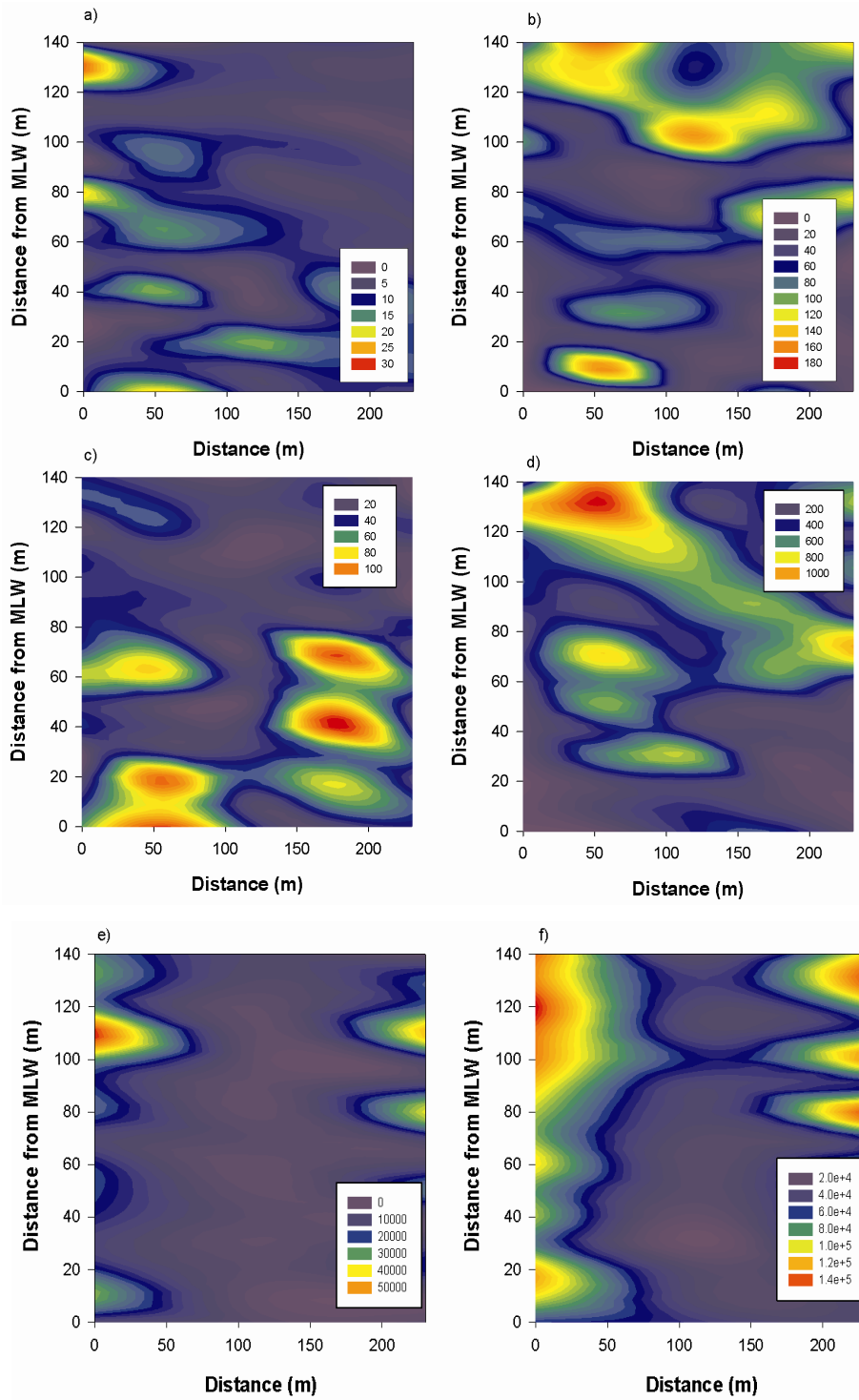


Fig. 2.2. Contour plots showing the distribution of bacteria across a mussel bed. *E. coli* distribution in mussels (a) and sediments (b), coliform distribution in mussels (c) and sediment (d), distribution of *Vibrio spp.* (e) and marine heterotrophic bacteria in mussels (f). Data points represent the mean number of colony forming units (CFU) expressed per gram, where $n = 3$ (mussels) and $n = 4$ (sediments). MLW = mean low water mark.

2.3.2 Spatial monitoring: comparison of bacterial concentrations in mussels and sediments

Although there was a weak relationship between the concentrations of *E. coli* in mussel flesh and the sediment these were not significantly correlated (Table 2.1; $P \geq 0.05$). The concentrations of total coliforms in the mussel flesh and in the sediment showed a weak, but significant, negative correlation (Spearman Rank Correlation Coefficient (r) = -0.14; $P = < 0.05$; Table 2.1). The concentrations of *E. coli* and total coliforms in both mussel flesh and sediment were not significantly correlated with lateral distance across the shellfish bed (east - west) ($P \geq 0.05$). However, *E. coli* and total coliform numbers in the sediment showed a significantly positive correlation with longitudinal distance up the shellfish bed from the MLW (north - south) ($P \leq 0.001$), whilst total coliforms in mussel flesh showed a weak, but significant, negative correlation with increasing distance from the MLW ($P \leq 0.001$); Table 2.1).

The concentrations of *E. coli* in the sediment, and of total coliforms within the sediment and the mussel flesh, were all positively correlated with concentrations of sediment NH_4^+ , PO_4^{3-} and TOC ($P \leq 0.05$; Table 1). However, *E. coli* within mussel flesh was not significantly correlated with either, NH_4^+ , PO_4^{3-} or TOC ($P \geq 0.05$; Table 2.1).

There was a relationship between the EC and FIB in both the mussel flesh and in the sediment ($P \leq 0.05$; Table 2.1); however, whilst EC was positively correlated with the FIB within the sediment, EC was negatively correlated with FIB within the mussel flesh. In contrast to the relationships observed between EC and FIB, the pH was negatively correlated with FIB in the sediment ($P \leq 0.05$) but positively correlated with FIB within the mussel flesh. However, whilst the relationship between pH and total coliforms in mussel flesh was determined to be statistically significant ($P \leq 0.05$) the relationship between pH and *E. coli* concentrations within mussel flesh was not significant ($P \geq 0.05$; Table 2.1).

In contrast to the distribution of FIB, *Vibrio spp.* and total marine heterotrophic bacteria were both significantly correlated with lateral distance across the mussel bed ($P \leq 0.05$; Table 2.1), but not with distance from MLW. Total marine heterotrophs and *Vibrio spp.* were positively correlated with each another ($P < 0.001$; Table 2.1). Both species also showed a significantly positive correlation with EC ($P \leq 0.01$; Table 2.1). A significant negative correlation was also observed between the numbers of total marine heterotrophs, *Vibrio spp.* and the concentration of PO_4^{3-} in the sediment ($P \leq 0.05$; Table 2.1). *Vibrio spp.* were significantly correlated with coliforms in sediment ($P \leq 0.05$; Table 2.1); however, there were no other significant relationships between either *Vibrio spp.* or total marine heterotrophs and *E. coli* and coliforms in either mussel flesh or sediment.

Table 2.1. Correlation coefficient (r) matrix demonstrating the relationships between bacterial concentrations, nutrients and physico-chemical parameters over a commercial mussel bed. Correlations significant at the $P \leq 0.05$ level (two - tailed) are marked with a (*). In all cases $n = 225$.

	Lateral distance (m)	Distance from MLW (m)	Sediment <i>E. coli</i> (g)	Sediment coliforms (g)	Mussel <i>E. coli</i> (g)	Mussel coliforms (g)	<i>Vibrio spp.</i> (g)	Marine heterotrophs (g)	EC (mS cm ⁻¹)	pH	Ammonium (µg/g)	Phosphate (µg/g)	Total organic carbon (µg/g)
Lateral distance (m)	1.000												
Distance from MLW (m)	0.000	1.000											
Sediment <i>E. coli</i> (g)	- 0.04	0.501 *	1.000										
Sediment coliforms (g)	0.000	0.530 *	0.693 *	1.000									
Mussel <i>E. coli</i> (g)	- 0.06	- 0.13	- 0.02	- 0.06	1.000								
Mussel coliforms (g)	- 0.08	- 0.26 *	- 0.100	- 0.14 *	0.442 *	1.000							
<i>Vibrio spp.</i> (g)	0.463 *	0.051	- 0.099	- 0.198 *	- 0.064	- 0.097	1.000						
Marine heterotrophs (g)	- 0.244 *	- 0.058	- 0.096	- 0.116	- 0.101	- 0.046	0.463 *	1.000					
EC (mS cm ⁻¹)	0.209 *	0.399 *	0.294 *	0.256 *	- 0.14 *	- 0.19 *	0.341 *	0.223 *	1.000				
pH	- 0.47 *	- 0.48 *	- 0.37 *	- 0.48 *	0.069	0.210 *	0.020	0.039	- 0.54 *	1.000			
Ammonium (µg/g)	0.019	0.651 *	0.544 *	0.651 *	- 0.04	- 0.190 *	0.075	- 0.059	0.470 *	- 0.57 *	1.000		
Phosphate (µg/g)	0.363 *	0.413 *	0.237 *	0.413 *	- 0.06	- 0.18 *	- 0.263 *	- 0.195 *	0.198 *	- 0.50 *	0.495 *	1.000	
Total organic carbon (µg/g)	- 0.01	0.497 *	0.423 *	0.497 *	- 0.05	- 0.21 *	0.004	- 0.141	0.49 *	- 0.49 *	0.691 *	0.052 *	1.000

2.3.3 Spatial monitoring: comparison of sediment characteristics and physico-chemical properties

Electrical conductivity and the concentrations of NH_4^+ , PO_4^{3-} and TOC in the sediment all significantly increased with increasing distance from MLW (Table 2.1). In contrast, sediment pH significantly decreased with both distance from MLW ($r = -0.48$; $P \leq 0.001$; Table 2.1) and with lateral distance across the mussel bed from west - east ($r = -0.47$; $P \leq 0.001$; Table 2.1). Electrical conductivity and concentrations of PO_4^{3-} also significantly increased with increasing lateral distance across the mussel bed from west - east. ($r = 0.209$; $P \leq 0.001$ and $r = 0.363$; $P \leq 0.001$ respectively; Table 2.1). No NO_3^- or NO_2^- were detected in any of the sediment samples and concentrations of total dissolved nitrogen were below the detection limit of 0.04 mg l^{-1} .

2.3.4 Intensive spatial sampling

Bacterial concentrations within mussel flesh showed no statistical difference over spatial scales less than 10 m (data not shown); *E. coli* ($P = 0.140$), coliforms ($P = 0.105$), *Vibrio spp.* ($P = 0.528$), marine heterotrophs ($P = 0.751$).

2.3.5 Temporal monitoring

Temporal monitoring was carried out over three tides per season to investigate variability (i) between two sequential tidal cycles (e.g. AM and PM tide) (ii) tidal state (e.g. spring or neap tide) and (iii) inter-seasonal variability.

No bacterial group showed any significant differences in concentrations over a single tidal cycle i.e. between AM and PM spring tides ($P \geq 0.05$). However, concentrations of total coliforms, *Vibrio spp.* and marine heterotrophic bacteria within mussel flesh varied significantly with tidal state ($P \leq 0.05$); however, there was no difference in *E. coli* numbers within the mussels between spring and neap tides (Fig. 2.3a). The concentrations of both total coliforms and marine heterotrophs in mussel flesh differed significantly between both AM and PM spring tides and the neap tide ($P \leq 0.001$; Fig. 2.3b) and ($P \leq 0.001$; Fig. 2.4b). *Vibrio spp.* differed significantly between the AM spring tide and the neap tide only ($P \leq 0.001$; Fig. 2.4a).

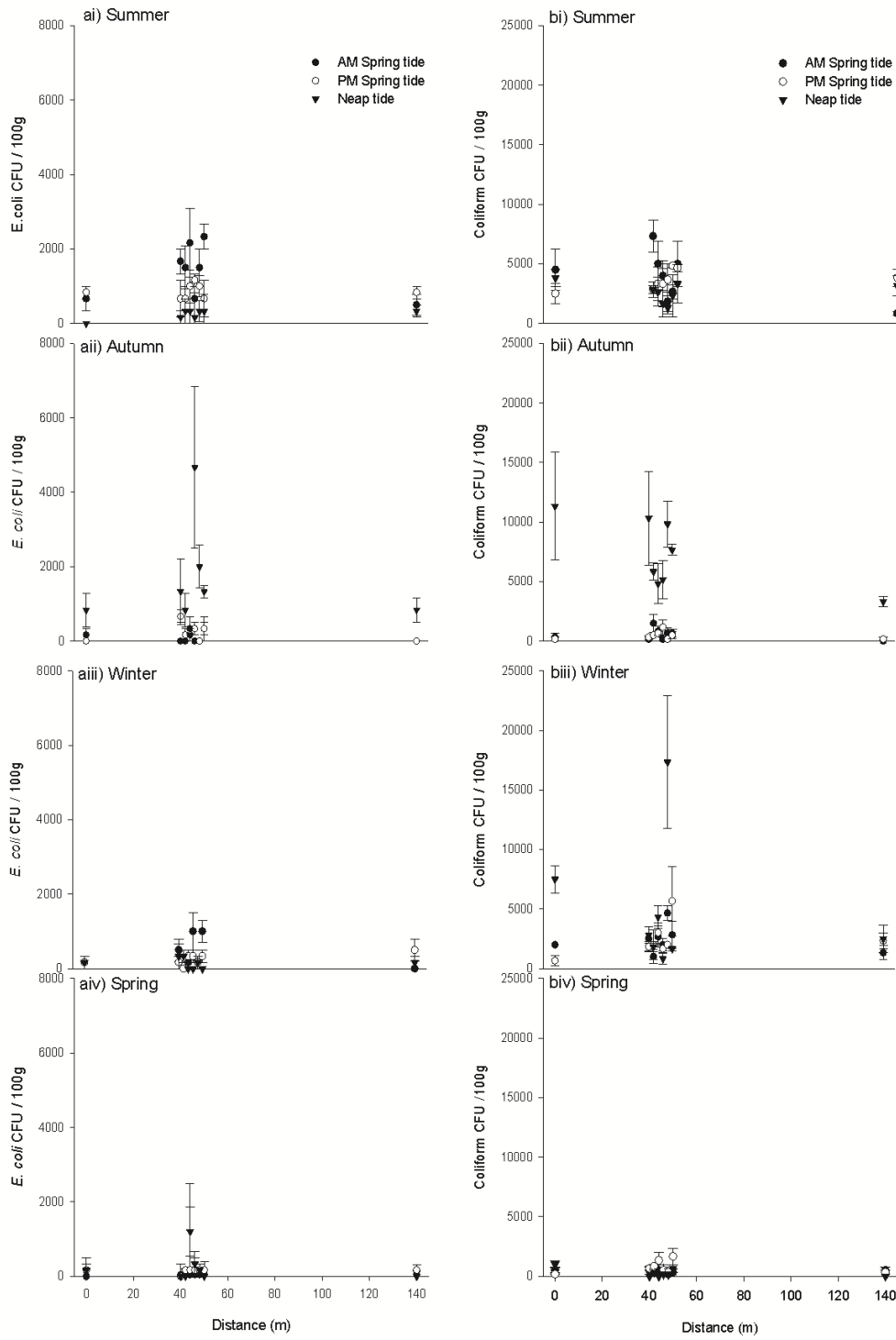


Fig. 2.3. Bacterial concentrations of a) *E. coli* and b) coliforms within mussel flesh, over differing temporal scales, demonstrating the effect of tidal state and seasonality. In all cases $n = 3$ where the data points represent the mean number of colony forming units (CFU) expressed per 100 g. Error bars represent the standard error (SE).

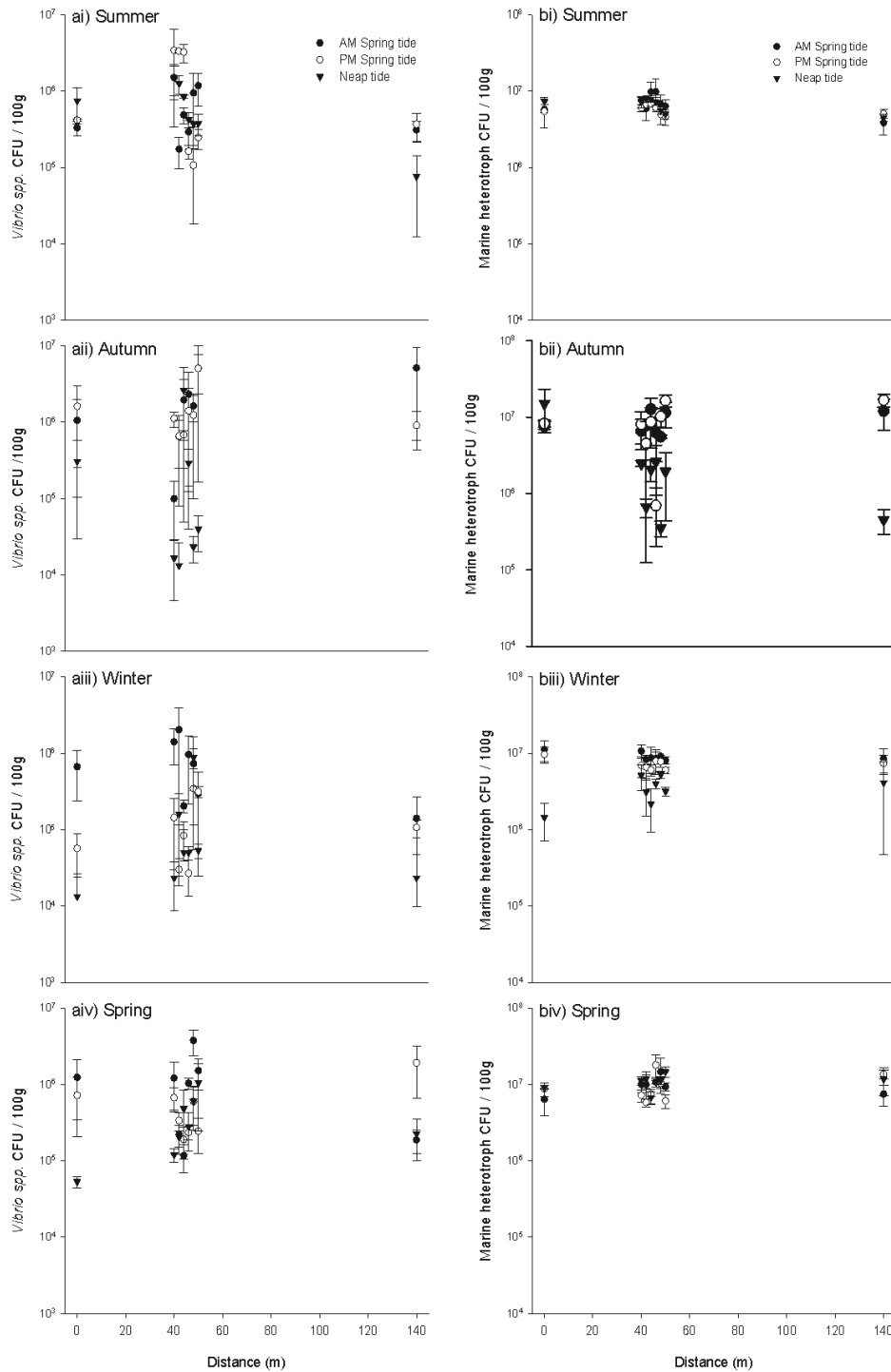


Fig. 2.4. Bacterial concentrations of a) *Vibrio* spp. and b) marine heterotrophs within mussel flesh, over differing temporal scales, demonstrating the effect of tidal state and seasonality. In all cases $n = 3$ where the data points represent the mean number of colony forming units (CFU) expressed per 100 g. Error bars represent the standard error (SE).

Despite remaining spatially consistent, concentrations of *E. coli* within mussel flesh were significantly influenced by season ($P \leq 0.05$; Fig. 2.3a). Significant differences in concentrations of coliform bacteria within mussel flesh were observed between spring and all other seasons ($P \leq 0.01$; Fig. 2.3b). Concentrations of *Vibrio spp.* differed significantly between spring and all other seasons ($P \leq 0.01$; Fig. 2.4a) as well as between summer and autumn ($P = 0.005$) and autumn and winter ($P \leq 0.001$). Marine heterotrophic bacteria showed a significant difference in concentrations between spring and all other seasons ($P \leq 0.001$; Fig. 2.4b).

2.4 Discussion

2.4.1 Spatial and temporal heterogeneity of faecal indicator bacteria in mussel tissue

The mussel bed surveyed in this study is classified as “Class B” according to EC/854/2004 (EU 2004a) containing 230 – 4,600 *E. coli* per 100 g of sampled mussel flesh in 90% of cases. Our findings concur with the assigned classification grade and this study did not find levels of *E. coli* within mussel tissues that exceeded the maximum value for the “Class B” classification (4,600 *E. coli* / 100 g) in any of the samples analysed. However, the spatial distribution of *E. coli* over the mussel bed was shown to be patchy and displayed clear ‘hotspots’ of contamination, and although the mussel bed has been declared ‘safe’ for mussel harvesting, it still contains hotspots of potentially hazardous pathogens. These irregular spikes of increased microbial cell numbers were not shown to be correlated with either lateral distance or distance from MLW and cannot be attributed to any known point sources of contamination. Importantly, although *E. coli* concentrations did not exceed the upper limit for “Class B” classification, several sampled points showed *E. coli* concentrations to be below the minimum limit for “Class B” (230 *E. coli*/100 g). These findings have important implications for routine monitoring, as the spatial location of the sampling point(s) will have a direct impact on the classification grade assigned to the harvesting area.

Previous research has suggested that the distance from MLW over an intertidal shellfish bed plays a key role in determining the bacterial concentrations of shellfish at the time of harvesting as shellfish closest to the MLW have increased immersion times relative to their con-specifics at higher positions on the bed (Charles & Newell 1997). Our study suggests that the spatial distribution of total coliform bacteria is more likely to be correlated with environmental variables than the distribution of *E. coli*. Spatial patterns of both *E. coli* and total coliforms could also be due to differential uptake or elimination dynamics of the shellfish (Ho and Tam 2000). However, the total *E. coli* contamination of the harvesting area was low and the patchy distribution may have simply been an artefact of previous contamination events whereby individual shellfish were yet to eliminate *E. coli* from their digestive tract.

Seasonality is a phenomenon that has been well documented for FIB (Riou et al. 2007; Faust 1976; Kay et al. 2008). Concentrations of bacterial indicator species increase over the warmer months of spring and summer, and decline over the cooler months of autumn and winter (Chigbu et al. 2005). In light of this finding, seasonal effects have been incorporated into routine monitoring protocols. In our study, the numbers of culturable *E. coli* and total coliforms in mussel flesh were five to ten times higher in the summer compared to the winter,

which endorses the current local practice in the Conwy Estuary of not commercially harvesting shellfish during the summer months. The concentration of *Vibrio* spp. and marine heterotrophic bacteria did not show a similar seasonality, which could indicate a better adaptation of these naturally marine dwelling organisms to seawater in comparison to *E. coli* or coliforms detected in this study which may be largely anthropogenic in origin. It is also important to note that this *Vibrio*-specific technique does not differentiate between non-pathogenic and pathogenic species in this group, i.e. *V. cholera* and *V. vulnificus*.

The effect of tidal state on the concentrations of FIB is poorly understood. This is largely due to logistical difficulties in sampling especially for intertidal shellfish beds. The findings from this study indicate that although there were no differences in FIB concentrations over a single tidal cycle, the concentration of coliform bacteria was influenced by the tidal state. These differences may not be apparent for sub-littoral shellfish beds, where the animals are permanently submerged, but for intertidal shellfish beds the tidal state will determine the length of either immersion or exposure of the shellfish. During spring tides, shellfish closest to the MLW will be submerged at high water for longer periods than during neap tides, but exposed for a longer period during low water than during neap tides. Therefore, tidal state will dictate the amount of feeding time available for intertidal shellfish and hence the amount of potential time available for shellfish to accumulate or eliminate micro-organisms. In contrast, the concentration of *E. coli* within the mussel flesh was not consistently higher during a single tidal state, however each season showed a tidal state that appeared to show elevated *E. coli* concentrations relative to the other observed tidal states. Current routine monitoring protocols discourage sampling during the same tidal state, instead suggesting a minimum of 7 days between sampling events, which is supported by our study.

Recent research has suggested that tidal state may not be the dominant factor in determining bacterial concentrations of shellfish. This can rather be attributed to sporadic and unpredictable rainfall events (Stapleton et al. 2007; Laws et al. 2008; Kay et al. 2005). A “rainfall event” is considered to be greater than 10 mm of rainfall (Riou et al. 2007). Rainfall events can increase the levels of agricultural run-off into shellfish harvesting waters (Henroth et al. 2002) and can also trigger the release of raw or partially treated sewage into the sea (storm water discharge; Lessard & Beck 1990) potentially contaminating shellfish beds in the vicinity, producing a “risk period” for consumers and the shellfish industry. The spatial pattern of contamination may therefore reflect the hydrodynamic movement of effluent plumes over the shellfish beds, an area that has received little research attention.

2.4.2 Spatial and temporal heterogeneity of naturally occurring bacteria

Whilst FIB such as *E. coli* provide an estimation of the level of contamination affecting shellfish harvesting areas, it has been widely documented that they are not representative of the risk posed to shellfish consumers from naturally occurring pathogenic bacterial strains such as *Vibrio parahaemolyticus* and *Vibrio vulnificus* (Barile 2009; Marino et al. 2005). Temporal heterogeneity of naturally occurring bacteria across the mussel bed was evident in this study, with differences in bacterial concentrations over differing tidal states. Naturally occurring marine bacteria are better adapted for survival in the marine environment than indicator species such as *E. coli* therefore different factors will govern both their distribution and concentration. Teplitski et al. (2009) previously noted that *Vibrio spp.* formed stable, commensal relationships with shellfish and that a greater understanding of these relationships is needed to reduce the ‘risk’ to human health as current monitoring regimes only focus on FIB species. Consequently, shellfish contaminated by pathogenic *Vibrio spp.* may be “missed” by current routine monitoring protocols. Human illness attributed to *Vibrio spp.* infection has risen by 40% over previous years and currently 75% of all seafood associated diseases have been attributed to *Vibrio spp.* infection (Teplitski et al. 2009). The risk to consumers posed by naturally occurring pathogenic bacterial strains is large. Wittman & Flick (1995) noted that 99% of fatalities associated with shellfish related illnesses (mainly from eating raw oysters) could be attributed to *Vibrio spp.* The results from this study suggest that *Vibrio spp.* should be incorporated into monitoring strategies as current practices do not consider naturally occurring bacteria, and faecal indicator organisms such as *E. coli* are not representative of the level of shellfish contamination from naturally occurring bacteria such as *Vibrio spp.* There is therefore, an urgent need to understand the association between naturally occurring bacteria and FIB in order to provide an accurate assessment of risk to consumers.

2.4.3 Spatial contamination patterns of faecal indicator bacteria in sediment and mussel tissue

Although significant reservoirs of FIB on mussel beds have been identified in the sediment (Martinez-Manzanarez et al. 1992) and in epizoic barnacles (Clements et al. 2013), information about the relevance of these sources for shellfish contamination is limited. However, this study demonstrated no correlation in the spatial distribution of FIB in the sediment with that in the mussel tissue. Spatial distribution of FIB within the sediment was patchy, with hotspot areas of high concentrations not corresponding to those areas of mussels containing high contamination levels.

Mussels are capable of filtering up to 10 l h^{-1} of water (Teplitski et al. 2009). The findings from this study suggest that these animals may be capable of effectively ‘stripping’ the bacteria from the water column before they are absorbed into the sediment reservoir. However, localised hydrodynamics may re-suspend FIB from the sediment allowing for absorption into the mussel reservoir. Higher concentrations of faecal indicator organisms were observed in the sediments respective to the mussels. This may be due to the bioaccumulation of bacteria by mussels and the subsequent release of bacteria (in higher concentrations) via the production of pseudo-faeces (Kooijman 2006). The interactions between these two important bacterial reservoirs are poorly understood, highlighting the need for further research in this area.

2.4.4 Correlation of bacterial concentration patterns with nutrient levels and physico-chemical parameters

Contamination events not only increase the concentrations of microorganisms into shellfish harvesting areas, they also increase the nutrient loading into these areas (Lessard & Beck 1990). This increase in nutrient load (particularly C, N and P) may ‘reactivate’ bacterial cells already present in the environment but are currently in a dormant state (Oliver 2010) potentially elevating the concentrations of cultureable bacterial cells. The findings from this study indicate that bacterial reservoirs present within sediments may be much more susceptible to changes in nutrient concentrations and strong positive correlations were observed between N, C and P availability and increases in concentrations of faecal indicator bacteria within sediment. The concentration of *E. coli* within mussel tissues showed no apparent correlation with these nutrient concentrations in the corresponding sediment; however the concentration within the mussel flesh were negatively affected by higher levels of nutrients in the sediment.

Bacteria present within the digestive tract of mussels are buffered from sudden physico-chemical changes in the environment, however an increase in salinity externally could lead to a competitive advantage for naturally occurring bacteria which could then become taken up in higher concentrations by the mussels. This would allow the naturally occurring species to effectively outcompete the native *in situ* bacterial population and lead to differential accumulation of bacterial species by the mussels (Hibbing et al. 2010). Currently, there is a lack of understanding of the interactions between the environment, microbial competition and anthropogenic disturbances within the mussel bed ecosystem. This lack of research hinders our ability to predict pathogen dispersion within the mussel bed with certainty, especially with

regard to effectively managing risk associated with shellfish consumption and indicates a clear need for further investigations.

Nutrient analysis and the analysis of physico-chemical parameters has the potential to act as a more rapid and cost effective measure of contamination of shellfish harvesting areas and could be used to identify areas of contamination within the environment, however the findings from this study do not yet support their use as the sole indicator for contamination on shellfish beds as neither nutrient levels nor physico-chemical parameters act as a reliable indicator of bacterial contamination within shellfish tissues.

2.4.5 Conclusions and future management recommendations

In conclusion, the distribution and abundance of faecal indicator bacteria within mussel flesh did not vary significantly over smaller spatial and temporal scales, but did vary significantly over larger spatial and temporal scales. This is significant in terms of legislation as the location of the sampling points for routine bacterial monitoring have a direct impact on the final classification grade awarded for the shellfish bed; this in turn has important implications for the economic prosperity of the shellfish industry and the assessment of 'risk' to shellfish consumers. Using FIB is insufficient for safeguarding consumers against the wider risk posed by naturally occurring bacterial species such as *Vibrio* spp. and this study suggests that other organisms should be included in routine monitoring programs. The drawback to this is the costs associated with current sampling and analysis techniques which tend to rely on culture methods for enumeration. If the scope of pathogen testing was expanded it may not prove cost effective to undertake routine monitoring when balanced against healthcare costs associated with shellfish poisoning. However, new technologies which do not rely on cultureability for rapid pathogen enumeration are rapidly emerging, particularly in the field of food safety testing and these could be readily applied to wider water/shellfish pathogen testing in the future (Ripp et al. 2012; Yoon et al. 2012). The sediment is clearly a significant reservoir for FIB and the differential distribution patterns between the concentrations of FIB in sediment and mussel flesh highlights the need for a better understanding of the *in situ* bacterial flux between these reservoirs. Weak correlations were observed between concentrations of faecal indicator bacteria and nutrient concentrations, rendering nutrient measurements unreliable as a proxy for bacterial contamination.

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CHAPTER 3

Epizoic barnacles act as pathogen reservoirs in shellfish beds

K. CLEMENTS,¹ L. GIMÉNEZ,¹ D.L. JONES,³ J. WILSON,⁴ AND S.K. MALHAM²

¹*School of Ocean Sciences, Bangor University, Menai Bridge, Anglesey, LL59 5AB, UK;*

²*Centre for Applied Marine Sciences, Bangor University, Menai Bridge, Anglesey, LL59 5AB*

UK; ³*School of Environment, Natural Resources and Geography, Bangor University,*

Gwynedd, LL57 2UW, UK; ⁴*Deepdock Ltd, Bwthyn Y Mor, Llanfaethlu, Holyhead, Anglesey,*

LL65 4HD, UK

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

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Abstract

Bivalve shellfish are well known vectors for human pathogens. Recent research on commercial shellfish beds has shown that the bacterial reservoir contained within the shellfish flesh, *in situ*, is often not representative of the microbial quality of the shellfish at the point of sale. This study investigates whether barnacles living on the surface of mussels represent a potential bacterial reservoir within shellfish beds and assesses their potential as a vector for human pathogen transfer. Barnacle and mussel samples were collected from three independent intertidal mussel (*Mytilus edulis* Linnaeus, 1758) beds and subjected to standard microbiological testing and ecological evaluation. Our results showed that coliform concentrations were significantly higher in barnacles than in the corresponding mussels, per unit area, across all surveyed sites. The dominant observed barnacle species was the invasive barnacle *Austrominius (Elminius) modestus* (Darwin, 1854) which has out-competed native species in the region and contains increased coliform bacterial concentrations relative to other observed barnacle species. This study concludes that, where present, epizoic barnacles represent a significant reservoir for bacteria within shellfish beds and therefore the capacity to act as vectors for human pathogens. Further work is needed to quantify the subsequent viability/pathogenicity of the epizoic bacterial reservoir after shellfish harvesting and food processing.

KEY WORDS: *E. coli*, Faecal coliform, Vibrio, Marine water, Microbiological contamination

3.1 Introduction

The global demand for shellfish products continues to rise to meet the needs of an ever increasing human population (Naylor et al. 2000). The nutritional benefits of shellfish consumption have been well publicised, further adding to the global demand for this nutritionally beneficial and relatively cheap food source (Gjedrem et al. 2012). This increase in shellfish cultivation is most apparent in developing countries where environmental legislation and protection of the wider shellfishery environment is currently lacking and where contamination of marine waters from sewage and agricultural runoff is particularly problematic (Oliveira et al. 2011). Even in developed nations, however, contamination of coastal waters from sewage discharge is still common, especially during storm events where sewage treatment facilities exceed capacity, leading to the release of un- or partially treated sewage directly to the sea (Lessard and Beck 1990). This release of sewage raises both the nutrient and microbiological load within shellfisheries and can alter the growth of the indigenous microbial community. Traditionally, shellfish are consumed either raw or lightly cooked and are often cited as a vector for outbreaks of bacterial and viral food poisoning in humans (Rippey 1994, Potasman et al. 2002). Consequently, there is an increasing challenge for the shellfish industry to supply a product of good quality that is safe for human consumption (Lee and Younger 2002).

Previous research on shellfish quality has largely focused on determining the biochemical quality of harvested shellfish flesh and the development of rapid diagnostic screening techniques for quantifying bacterial and viral indicator species within shellfish tissues (e.g. *E. coli*, faecal streptococci; Svärth 1999, Romalde et al. 2002, Field and Samadpour 2007, Kay et al. 2008). These indicators provide a proxy for the presence of potential disease causing agents (e.g. *Vibrio vulnificus*, *Salmonella* spp., Norovirus) which would be too costly to screen individually within routine monitoring programmes. Within the European Union this has led to the formulation of stringent legislation based on the use of indicator species (e.g. EC 854/2004 and EC 853/2004) to safeguard against contaminated shellfish products entering the food chain (EU 2004ab).

Bivalve shellfish are filter feeders capable of effectively bio-accumulating human pathogenic bacteria, protozoa and viruses (Roslev et al. 2009). Research, however, has suggested that the bacteria contained within the shellfish tissue itself may be overshadowed by larger bacterial reservoirs present within the wider shellfish production area (e.g. within sediments, particulate organic matter in the water column or on the surface of the shells;

Martinez-Manzanares et al. 1992; Fries et al. 2008; Wahl 2008). Of particular concern are pathogens associated with the shell surface as this effectively bypasses routine screening procedures which focus on the analysis of the internal shellfish tissue only.

Epizoic (surface dwelling) barnacle species are commonly associated with shellfish and have been recognised as a potential contaminant for commercially harvested mussels (Clegg and Sherwood 1947), but only by secondary contamination, post harvesting. The bacterial reservoir contained within barnacles, however, has to date not been quantified and the *in* and *ex situ* bacterial flux between the two species remains undetermined. Studies on bacterial biofilms present in barnacles have been restricted to ecological studies only (Bacchetti de Gregoris et al. 2012) and little data exists on the bacterial content of different barnacle species. The primary aim of this work was to investigate the bacterial load of barnacle species attached to commercially harvested mussels (*Mytilus edulis*) with a view to assessing the potential risk of shellfish contamination and transfer to the human food chain.

3.2 Materials and methods

3.2.1 Sampling Location

Three intertidal, commercial mussel (*Mytilus edulis* L.) beds were sampled between 1st April and 10th April 2011. Located in the Conwy region (North Wales, UK), Conwy Bridge (53.280279N, -3.838767W), Llanfairfechan (53.259132N, -3.980289W) and Conwy Morfa (53.298015N -3.854535W) represented three commercially harvested shellfish beds that are routinely monitored for bacterial contamination and have been classified as “Class B” (containing between 230 - 4,600 *E. coli* per 100g) in accordance with regulation EC 854/2004 (EU 2004a). Approximately 6 - 10 individual mussels and their associated barnacles were collected by hand from 15 random sample points per mussel bed and subsequently pooled prior to laboratory analysis for bacterial determination. Subsequent processing required 50 g of both mussel and barnacle, the variation in the number of animals collected per sample, is a result of mussel size and degree of ‘fouling’ i.e. the number of barnacles present, per sample point. In addition, 10 further samples were collected, per bed, to enable the analysis of various ecological parameters. All samples were transported and stored at 4°C and processed within 6 h of collection.

3.2.2 Determination of Bacterial Load

Only live shellfish were chosen for evaluation. Shellfish samples were washed with sterile seawater to remove any residual sediment and debris before surface swabbing with 100% methanol to eliminate the surface biofilm. Samples were left to dry for 30 min at room temperature to allow the methanol to fully evaporate before aseptically removing 50 g (wet weight) of the encrusting barnacles and adding them to 50 mL of 25% strength Ringer’s solution. The mussel shells were re-sterilised by swabbing with methanol to remove any residual bacteria from the barnacle removal process and left to dry at room temperature for 10 minutes. Once dry, the mussels were then opened aseptically and 50 g (wet weight) of flesh and extra cellular fluid was obtained. Barnacle and 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 was plated onto Brilliance® selective agar (#CM1046; Oxoid Ltd, Basingstoke, UK) to determine total coliform counts. In addition, 10 µL of the homogenate was added to agar plates containing TCBS cholera agar to determine total *Vibrio spp.* (#CM0333; Oxoid, UK) and to marine agar (#1059; Laboratorios Conda, Madrid, Spain) to determine total marine heterotrophic bacteria. All plates were inverted and

incubated at 37°C (Brilliance® agar) or 25°C (TCBS and marine agar) and bacterial colony forming units (CFU) enumerated after 24 h.

3.2.3 Assessment of Ecological Parameters

Ecological parameters for each mussel bed were surveyed using a 10 cm² quadrat randomly thrown 10 times per mussel bed. Total epifaunal biomass was collected for subsequent laboratory analysis. Samples were washed with seawater to remove any debris including organisms other than the mussels and barnacles. Only extant mussels were selected for this portion of the analysis. The total wet weight biomass, mussels, mussel flesh and barnacles were weighed and recorded. Mussel and barnacle flesh samples were then dried (80°C, 48 h) to determine their dry weight. In addition, the total number of both living and dead barnacles per 10 cm² was determined and each barnacle identified to species level.

3.2.4 Statistical Analysis

Data were analysed using PASW statistics v18 (IBM Corp., Armonk, NY). Normality was assessed using a one sample Kolmogorov-Smirnov test ($P \geq 0.05$). Bacterial count data were analysed using the Independent Samples Kruskal-Wallis test and any significant differences ($P \leq 0.05$) were investigated further using independent samples Mann-Whitney U test, with fixed factors of either site or bacterial species and three replicate units per analysis. Ecological data were analysed using a series of one way Analysis of Variance (ANOVA) and significant differences were investigated using the Least Significant Difference (LSD) *post hoc* test.

3.3 Results

3.3.1 Bacterial Concentrations in Mussels and Barnacles

Total coliform concentrations were significantly higher in barnacles compared to mussels across all three sample sites ($P < 0.05$; Fig. 3.1). There was also a significant difference in coliform concentration observed in both barnacles ($P = 0.001$) and mussels ($P = 0.033$) between the three sampling sites. Total coliform levels in mussel tissues were all below the upper threshold for European Union “class B” classification (4,600 *E. coli* CFU 100 g⁻¹). In contrast, barnacles at all sites showed total coliform concentrations in exceedance of the upper threshold for this critical classification.

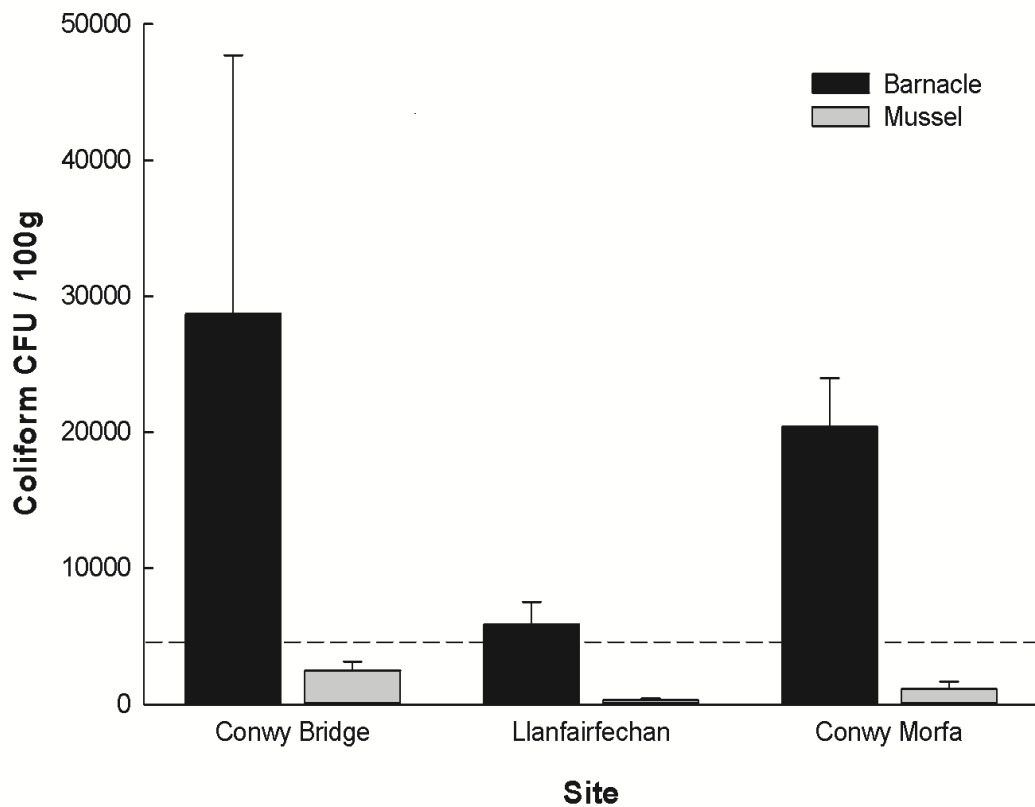


Fig. 3.1. Total coliform population observed in mussels and barnacles across three commercial shellfish beds. For reference the dashed line represents the upper European Union threshold for ‘class B’ grading of mussels (4,600 *E. coli* CFU 100 g⁻¹) (EU 2004a). In all cases $n = 15$ where data points represent the mean \pm SE.

Total heterotrophic bacterial counts in the barnacle and mussel tissue are shown in Figure 3.2 and are used here to provide a measure of Total Viable Counts (TVC). Enumeration of *Vibrio spp.* indicated that they represented the majority of the TVC (Fig. 3.2). Significant differences were observed in both *Vibrio spp.* and total heterotroph concentrations in both barnacles ($P < 0.001$) and mussels ($P < 0.001$) across all three sample sites. Results for the Llanfairfechan and Conwy Morfa sample sites showed significantly higher *Vibrio spp.* ($P < 0.001$) and heterotrophs ($P < 0.001$) in mussels compared to barnacles. This trend, was reversed at the Conwy Bridge site where total heterotrophic bacteria were significantly higher in barnacles than in mussels ($P < 0.001$) whilst *Vibrio spp.* numbers did not differ significantly between the two shellfish types ($P = 0.624$).

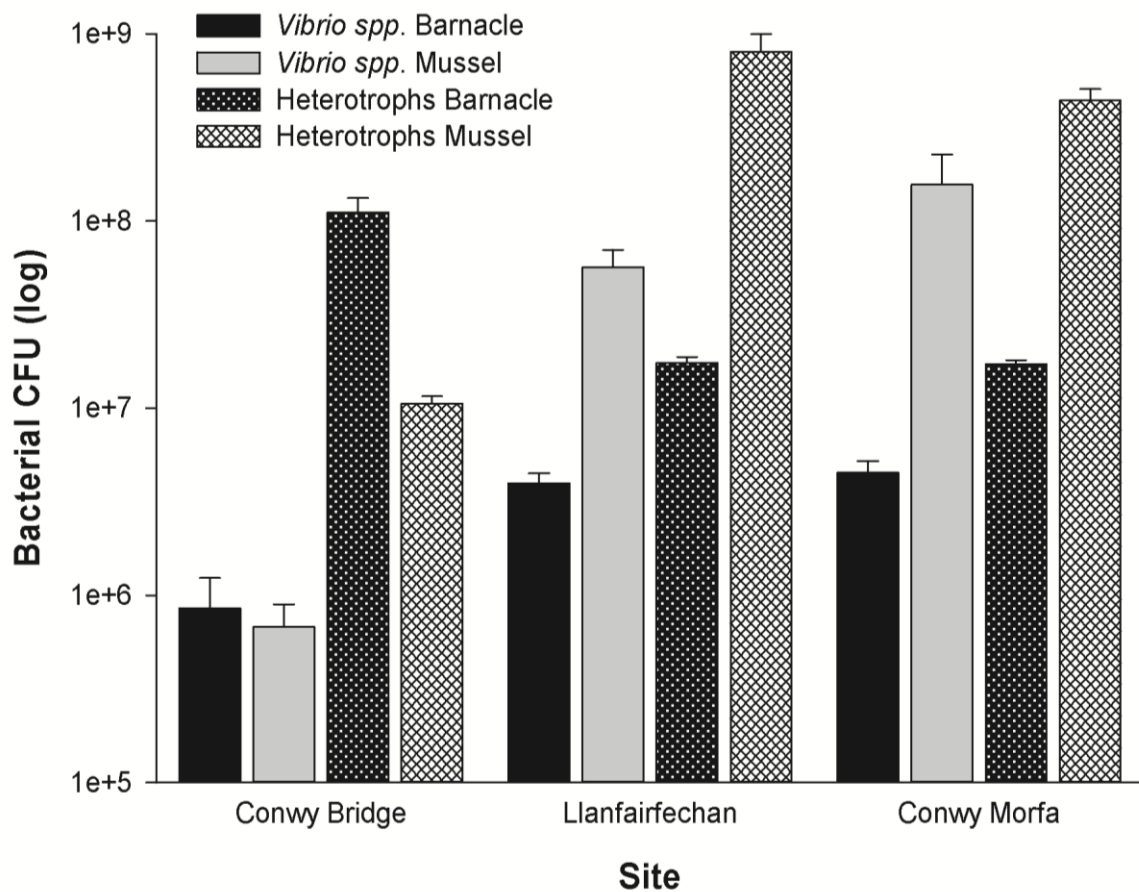


Fig. 3.2. *Vibrio spp.* and total marine heterotroph (TVC) population size observed in both mussels and barnacles across three commercial shellfish beds. In all cases $n = 15$ where data points represent the mean \pm SE.

3.3.2 Ecological Parameters

Barnacle communities across all sites consisted of four species, but they were dominated by *Elminius modestus* (Table 3.1). Site specific species composition varied among the three sites for all barnacle species (*Elminius modestus*, $P = 0.02$; *Cthalamus montagui*, $P < 0.01$; *Balanus crenatus* $P = 0.005$) with the exception of *Semibalanus balanoides* ($P = 0.455$). The total number of barnacles per unit area at Conwy Bridge was significantly higher than at both Llanfairfechan and Conwy Morfa ($P = 0.002$), but did not differ significantly between the latter two sites ($P = 0.968$) (Table 3.1).

Table 3.1 Species composition of barnacles on the surface of mussels obtained from three commercial shellfish beds. Values are expressed as a percentage of the mean number of barnacles per site. In all cases $n = 10$.

Species composition	Shellfishery		
	Conwy Bridge	Llanfairfechan	Conwy Morfa
<i>Elminius modestus</i>	98.1	93.4	91.7
<i>Semibalanus balanoides</i>	0.7	1.9	4.7
<i>Cthalamus montagui</i>	1.1	5.0	0.0
<i>Balanus crenatus</i>	0.0	0.0	3.2

In contrast to barnacle numbers, total barnacle dry weight per unit area was not significantly different between sites ($P = 0.189$), however, the mean weight of barnacles per mussel (Table 3.2) differed significantly among sites ($P = 0.002$). Barnacles living on mussels in Conwy Bridge were significantly smaller in comparison to encrusting barnacles located in Llanfairfechan and Conwy Morfa. Mussels at Conwy Bridge also had significantly higher numbers of encrusting barnacles relative to the other sites ($P < 0.001$; Table 3.1). Mussel flesh weight also differed significantly between sites ($P < 0.01$).

Table 3.2 Comparison of ecological parameters across three commercial mussel (*M. edulis*) beds. In all cases $n = 10 \pm$ Standard Error (SE).

Ecological parameters	Shellfishery		
	Conwy Bridge	Llanfairfechan	Conwy Morfa
Barnacle number (10cm ²)	803 ± 5	259 ± 58	253 ± 66
Number of barnacles per mussel	52.8 ± 2.3	12.1 ± 0.2	8.3 ± 0.9
Barnacle weight (g barnacle ⁻¹)	0.03 ± 0.002	0.17 ± 0.01	0.10 ± 0.006
Mussel flesh weight (g shell ⁻¹)	41.9 ± 5.0	25.9 ± 3.9	81.8 ± 10.2
Total barnacle weight (g mussel ⁻¹)	23.0 ± 5.5	42.8 ± 11.7	23.0 ± 5.5

Coliform concentrations corrected by weight per unit area (Table 3.3) and expressed as a percentage of the total, show that across all sites the epizoic barnacles are a much larger reservoir for coliform bacteria than their associated mussels. The bacterial reservoir contained within the mussel flesh is less than 20% of the reservoir contained within the barnacles attached to the shell of the mussel. Llanfairfechan showed the lowest coliform concentrations (Fig. 3.1) and also the lowest relative coliform reservoir present within the mussel flesh.

Table 3.3 Proportion of the coliform reservoir associated with mussels and their epizoic barnacles in three commercial shellfisheries. Values are expressed as a percentage of the total coliform reservoir observed for both species.

Coliform reservoir	Shellfishery		
	Conwy Bridge	Llanfairfechan	Conwy Morfa
Mussel	13.6	3.0	16.6
Barnacle	86.4	97.0	83.4

3.4 Discussion

Our findings indicate that barnacles represent a significant reservoir for potential human pathogenic bacteria on commercial shellfish beds. On a per unit area basis, approximately 80% of the bacteria within the commercial shellfish bed were associated with the epizoic barnacles. Although we measured total coliform bacteria, it is important to note that we cannot confirm whether the individual coliform species differed between the barnacles and mussels. Nevertheless, our results have important implications for the management, harvesting and monitoring of mussel beds as well as subsequent processing of the harvested material. We did not investigate the factors that led to the differential accumulation of coliforms and *Vibrio spp.* by barnacles relative to mussels; however, our results suggest that the differential bacterial accumulation is dependent upon community composition of the epizoic assemblage as well as differences in physiological and morphological traits between the two shellfish types.

In terms of monitoring the environmental quality of shellfisheries and the degree of pollution derived from agricultural and human sewage discharges, current legislation is concerned only with the bacterial quality of the shellfish; thus, other potential *in situ* pathogen reservoirs are excluded. In this case, we examined barnacles; however, contamination may also occur from other elements of the surface biofilm in addition to co-harvested sediment and water. Depending upon shellfish preparation, these elements might be transferred to food processing environments where the outside of the shellfish may come into contact with food preparation surfaces allowing for cross contamination with other foods or shellfish batches (Lee et al. 2008). Our results call for a more extensive monitoring of the benthic community living on commercial shellfish beds to evaluate and quantify the pathogen flux between epizoic species and bivalve shellfish during standard commercial handling procedures.

Increased global demand for shellfish products (Wijkstrom 2004, Børresen 2008, Teplitski et al. 2009) has led to exploitation of ‘poorer quality’ mussels (mussels encrusted with barnacles). Likewise, a shift in consumer preference for more ‘natural’ foods has also increased the demand for mussels retailed complete with their associated barnacles (Acebron and Dopico 1999, Oliveira et al. 2011). Although most species of encrusting barnacles are not consumed directly, little is currently known about the behaviour of these barnacles during storage/transport, their physiological state, their susceptibility to damage post-harvest, and the fate of bacteria contained within them. Secondary bacterial contamination of mussels by their associated barnacles during transport and storage was noted in 1947 (Clegg and Sherwood 1947), but to our knowledge it has never been quantified. Sagoo et al. (2007) noted that of 682

batches of shellfish deemed fit for human consumption at the time of harvesting, 4% failed re-testing at the point of retail, suggesting secondary bacterial contamination during transit to the point of sale. No attempt has been made to systematically quantify the levels or identify sources of secondary contamination of bivalve shellfish on a commercial scale. Consequently, the implications to the shellfish industry in relation to the current practice of transportation and storage of live bivalve shellfish remain unknown.

Our study also suggest that not all bacterial species are preferentially accumulated by epizoic barnacles respective to their mussel counterparts and a degree of site specificity exists, highlighting the need for individual assessments of shellfish harvesting areas. *Vibrio spp.* represented a large proportion of the total culturable bacteria found in both mussels and barnacles. Species such as *Vibrio parahaemolyticus*, *V. cholera* and *V. vulnificus* are well documented human pathogens (Rippey 1994, Potasman et al. 2002, Teplitski et al. 2009) responsible for several outbreaks of shellfish-related gastro-enteric food poisoning in humans (Potasman et al. 2002, Lee et al. 2008). This study has shown that *Vibrio spp.* are present in higher concentrations within mussels as opposed to barnacles; this could be explained by the selective filter feeding in mussels (Shumway et al. 1985). Shellfish beds with higher concentrations of total coliforms also show lower concentrations of total *Vibrio spp.* This shift in the microbial community may be due to differential uptake or depuration of the bacterial species (Marino et al. 2005) or competition between the bacterial species (Hibbing et al. 2009). Naturally occurring *Vibrio spp.* contaminating bivalve molluscs have also been shown to be less easily removed by depuration than faecal bacterial indicators such as *E. coli* (Rodrick and Schneider 1991). Whether this also applies to epizoic organisms remains unknown.

If bacteria are accumulated in important quantities in epizoic organisms, it is important to understand the process that leads to their ingestion. Previous research into the differential bio-accumulation rates of mussels (as selective filter feeders) and barnacles (as suspension feeders) concluded that differential feeding mechanisms could be responsible for the differences in bio accumulation of bacteria, or that differences in the bacterial content may reflect the different nutritional needs of the organisms (Shumway et al. 1985, Dolmer 2000, Newell et al. 2001). Buschbaum (2001) and Buschbaum and Saier (2001) noted that barnacles preferentially choose to settle on live mussels, close to the inhalant siphon, where they utilise the feeding current generated by the mussels, but utilise different nutritional sources. Bacteria present in the water column can therefore be taken up by both barnacles and mussels. No direct data exists on differential bacterial up take rates between the two species (Riisgård and Larsen 2000). Mussels reject particles via the production of pseudo faeces (Kooijman 2006)

and are capable of 'self purifying' (de Mesquita et al. 1991) whilst barnacles lack this capacity. This may partially explain the higher bacterial concentrations present in barnacles compared to mussels. Bioaccumulation of bacteria in barnacle flesh is under researched; however, the capacity of barnacles to accumulate compounds such as heavy metals has been well documented (Powell and White 1990, Aydin Onen et al. 2011, Reis et al. 2011)

Wahl (2008) discovered that the properties and functions of a body surface play a crucial role in the uptake of particulate matter in aquatic organisms. Therefore the dominant barnacle species may have an important role in determining the bacterial community based simply on its shell morphology (Verran and Boyd 2001). The dominant barnacle was the invasive Australasian barnacle *Elminius modestus*. First observed in the UK in 1947, *E. modestus* was first documented in our wider study area in 1956 (Crisp 1958). It is distinguished from other native barnacle species by the presence of four basal plates instead of six (Hayward and Ryland 1995). Generally, smaller and more dorso-ventrally flattened than native UK barnacle species (*Semibalanus balanoides*, *Balanus crenatus*), *E. modestus* has the capacity to rapidly colonize surfaces within one year of the settling of a few scattered adults (Crisp 1958). Little data exists on the bacterial composition of *E. modestus* compared to native barnacles; however, our data suggests that *E. modestus* may represent a significant reservoir for human pathogens in comparison to native barnacle species.

In conclusion, barnacles represent a significant *in situ* bacterial reservoir in commercial mussel beds and as such have the potential to act as secondary contaminants to mussels during transit to retail outlets. Barnacles should therefore be incorporated into monitoring programmes of bacterial communities since they could contribute to secondary contamination of commercial shellfish. Further work is needed to quantify the bacterial reservoir present in barnacles and to understand the bacterial flux both *in situ* and *ex situ* between the two species. Research is also needed to determine the accumulation efficiency of bacteria and other pathogenic micro-organisms in both native and non-native barnacle species and to establish whether barnacles preferentially feed on specific bacterial species or compete for food resources with the associated mussels.

3.5 Acknowledgements

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CHAPTER 4

Differential depuration dynamics of bivalve shellfish and epizoic barnacles: Implications for current depuration practices

KATIE CLEMENTS,¹ LUIS GIMÉNEZ¹, DAVID L. JONES² AND SHELAGH K. MALHAM³.

¹*School of Ocean Sciences, Bangor University, Menai Bridge, Anglesey, LL57 5AB, UK;*

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

*Corresponding author. Email: ktclements@hotmail.co.uk; ospc02@bangor.ac.uk

Running head: Differential depuration dynamics of bivalve shellfish and epizoic barnacles

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Abstract

Depuration of bivalve shellfish is a common practice utilised worldwide to purify shellfish contaminated from anthropogenic sources. Historically, research efforts have focused almost entirely on quantifying the bacterial content of the bivalve shellfish flesh. Recent research, however, has suggested that the epizoic barnacles often associated with bivalve shellfish may act as a larger reservoir for potential human pathogens. This study investigated the elimination of bacteria from both bivalve shellfish and epizoic barnacles undergoing depuration, and quantifies the elimination rates for both shellfish species. Bivalve shellfish and barnacle samples were collected from three commercial mussel (*Mytilus edulis*) beds and were subjected to standard microbiological evaluation during treatment in a scaled-down commercial depuration facility. Our results showed that a 42 hour depuration treatment was sufficient to eliminate bacterial indicator organisms from the mussel flesh, but was insufficient to completely eliminate the same indicator species from the epizoic barnacles. Despite this, calculation of the elimination rates over the course of depuration treatment demonstrated that epizoic barnacles were capable of eliminating total coliform bacteria up to five times faster than the corresponding mussels. This study concludes that epizoic barnacles and bivalve shellfish show differential bacterial elimination rates and whilst barnacles are able to eliminate bacteria at a higher rate, they still retain high bacterial levels post-depuration treatment. Further work is needed to assess the potential human health risks associated with secondary contamination of purified bivalve shellfish from epizoic organisms post purification treatment.

KEY WORDS: Barnacle, *Mytilus edulis*, purification, public health, microbial quality.

4.1 Introduction

Bivalve shellfish are often implicated in outbreaks of food poisoning in humans (Potasman et al. 2002). Despite this, the global demand for shellfish products continues to increase to supply an ever expanding human population (Naylor et al. 2000). Shellfish harvesting areas are often located in shallow coastal waters where they are subject to anthropogenic contamination from raw or partially treated sewage released into the sea, or from agricultural diffuse pollution entering coastal waters (Selegen et al. 2001). Bivalve shellfish are filter feeders capable of filtering large volumes of water per hour (Teplitski et al. 2009), and have the capacity to bio-accumulate pathogenic micro-organisms present in low concentrations in the surrounding water (Lees 2000). As shellfish are often consumed raw or lightly cooked they are able to act as vectors for both viral and bacterial outbreaks of food poisoning in the human population (Rippey 1994). The challenge for the shellfish industry is to provide a good quality, nutritious product, which is also safe for human consumption (Lee & Younger 2002).

The monitoring of shellfish contamination is achieved through the use of indicator species designed to act as a proxy for pathogenic bacteria and viral species such as *Vibrio parahaemolyticus* and norovirus. Previous research has focused on the development of robust, inexpensive, diagnostic and screening techniques for indicator species such as *Escherichia coli*, coliforms and faecal streptococci (Field & Samadpour 2007), as direct screening for individual pathogens has proven to be too costly for routine monitoring purposes.

Bivalve shellfish harvested from contaminated waters where accumulation of potentially pathogenic organisms is likely to occur, should undergo a purification treatment prior to retail. In many countries worldwide, the natural ability of bivalve shellfish to ‘self purify’ is utilised, and harvested animals are subjected to a depuration procedure in which the animals are held within a ‘clean water’ environment for a given time period and allowed to naturally relieve themselves of their pathogenic load. Many industrialised countries have stringent legislation, based upon scientific research, which dictates the physical parameters of this process.

Within the European Union, legislation (EC854/2004) stipulates that bivalve shellfish harvested from class ‘B’ areas (<4,600 *Escherichia coli* per 100 g flesh) must be purified in an approved depuration system (EU 2004a) and the end products must comply with EC 854/2004 microbial standards of less than 230 *E. coli* per 100 g flesh (EU 2004a,b). In England and Wales all bivalve shellfish, harvested from class ‘B’ areas, are self purged or ‘depurated’ in

closed 'recirculation' systems, with UV water treatment. All commercial depuration systems must meet the conditions of approval as determined by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) (Lee 2008). Currently in the UK there are five approved commercial depuration systems which have been validated by stringent bacteriological testing and are now regarded as 'proven' designs which require less stringent on-going bacteriological testing (Lee 2008). Despite this, "zero contamination risk" to public health, is not achievable due to high variability of naturally occurring pathogens (Kay et al. 2004).

To date, research efforts have been concerned with the potential pathogen concentration present within the bivalve shellfish flesh. Whilst this is fundamental to achieving food safety and protecting public health, other sources of contamination must also be considered. Increasing global pressure on seafood resources has led to the exploitation of previously under-utilised shellfish resources. This, combined with a shift in consumer demand for more "natural-looking" food products (Acebron & Dopico 1999) has led to the increase in the number of producers marketing bivalve shellfish complete with any associated organisms such as barnacles.

The primary aim of this study was to build upon previous work that had identified epizoic barnacles attached to bivalve shellfish as a potential pathogen reservoir (Clements et al. 2013). This study aimed to assess the efficiency of a standard depuration treatment in the removal of potential pathogens from epizoic barnacles and to quantify the elimination rates of both the bivalve shellfish and epizoic barnacles with a view to determining whether epizoic barnacles posed a threat to human health through secondary contamination of purified bivalve shellfish.

4.2 Materials and methods

4.2.1 Sampling Location

Field sampling followed the procedure as described in Clements et al. (2013). Three intertidal, commercial mussel (*Mytilus edulis*, Linnaeus; 1758) beds were sampled between 1st April and 10th April 2011. Located in the Conwy region (North Wales, UK), Conwy Bridge mussel bed (53.280279N, -3.838767W) was sampled on 1st April, Llanfairfechan (53.259132N, -3.980289W) was sampled on 6th April and Conwy Morfa (53.298015N -3.854535W) was sampled on 10th April. All three beds are commercially harvested and routinely monitored for bacterial contamination. Mussels and their associated barnacles were collected by hand from 15 random sample points per mussel bed and subsequently pooled prior to laboratory analysis for bacterial concentration determination. In addition, 15 individual mussel samples were randomly collected at each bed to enable the analysis of various ecological parameters. All samples were transported and stored at 4°C and either processed within 6 h of collection or introduced into a scaled down commercial depuration facility and subsequently analysed at pre-determined time periods.

4.2.2 Determination of Bacterial Content

Preparation of shellfish samples followed the procedure of Clements et al. (2013). Shellfish samples were washed with sterile seawater to remove any residual sediment and debris before rinsing in 100% methanol to sterilise the shell surface biofilms. Samples were left to dry for approximately 30 minutes to allow time for the methanol to fully evaporate, before aseptically removing 50 g of the encrusting barnacles and adding them to 50 mL of 25% Ringers solution. The associated mussels (approximately 10 individuals) were then re-swabbed with methanol to remove any residual bacteria and left to dry, before being opened aseptically and 50 g of flesh and extra cellular fluid obtained. Barnacle and mussel samples were homogenised for 60 seconds at 10,000 rev/min using a Bamix™ blender. From the resulting homogenate 200 µL was plated onto Brilliance® agar (#CM1046; Oxoid Ltd, Basingstoke, UK) to determine coliform counts. In addition, 10 µL of the homogenate was added to plates containing TCBS cholera agar (#CM0330; Oxoid, UK) and to marine agar (#1059; Conda Lab, Madrid, Spain) to determine total *Vibrio spp.* and total marine heterotroph counts respectively. All plates were inverted and incubated at 37°C (Brilliance agar) and 25°C (TCBS and marine agar) and bacterial Colony Forming Units (CFU) were enumerated after a 24 hr incubation period.

4.2.3 Depuration Facility

A scaled down version of a commercial depuration system containing approximately 800 L of seawater was used for shellfish depuration. In accordance with current UK depuration practices the water temperature was maintained at 16°C and the water recharge flow rate was maintained at a minimum of 30 L/h⁻¹. Dissolved oxygen was measured using a standard probe and was maintained at levels exceeding 50% saturation. Salinity was measured using a standard refractometer and levels were maintained at 35 parts per thousand. Water sterilisation was achieved via UV disinfection at a rate of 10,000 µws/cm². Water quality was tested at 12 hour intervals for nitrate, nitrite, ammonium and pH using an API saltwater master test kitTM (Mars fishcare, Chalfont, USA). Total coliform concentrations were determined using the vacuum filtration technique as described in Quilliam et al. (2011). Briefly, 50 mL of water was aseptically removed from the depuration system and immediately filtered through a 0.2 µm cellulose acetate membrane (Sartorius Stedium Biotech, Epsom, Surrey, UK) and the filter subsequently transferred onto an agar plate containing M-endo LES media (# MM0551 Oxoid, Basingstoke, UK). The plates were then inverted and incubated at 37°C for 24 hours. Any resulting metallic green colonies were enumerated as total coliform Colony Forming Units (CFU).

Mussels (complete with epizoic barnacles) were loaded into the system in 3 separate batches of approximately 20 kg. Each batch represented one sample site. Between batches the system was drained and cleaned to minimise any cross contamination between mussels from different sites. Prior to loading, the mussels were washed with sterile seawater to remove any debris and randomly separated into batches of ten animals. Mussels were loaded into the system in raised baskets, to minimise recontamination from pseudo-faeces, with 10 animals per basket. Three baskets were removed at 6 hours, 12 hours, 24 hours, 48 hours and 72 hours into the depuration process. The baskets were removed carefully so as not to re-suspend any pseudo-faeces and the mussels were processed for bacterial contamination as described above. Each basket represented one replicate, and three replicate baskets were analysed per time point.

To obtain an accurate baseline 15 replicate samples were analysed per site, immediately upon return to the laboratory, using the process described above. The resulting bacterial values were assumed to be indicative of the overall bacterial contamination level per site at the time of sampling. These values are described as time 0 hours (pre-depuration).

4.2.4 Ecological Assessment

Ecological parameters for each mussel bed were assessed by collecting a further 15 individual mussels per site at random. Only extant mussels were selected for this portion of the analysis. Wet weight measurements were taken of the total biomass and of both the individual mussels and the epizoic barnacles per mussel.

4.2.5 Statistical Analysis

Data were analysed using PASW statistics v18 (IBM Corp., Armonk, NY). Normality was assessed using a one sample Kolmogorov-Smirnov test ($P \geq 0.05$). Bacterial concentration data were analysed using a series of Independent samples Mann-Whitney U tests, with three replicate units per analysis. Correlations between bacterial concentrations and time were performed using a Spearman Rank Correlation Co-efficient. Ecological data were analysed using a series of One Way Analysis of Variance (ANOVA). Post Hoc tests were unable to be conducted due to the small number of cases within each analysis.

4.3 Results

4.3.1 Water Quality Parameters

Total coliform CFU were not detected in any of the water samples taken from the depuration system. Assessed water quality parameters all remained within acceptable limits nitrate (< 20 mg/L), nitrite (< 0.1 mg/L), ammonium (< 0.05 mg/L), pH (8.0-8.4) and salinity (34 - 36 ppt).

4.3.2 Bacterial Concentrations in Mussels and Barnacles Undergoing Depuration

Across all three sampling sites; initial total coliform concentrations were higher in barnacles than in mussels ($P \leq 0.05$; Fig. 4.1). Total coliform concentration in both mussels and barnacles decreased significantly over the course of the depuration process ($P \leq 0.05$; Fig. 4.1). After the 42 hours post depuration period (as required by European law for class 'B' mussels; EU 2004a), the total coliform concentration of the mussel flesh was barely detectable, whilst the total coliform concentration within the barnacles remained elevated above levels considered acceptable for shellfish products intended for human consumption under current European legislation (Fig. 4.1).

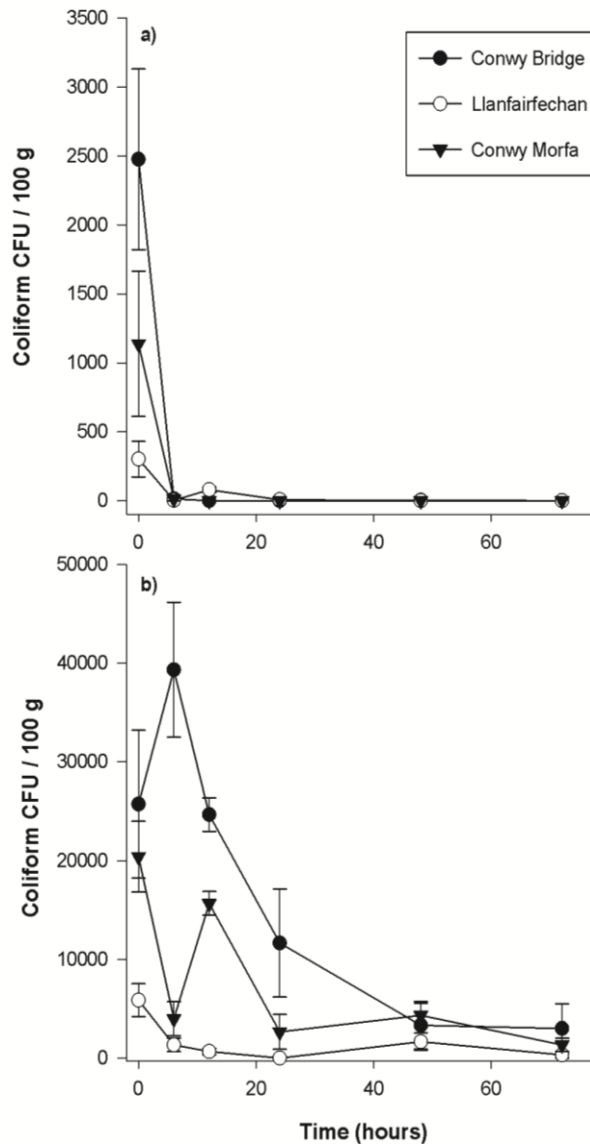


Fig. 4.1. Total coliform population observed in a) mussels and b) barnacles from three commercial shellfish beds held over a 72 hour time period in a depuration facility. In all cases $n = 3$ with the exception of time point '0' where $n = 15$. Data points represent the mean \pm Standard Error.

Significant differences in *Vibrio spp.* concentrations between mussels and barnacles were only evident for the Conwy Morfa site ($P \leq 0.001$; Fig. 4.2). Llanfairfechan and Conwy Bridge sites showed no such differences in *Vibrio spp.* concentrations between mussels and barnacles ($P \geq 0.05$; Fig. 4.2). Conversely, the Conwy Bridge and Llanfairfechan sites showed a significant increase in *Vibrio spp.* concentrations over the depuration period for both mussels and barnacles ($P \leq 0.001$; Fig. 4.2). No significant increase in *Vibrio spp.* concentration was observed for the Conwy Morfa site.

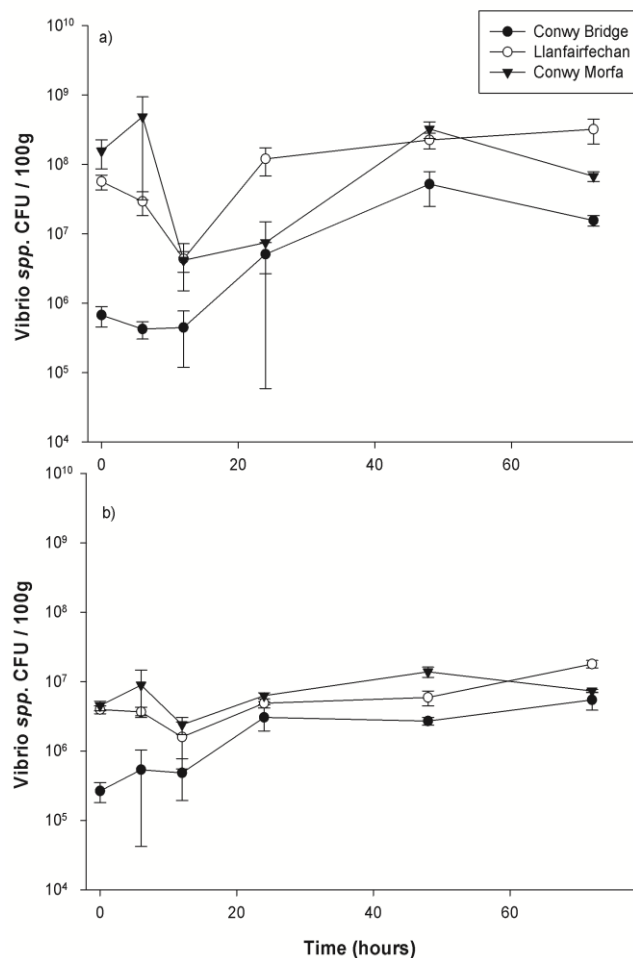


Fig. 4.2. Presumptive *Vibrio* spp. population observed in both a) mussels and b) barnacles from three commercial shellfish beds held over a 72 hour period in a depuration facility. In all cases $n = 3$ with the exception of time point '0' where $n = 15$. Data points represent the mean \pm Standard Error.

Total marine heterotrophic bacterial concentrations are displayed in Figure 4.3 and are used as an indicator of the Total Viable Counts (TVC). Significant differences in the bacterial concentration between mussels and barnacles were observed across all sample sites ($P \leq 0.05$; Fig. 4.3). Within the barnacles there was no significant reduction in TVC concentration over the depuration period ($P \geq 0.05$). Within the mussel flesh the TVC concentration increased over the course of the depuration period, but this was only statistically significant for the Conwy Bridge site ($P \leq 0.05$; Fig. 4.3).

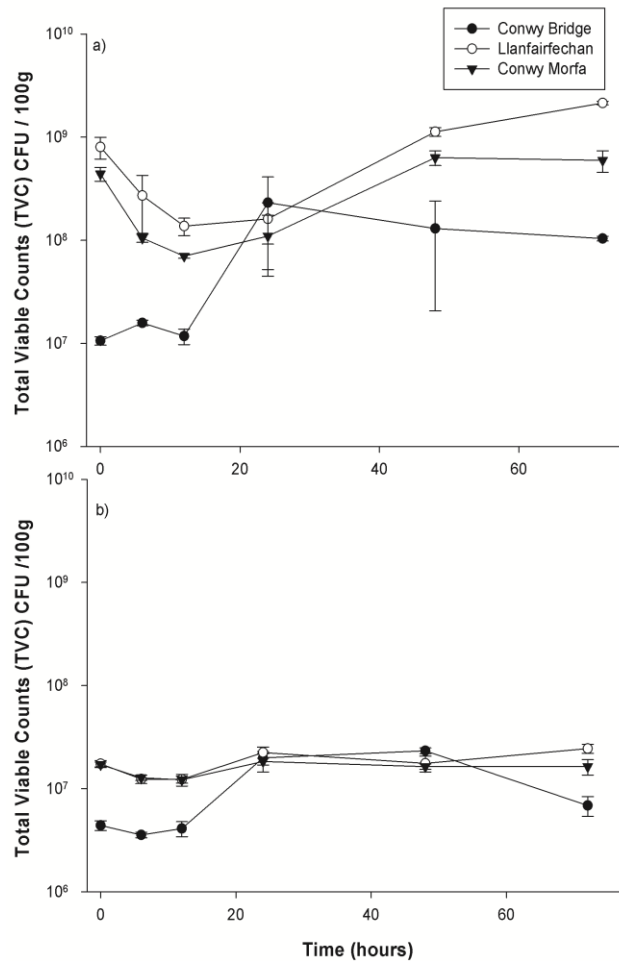


Fig. 4.3. Total viable count (TVC) of the marine heterotroph population observed in both a) mussels and b) barnacles from three commercial shellfish beds held over a 72 hour period in a depuration facility. In all cases $n = 3$ with the exception of time point '0' where $n = 15$. Data points represent the mean \pm Standard Error.

Calculations of the elimination rate of total coliform bacteria from both mussels and barnacles over a 48 hour depuration period (Table 4.1) show that across all sites, barnacles eliminated total coliform bacteria at an increased rate compared to the corresponding mussels. For both the Conwy Bridge and Conwy Morfa site the rate of bacterial elimination was five times higher in barnacles than in mussels.

Table 4.1. Comparison of the elimination rate of total coliform bacteria for mussels and barnacles from three commercial shellfisheries, over a 48 hour depuration procedure.

Coliform elimination rate (CFU / h)		
Shellfishery	Mussel	Barnacle
Conwy Bridge	51.5	294.2
Llanfairfechan	6.3	14.0
Conwy Morfa	23.7	118.7

4.3.3 Ecological Assessment

Of the three sites surveyed; Conwy Morfa and Conwy Bridge retained very similar ecological characteristics (Table 4.2). Conwy Bridge and Conwy Morfa had significantly higher mean mussel weights compared to Llanfairfechan ($P \leq 0.001$), but lower mean barnacle weight per mussel ($P \leq 0.05$; Table 4.2). The mussel-to-barnacle weight ratio was higher at Llanfairfechan compared to Conwy Bridge and Conwy Morfa.

The total coliform concentrations were significantly higher in barnacles than in mussels ($P \leq 0.001$; Table 4.2 and the relative contribution of barnacles to the total coliform concentration of both mussels and barnacles ranges from 79 - 99%.

Table 4.2. Ecological evaluation and bacteriological comparison of three commercial mussel (*M. edulis*) beds. In all cases n = 15, bracketed numbers represent the Standard Error.

	Shellfishery		
	Conwy Bridge	Llanfairfechan	Conwy Morfa
Mussel weight (g/mussel)	24.4 (± 1.1)	14.4 (± 1.2)	24.8 (± 1.7)
Total barnacle weight (g/mussel)	7.7 (± 0.7)	12.9 (± 1.5)	7.6 (± 0.9)
Mussel : Barnacle weight ratio	3.17 : 1	1.1 : 1	3.3 : 1
Mussel coliforms (CFU/100 g)	2475 (± 654)	302 (± 130)	715 (± 526)
Barnacle coliforms (CFU/100 g)	14349 (± 9503)	1138 (± 443)	5912 (± 2107)
Total coliform concentration (mussel + barnacle) (CFU/100 g)	16825 (±7101.4)	1440 (±116.5)	5944 (±638.0)
Total barnacle contribution (%)	85	79	99

4.4 Discussion

Our findings agree with previous research (Clements et al. 2013) showing that at the time of harvesting approximately 80% of the total coliform population was contained within the epizoic barnacles. Our findings also indicate that a standard depuration procedure of 42 hours, in accordance with European legislation (EU 2004a,b) was sufficient to eliminate the total coliform bacteria from mussel flesh, but insufficient in reducing the total coliform concentration within barnacles to beneath the accepted levels. This has important implications for the shellfish industry, particularly with regard to potential re-contamination of purified bivalve shellfish by coliforms and other microbial pathogens during transit, storage, and at the point of retail.

Coliform elimination rate over the depuration process were shown to be higher in barnacles compared to mussels, however, it is hypothesised that this is a direct result of higher initial bacterial concentrations within the barnacles. Mcleod et al. (2009) demonstrated that in oysters the initial pathogen (poliovirus) concentration positively correlated with the rate of elimination when compared to other pathogens such as norovirus and hepatitis A virus, which accumulated at lower concentrations and was eliminated more slowly from the oyster tissues. Further research needs to be undertaken to fully investigate the bacterial elimination efficiency of bivalve shellfish and epizoic barnacles and assess the potential impact (if any) to the shellfish industry.

Whilst it has already been established that epizoic barnacles can act as a potential bacterial reservoir *in situ* on shellfish beds (Clements et al. 2013), their potential to re-contaminate harvested shellfish remains unknown. The depuration process has been shown to be effective in the elimination of bacteria such as *Escherichia coli*, total coliforms, and faecal streptococci from bivalve shellfish (Marino et al. 2005; Barile 2009; Cusson et al. 2005). Research into the effectiveness of the depuration process on the elimination of bacteria and viruses from epizoic organisms is limited. The reason for this is two-fold; firstly, prior to entering depuration the majority of the epizoic organisms are removed, and secondly the epizoic organisms themselves are not consumed directly, nor are they of any commercial value. Recent years have seen a shift in consumer preference for more “natural looking” shellfish which are perceived to be more “wholesome and nutritious” (Acebron & Dopico 1999). Little evidence exists to support this perception; however, in a market-driven industry many shellfish producers have adapted their post harvesting procedures to cater for the consumer demand and bivalve shellfish are increasingly sold complete with epizoic organisms. Although not

directly consumed, the findings from this study suggest that epizoic organisms still represent a significant bacterial reservoir post-depuration and could pose a risk to consumers via cross-contamination during handling and surface contact in food processing environments or domestic kitchens.

Current legislation (EU 2004a,b) focuses only on quantifying the bacterial content of the bivalve shellfish flesh, through the use of indicator organisms, in order to minimise any risk to public health, as due to their filter feeding mechanisms they are potentially able to accumulate pathogenic microorganisms (Whittman & Flick 1995; Sunnotel et al. 2007). Other potential sources of contamination post-harvest, such as storage in contaminated water and sediment contamination (Clegg & Sherwood 1947), have been identified and protocols developed to minimise the risk of re-contamination (CEFAS 2008). Until recently epizoic barnacles had not been considered as a potential source of contamination of harvested shellfish, but, containing over 80% of the total coliform population it is important to understand the effects of adding these animals into commercial depuration systems.

Research into the effectiveness of various different methods of sterilisation during depuration is abundant (Crocì et al. 1992; Correa et al. 2007; Xu et al. 2002). What remains unclear is how well these methods perform when challenged by the addition of epizoic barnacles, an approximate 80% increase in initial bacterial loading. Whilst depuration may be effective at eliminating bacteria from bivalve shellfish, the results of this study show that it has limited effectiveness at eliminating bacteria held within the epizoic barnacles. The bacterial flux, post-harvest, between the two species is also poorly understood and although no evidence is presented here, there is the potential for bacteria eliminated from the barnacles to be taken up by the shellfish during the depuration process. The results of this study highlight the need for extensive research to be conducted into the efficiency of the current depuration process when challenged by bivalve shellfish complete with their epizoic organisms. Further research is also necessary to understand the *in situ* bacterial flux between bivalve shellfish and their associated barnacles within commercial depuration systems.

Our findings also indicate that not all bacterial species can be successfully depurated from either bivalve shellfish or their associated barnacles. Whilst depuration was successful in reducing the coliform concentration from both shellfish types, the depuration process failed to reduce the concentrations of *Vibrio spp.* and marine heterotrophic bacteria in both shellfish types. Extensive research has been conducted into the suitability of indicator species such as *E. coli* and coliforms (Field & Samadpour 2007; Teplitski et al. 2009; Muniain-Majika et al. 2002; Wolf et al. 2008; de Mesquita et al. 1991). Research has concluded that these species are poor

indicators for pathogenic bacteria species such as *E. coli* O157:H7 and pathogenic *Vibrio* species such as *Vibrio parahaemolyticus* (Barile 2009) and viruses such as Norovirus and Hepatitis A (Pommepuy et al. 2002). Nevertheless, research efforts have also failed to identify a suitable alternative indicator species and thus, current legislation still utilises *E. coli* as an indicator for contamination (Romalde et al. 2002).

Much debate exists over the depuration time frame. In England and Wales current legislation stipulates that shellfish must be held within depuration systems for a minimum of 42 hours (EU 2004b). Although for practical purposes many shellfish producers operate their depuration systems for 48 hours. Whilst the 42 hour time frame has proven to be effective at reducing the concentration of bacterial indicator organisms within bivalve shellfish, it has also proven to be ineffective at reducing concentrations of other bacterial species such as *Vibrio spp.* and salmonella (Marino et al. 2005; Barile 2009) and viruses such as adenoviruses (Hernroth & Allard 2007). Likewise, this study has shown that whilst the 42 hour depuration timeframe was more than sufficient to remove coliform bacteria from within bivalve shellfish flesh, it was not sufficient to reduce the coliform concentration within the barnacle flesh. It could then be argued that in the interests of shellfish safety, the depuration time should be extended for bivalve shellfish depurated complete with their epizoic barnacles.

The findings from this study also support the notion that a degree of site specificity exists between different shellfish beds. Previous research has shown that the barnacle species differed significantly between shellfish beds (Clements et al. 2013) and the results from this study support this conclusion. This study shows that the degree of 'fouling' i.e. the mussel to barnacle weight ratio is not representative of bacterial contamination levels found within the barnacles. Bacterial concentrations within the barnacle flesh are more likely to be dependent on ecological and anthropogenic factors that differ between shellfish harvesting areas.

In conclusion, the current depuration procedure is effective for reducing the concentration of bacterial indicator species from bivalve shellfish flesh; however, it is insufficient in reducing the concentration of bacterial indicator species within epizoic barnacle flesh to beneath acceptable levels. Barnacles have been shown to eliminate bacteria more effectively over the course of the depuration procedure than their corresponding bivalve shellfish. It is likely that this is due to the elevated initial concentrations. This study raises questions over the effectiveness of the standard depuration procedure when challenged with higher initial bacterial loading, due to the significant bacterial reservoir contained within epizoic barnacles. It also questions whether current legislative standards are suitable for bivalve shellfish entering into depuration complete with epizoic barnacles. Finally, this study also highlights the

need for further research into the bacterial flux between bivalve shellfish and epizoic barnacles within the depuration system and to determine if the epizoic barnacles are capable of secondarily contaminating purified shellfish post depuration.

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CHAPTER 5

Assessment of the potential for offshore shellfish cultivation to mitigate against bacterial and viral contamination

KATIE CLEMENTS,^{1†} JAMES B. WINTERBOURN,^{2†} LUIS GIMÉNEZ,¹ DAVID L. JONES,² SHELAGH K. MALHAM³ AND JONATHAN W. KING³

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

Running head: Assessing the potential of offshore shellfish cultivation

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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 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, relocation offshore may offer an alternative mitigation strategy against shellfish contamination. The objective of this study was to identify the effect of distance 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 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*,

5.1 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-vectoring 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 vectoring 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 Directive) 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-vectoring 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-vectoring 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. 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 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 offshore cultivation 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 accumulated within common mussels (*Mytilus edulis*) experimentally deployed 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 would both reduce with distance from shore. Parallel water samples were collected to investigate whether concentrations of FIB in mussels correlate with those in water to help explain any effect of distance observed along the transect; *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 cultivation of *Mytilus edulis* with respect to survival and growth. These four objectives were ultimately designed to help guide the optimal location for offshore shellfish cultivation in terms of balancing shellfish biomass with pathogen reduction potential.

5.2 Materials and methods

5.2.1 Sampling Location

A single, linear transect, was established in February 2012, running 12 km north from the Great Ormes Head (North Wales, UK; Fig. 5.1). 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.

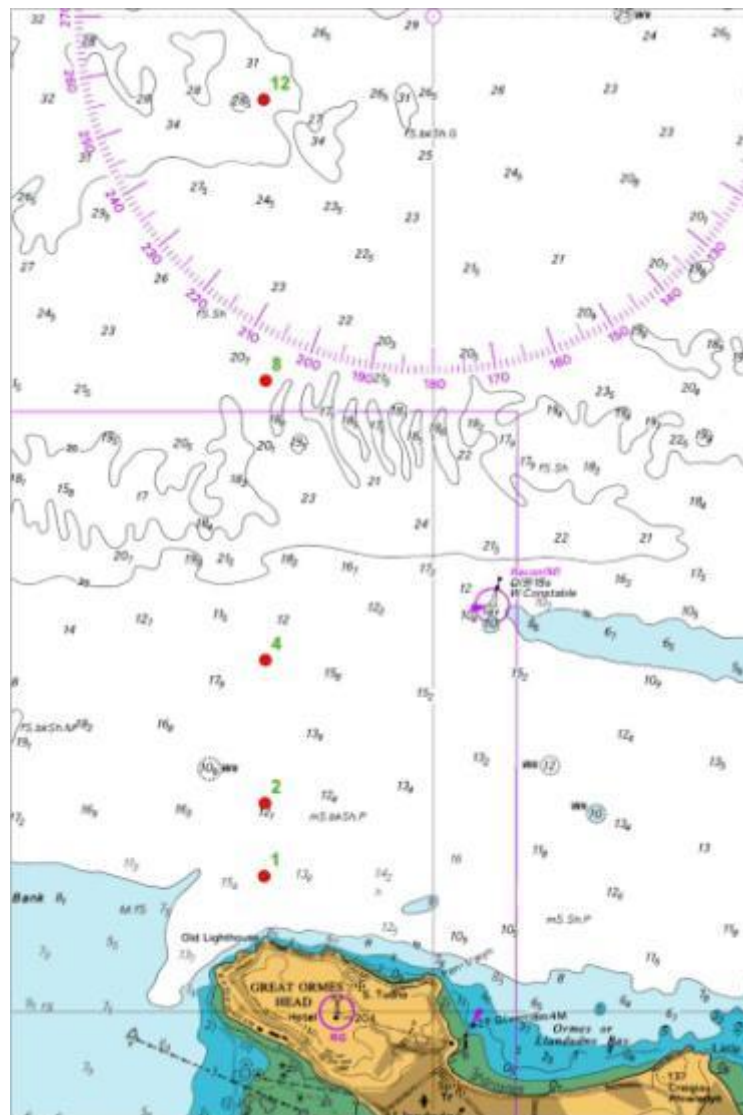


Fig. 5.1. Map showing the location of the offshore transect. Sampling points are represented by filled circles and numbered according to their respective distance offshore (in km).

5.2.2 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.

5.2.3 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.

Concurrently, at each monitoring point, three replicate 1.5 L water samples were collected from 1 m depth, in sterile containers, for the determination of bacterial concentrations within the surrounding sea water. Water samples were stored and transported at 4°C and processed within 6 h of collection.

5.2.4 Determination of bacterial concentrations in mussels

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.

5.2.5 Determination of bacterial concentrations in seawater

Determination of total coliform concentrations in seawater samples was based on the procedure described in (Quilliam et al. 2011). Briefly, 300 ml was aseptically vacuum filtrated through a 0.2 µm cellulose acetate membrane (#11107-47-N Sartorius Stedim Biotech, Epsom, Surrey, UK). Membranes were removed aseptically and placed onto a plate containing M-endo LES agar (#MM0551 Oxoid, Basingstoke, UK). Plates were inverted and incubated at 37°C. Total coliform colony forming units (CFU) were enumerated after 24 h.

5.2.6 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 vMC₀ 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).

5.2.7 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.

5.2.8 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 Kruskal-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.

5.3 Results

5.3.1 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 \pm SE) and 6540 ± 1021 respectively to below quantifiable limits for both GI and GII in 9 out of 10 replicate samples. GI was detected $< \text{LOQ}$ 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 5.1).

Table 5.1 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 $n = 10 \pm$ Standard Error (SE).

	Length of Deployment	
	(T_0)	(T_{28})
Bacterial concentrations		
<i>E. coli</i> (CFU / 100g)	1400 \pm 470.2	0.00 \pm 0.00
Coliforms (CFU / 100g)	13350 \pm 2751.8	0.00 \pm 0.00
Viral concentrations		
NoV GI (gc / g)	163.59 \pm 17.57	100% $< \text{LOQ}$ (33% N.D)
NoV GII (gc / g)	6540 \pm 1021.0	90% $< \text{LOQ}^*$ (33% N.D)

* 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*.

5.3.2 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 or collisions with marine traffic.

5.3.3 Bacterial concentrations in mussels and seawater

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. 5.2a). *E. coli* was not detectable at any distance offshore after 28th March 2012 (Fig. 5.2a).

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. 5.2b). 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. 5.2b). 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. 5.2b).

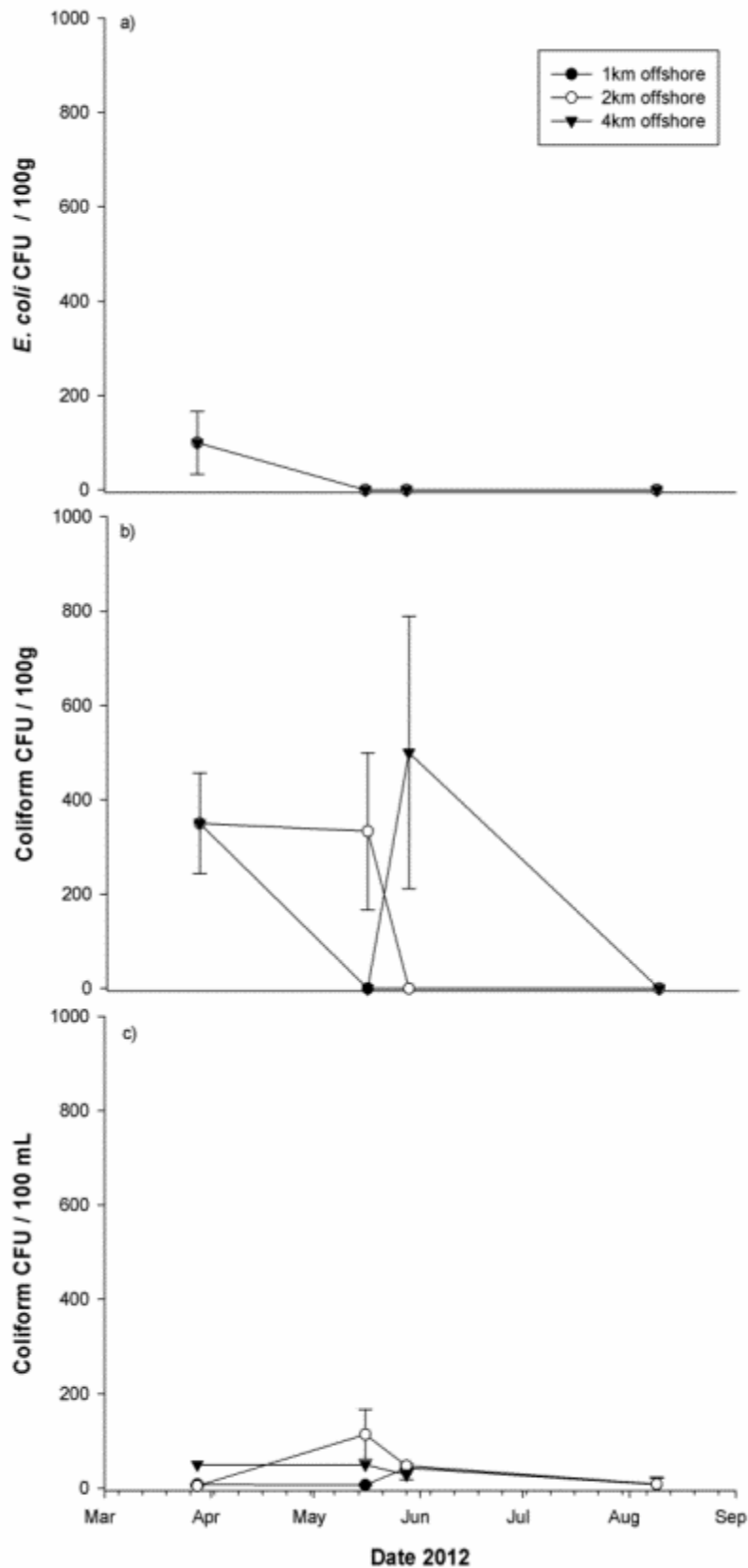


Fig. 5.2. Concentrations of (a) *E. coli* and (b) total coliforms observed in offshore relayed mussels from differing distance offshore over 124 days. For comparison (c) total coliform concentration observed in seawater at differing offshore distances over 124 days. In all cases $n = 3$ where the data points represent the mean \pm Standard Error (SE).

Total coliform concentrations within mussel tissue was strongly positively, but not significantly, correlated with total coliform concentrations in seawater ($r = 0.83$; $P = 0.653$; Fig. 5.2c). Total coliform concentrations in seawater varied over different offshore distances (Fig. 5.2c). At T_0 no significant difference in coliform concentration was observed between 1 km and 2 km offshore ($P = 0.275$), however, total coliform concentrations were significantly greater at 4 km offshore, relative to concentrations at both 1 km and 2 km ($P = 0.037$ in both cases). On 16th May 2012, total coliform concentrations differed significantly between both 1 and 2 km offshore ($P = 0.043$) and between 1 and 4 km offshore ($P = 0.043$), but did not differ significantly between 2 and 4 km offshore ($P = 0.099$) with the highest concentration observed at 2 km and the lowest observed coliform concentration at 1 km offshore. No significant differences in total coliform concentrations at different offshore distances were detected on either 28th May 2012 or 9th August 2012 ($P \geq 0.05$ in all cases).

Total coliform concentrations observed in seawater were all below the maximum threshold for “excellent” water quality (2500 *E. coli* CFU L⁻¹) as defined by the revised bathing water directive 2006/7/EC (EU 2006).

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 \pm SE of 830 \pm 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.

The high frequency of results below the LOQ prevented any quantitative statistical analysis. However, the data for each genogroup were interpreted using three categorical qualities; 1) detected above LOQ, 2) detected below LOQ, and 3) Not detected (Fig. 5.3).

For the first sample collection of 16th May 2012, within the three replicate samples for each site, non-detection of GI NoV occurred with increasing frequency as the distance variable increased. GI NoV was not detected in any replicate at 4 km but was detected <LOQ in one replicate sample at 2 km and two replicate samples at 1 km offshore (Fig. 5.3a). For GII NoV,

non-detection occurred exclusively at 4 km offshore and did so in 3/3 replicate samples. At both 1 and 2 km offshore, GII NoV was detected at quantifiable levels in 1/3 replicates (146 gc g⁻¹, 114 gc g⁻¹ respectively) and at <LOQ levels in other replicate samples (Fig. 5.3b).

For the sample collection of 28th May 2012, GI NoV was detected <LOQ in all three replicate samples at 1 km offshore. Non-detection occurred in 1 of three replicate samples at both 2 and 4 km moorings with GI NoV being detected <LOQ in all other replicates (Fig. 5.3a). Non-detection of GII NoV on this date occurred in 2/3 replicate samples at the 4 km mooring but was detected <LOQ in the third replicate at the 4 km mooring and in all replicate samples from 1 and 2 km moorings (Fig. 5.3b).

For the sample collection 9th August 2012, NoV GI was detected <LOQ in all 3/3 replicates at both 1 and 2 km moorings (Fig. 5.3a). The mooring at 4 km could not be located. NoV GII was not-detected (1/3 replicates) and detected <LOQ (2/3 replicates) with the same frequency at both 1 and 2 km moorings (Fig. 5.3b).

Three replicate samples for each site / collection event, intended to allow for calculation of error in quantitative statistical analysis, provided insufficient data for categorical analysis. Chi-square test of association could not determine the distance to be associated with categorical detection statuses for either genogroup due to low expected counts for each contingency.

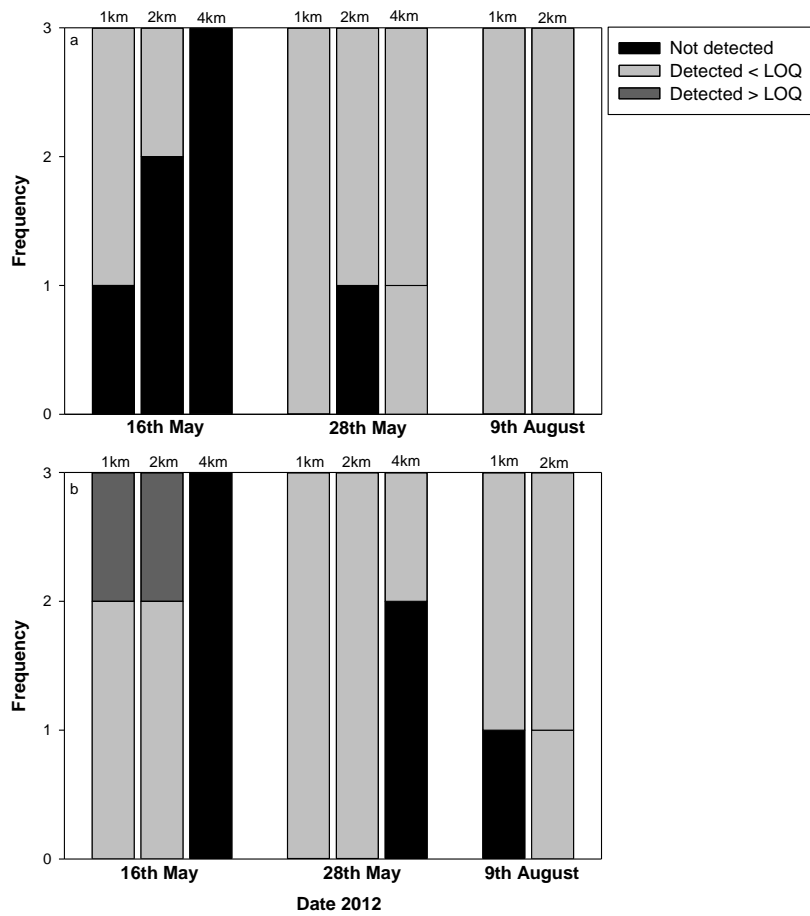


Fig. 5.3. Results for (a) norovirus GI and (b) norovirus GII detected in replicate mussel samples from different offshore distances for each time point. Data are shown as frequencies of detection status. In all cases $n = 3$.

5.35 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. 5.4) 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. 5.4). 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. 5.4). 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. 5.4).

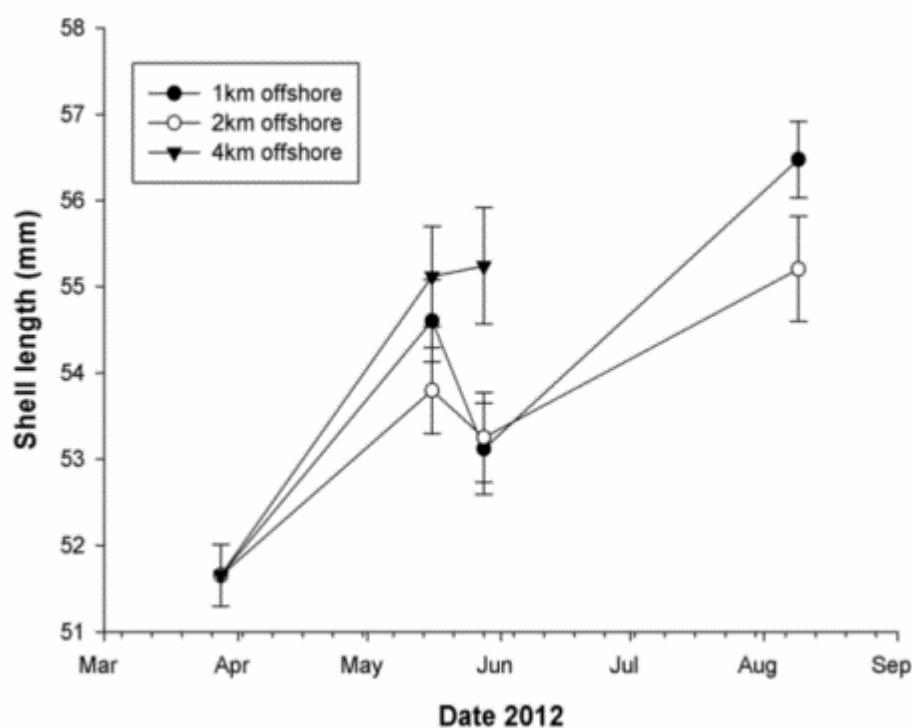


Fig. 5.4. Mussel shell length as observed in offshore relayed mussels from differing distance offshore over 124 days. $n = 60$ (with the exception of 28th March 2012 where $n = 100$). The data points represent the mean \pm Standard Error (SE).

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. 5.5). 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. 5.5).

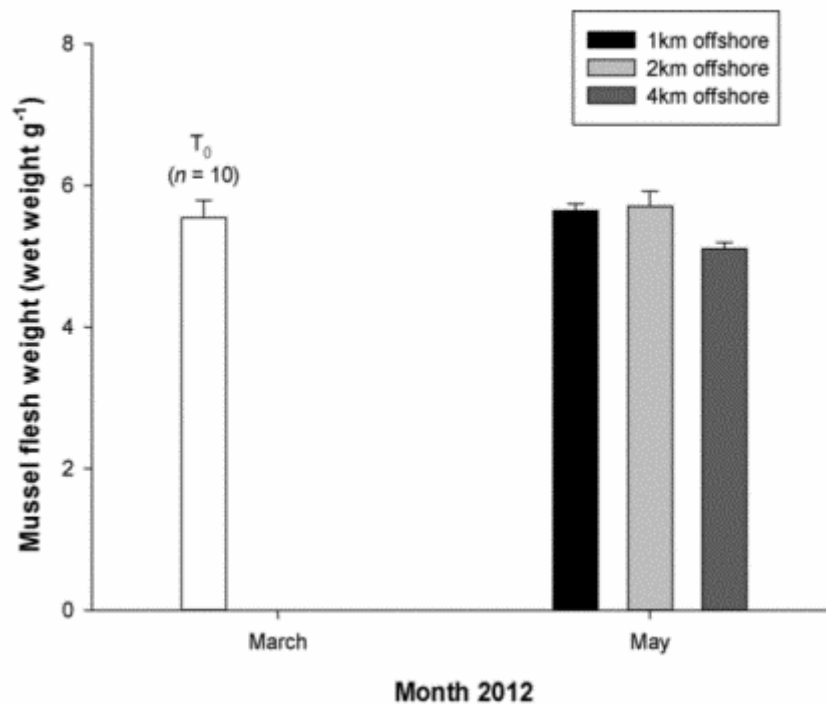


Fig. 5.5. Mussel flesh weight measured pre-deployment on 28th March 2012 and post-deployment on 28th May 2012 over differing distance offshore. In all cases $n = 5$ except at T₀ where $n = 10$. Data points represent the mean \pm Standard Error (SE).

At all points along the transect, mussel mortality was low. Mussels held 2 km offshore demonstrated the highest percentage mortality compared to mussels held at both 1 km and 4 km offshore. Mussel mortality was approximately 10% higher at 2 km offshore compared with mussels 1 km offshore on the 9th August 2012 (Fig. 5.6) and approximately 6% higher compared to mussels held at 4 km on 28th May 2012.

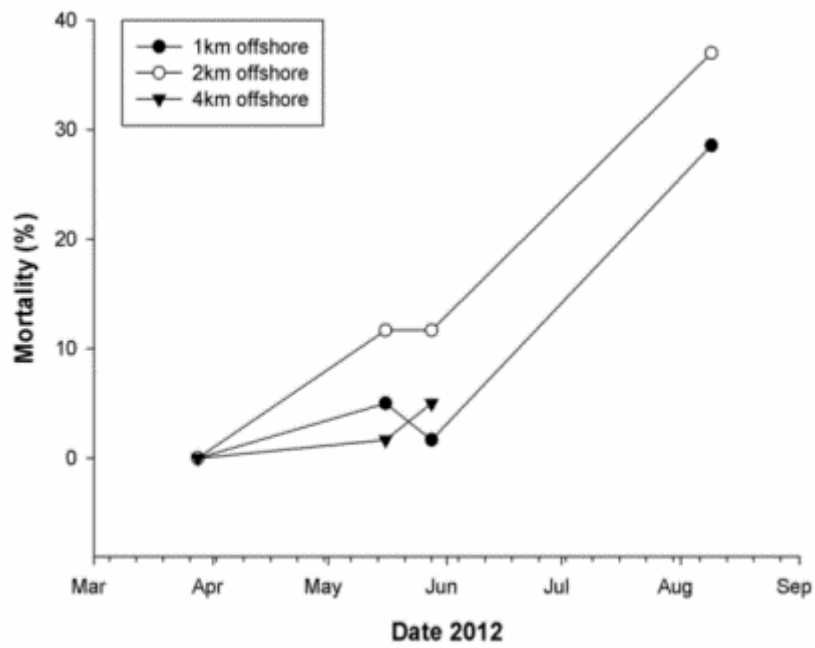


Fig. 5.6. Percentage mortality observed in offshore relayed mussels from differing distance offshore over 124 days. In all cases $n = 20$ (with the exception of 28th March 2012 where $n = 100$). The data points represent the mean \pm Standard Error (SE).

5.4 Discussion

5.4.1 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. Total coliform levels in mussel tissue did appear to correlate with total coliform levels derived from the immediate surrounding waters; however, this relationship did not prove statistically significant.

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 production 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, and that adopted herein, 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.

One observation 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: 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 contamination 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.

5.4.2 M. edulis productivity after offshore deployment

Assessments on survival and growth of offshore relocated *M. edulis* indicated low percentage mortality 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.

5.4.3 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 (Gouletquer 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).

5.4.4 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, 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 cultivation 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 cultivation of mussels in the selected area may be sufficient to mitigate against both FIB and NoV contamination. 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 cultivation 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, however, 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.

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CHAPTER 6

Use of *Mytilus edulis* biosentinels to evaluate spatial contamination patterns of norovirus and faecal indicator organisms in close proximity to a coastal sewage discharge.

JAMES B. WINTERBOURN^{*†}, KATIE CLEMENTS^{2†}, JAMES LOWTHER⁴, SHELAGH K. MALHAM², JAMES E. MCDONALD³, DAVEY L. JONES¹

¹*School of the Environment, Natural Resources & Geography, Bangor University, Bangor, Gwynedd, LL57 2UW, 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*

† Authors contributed equally.

*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

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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.

6.1 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 partially or untreated 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.

6.2 Materials and methods

6.2.1 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 in 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.

6.2.2 Site selection.

The offshore submarine sewage outfall pipe at Kimmel Bay, North Wales (53.336901N, 3.569200W (WGS84); Fig. 6.1), which serves a total population equivalent of 77,953 people, was selected for this study. The discharge is consented for up to 38,860 m³/d with a dry weather flow not exceeding 15,941 m³/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 log₁₀ 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.

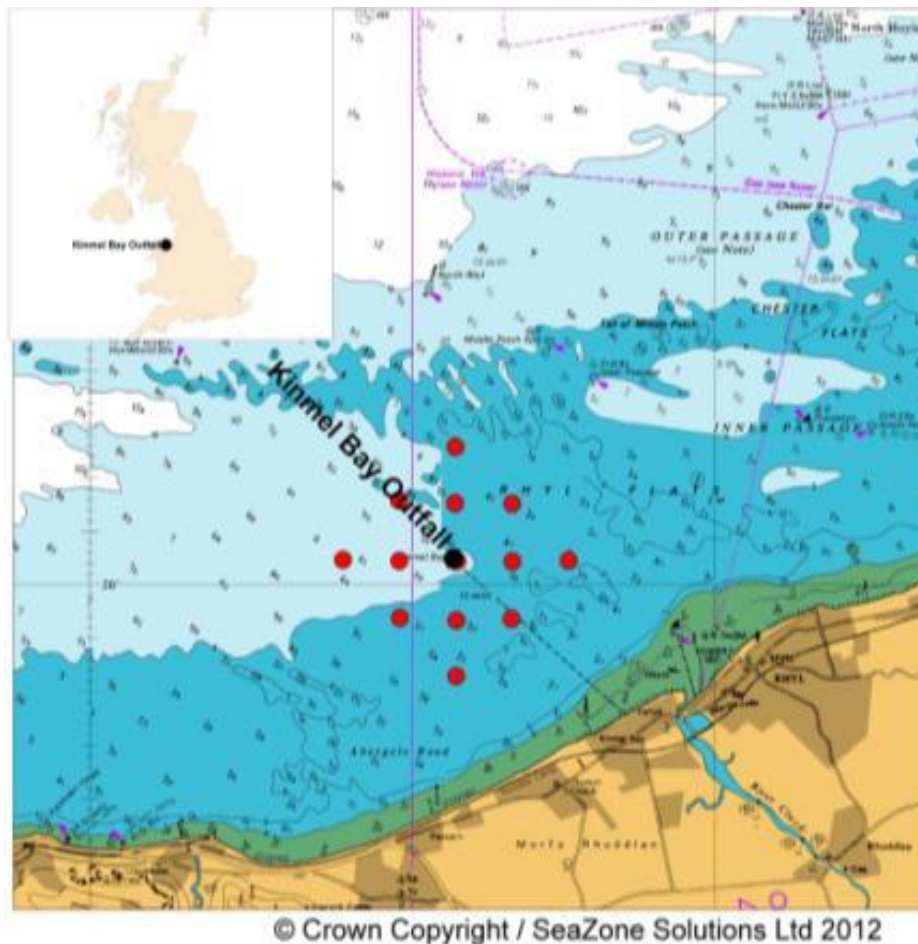


Fig 6.1. Map showing the location of the study site and location of the outfall.

6.2.3 Sampling Regime and Shellfish Biosentinels.

A diamond-shaped array of 13 independent sampling points was selected based on model simulations of sewage plume behaviour (Fig. 6.1). 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.

6.2.4 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 vMC₀ 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 (sequences and cycling parameters in supplementary information, Tables 1 and 2, respectively). 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 vMC₀ 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).

6.2.5 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). 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.

6.2.6 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.

6.2.7 Hydrodynamic Model.

A hydrodynamic simulation model was already available which described the dispersion of the effluent plume from the Kinnel Bay offshore outfall. The model was prepared by Metoc (Intertek Ltd, Liphook, Hampshire) for Welsh Water (Dŵr Cymru Ltd, Treharris, UK) who are responsible for maintaining the Kinnel 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 m³/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. 6.1). 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.

6.3 Results

6.3.1 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 6.1. 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 the on-line supplementary information (Table S1).

Table 6.1 Baseline (T_0) levels of NoV GI, GII, *E. coli* and total coliforms in mussels.

Target	Value
NoV GI	52.2 ± 6.29
NoV GII	3311 ± 167
<i>E. coli</i>	400 ± 163
Coliforms	3400 ± 670

NoV GI and GII are expressed as detectable genome copies/ g digestive gland. *E. coli* and coliforms are expressed as CFU / 100g shellfish flesh and intravalvular fluid. $n = 10$ in all cases.

6.3.2 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 g-cop/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 $20,833 \pm 1764$ 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 FIOs in mussel samples (Fig. 6.2). 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.

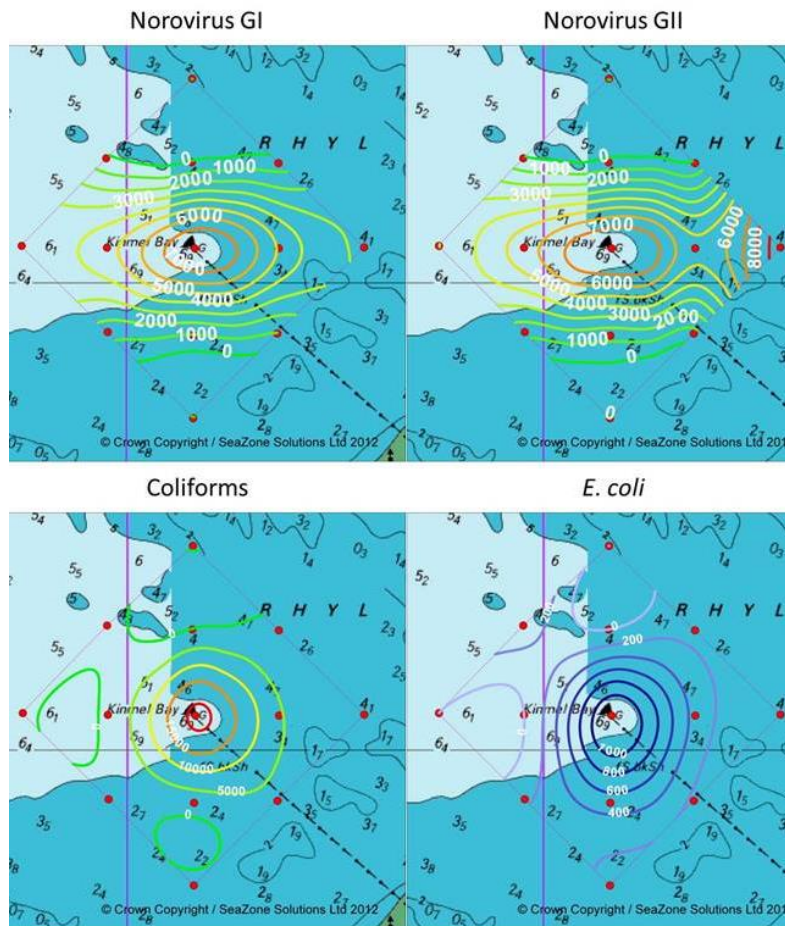


Fig. 6.2. Contour plots showing the concentrations of norovirus GI, GII, *E. coli* and coliforms from experimentally deployed mussels after 1 month in situ. For norovirus GI and GII, contours represent scored data as detectable genome copies / g digestive gland. ‘Not detected’ scores 20 gc / g. 1-100 (<LOQ) scores 50 gc / g. $n = 1$. For coliforms and *E. coli*, contours represent the mean CFU / 100 g shellfish flesh and intervalvular 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, 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 6.2. 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.

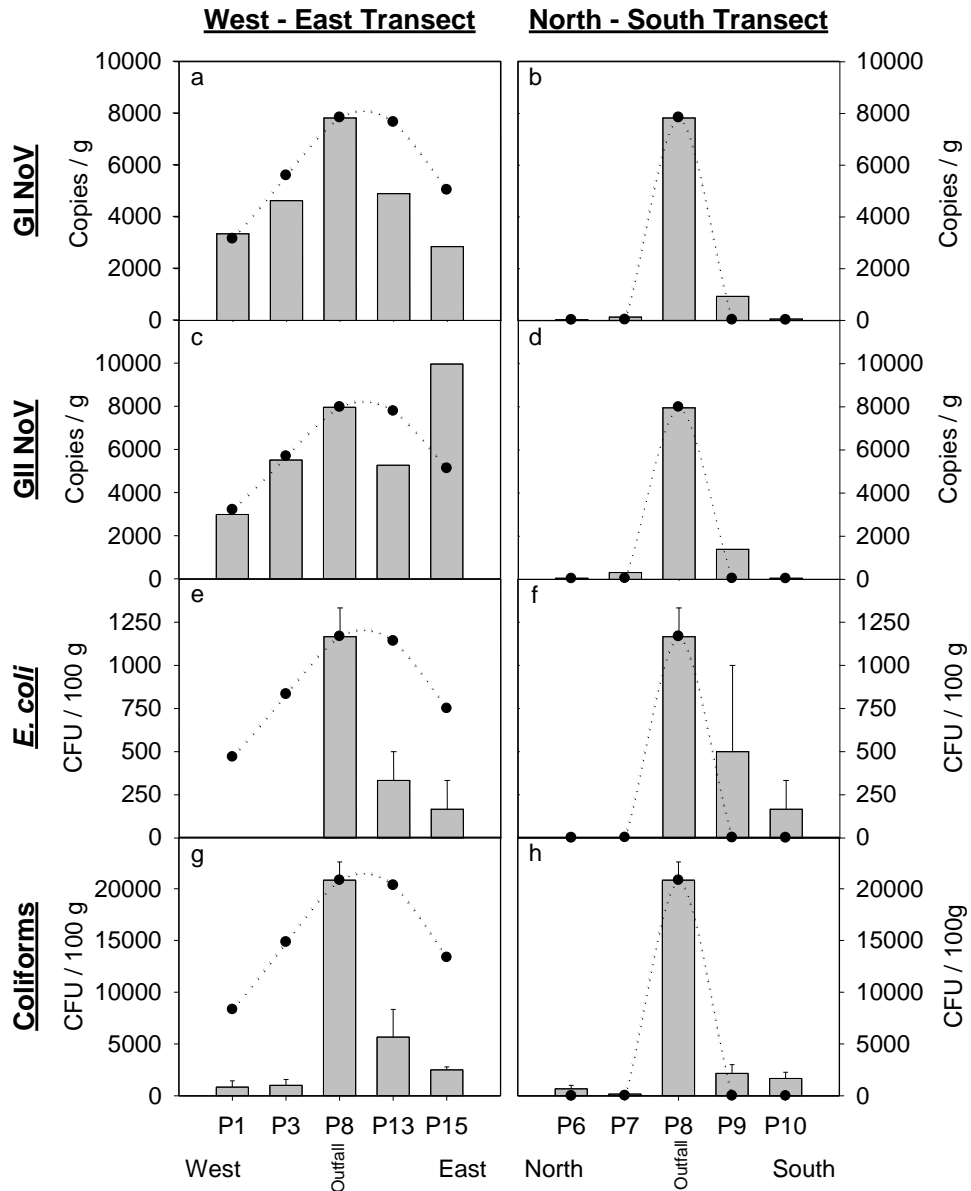


Fig. 6.3. Distance between adjacent sites 1km. For NoV GI and GII bars represent scored data as detectable genome copies / g digestive gland. Not detected scores 20 gc / g. 1-100 (<LOQ) scores 50 gc / g. For *E. coli* and coliforms bars represent mean CFU / 100 g shellfish flesh and intravalvular fluid. 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.

6.3.3 Comparison of experimental results with hydrodynamic model predictions.

Spearman's rank-order correlation coefficients (r) 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 6.2). 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.

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. 6.3). 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. 6.3c). 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 6.3ac). 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. 6.3eg). However, higher levels than the model would predict were found to the South (onshore) of the outfall (Fig. 6.3fh).

Table 6.2 Correlation co-efficient matrix showing the Spearman's rank -order correlations between NoV GI, GII, *E. coli*, coliforms and the predicted values of the model.

	<i>E. coli</i>	Coliforms	NoV GI	NoV GII	Model
Coliforms	.747** .003	-	-	-	-
NoV GI	.296 .326	.601* .030	-	-	-
NoV GII	.220 .470	.543 .055	.905** <.001	-	-
Model	.217 .477	.349 .242	.779** .002	.752** .003	-

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed). Numbers in bold represent the spearman's rank order correlation co-efficient (R) values beneath represent the level of significance (P).

6.4 Discussion

6.4.1 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 be 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.

6.4.2 NoV contamination patterns.

A relatively high T_0 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 <20 g-cop/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. 6.1), 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. 6.1) 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. 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.

6.4.3 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 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 / re-suspension 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.

6.4.4 NoV GI and GII ratios.

To 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*.

6.4.5 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.

6.5 Acknowledgements

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SUPPLEMENTARY INFORMATION

Table S1 - Primer Sequences

GI PRIMERS AND PROBE

IFRG1 FWD	CGC TGG ATG CGN TTC CAT
NEDG1 REV	CCT TAG ACG CCA TCA TCA TTT AC
TM9 PROBE	TGG ACA GGA GAT CGC

GII PRIMERS AND PROBE

IFRG2 FWD	ATG TTC AGR TGG ATG AGR TTC TCW GA
COG2R	TCG ACG CCA TCT TCA TTC ACA
IFRG2-TAMRA PROBE	AGC ACG TGG GAG GGC GAT CG

MENGO VIRUS PRIMERS AND PROBE

MENGO FWD	GCG GGT CCT GCC GAA AGT
MENGO REV	GAA GTA ACA TAT AGA CAG ACG CAC AC
MENGO PROBE	ATC ACA TTA CTG GCC GAA GC

N=A/T/C/G, R=A/G, W=A/T

TM9 probe and Mengo probe labelled 5' 6-carboxyfluorescein (FAM), 3' MGB (minor groove binder)

IFRG2-TAMRA probe labelled 5' 6-carboxyfluorescein (FAM), 3' carboxytetramethylrhodamine (TAMRA)

Table S2 - Cycling parameters

Step description	Temperature and time	Number of cycles
RT	55 °C for 1 h	1
Preheating	95 °C for 5 min	1
	Denaturation	95 °C for 15 s
Amplification	Annealing	60 °C for 1 min
	extension	65 °C for 1 min

Table S3. 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 g-cop/g. GI was not detected in 1/10 replicates, which was scored with 20 gen-c/g.

Table S4. 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	334	230	46	1453	20	127	7825	928	50	50	490	4899	964	2853
GI	5		31		[0]				[35]	[15]				
NoV	298	150	55	1367	50	317	7954	1392	50	114	187	5264	662	9958
GII	3		08		[11]				[75]					
<i>E. coli</i>	0	333±	0	167	0	0	1167±	500	167	0	167	333±	167	167
		333		±16			166	±50	±16		±16	167	±16	±16
				7				0	7		7		7	7
Colif	833	1500	10	1667	667	167	20833	2167	1667	1667	667	5667	4000	2500
orms	±60	±289	00	±33	±33	±16	±1764	±83	±60	±88	±16	±268	±50	±28
	1		±5	3	3	7		3	1	2	7	2	0	9
			77											

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 7

General Discussion

7.1 Discussion

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

- 1. Identification and quantification of bacterial reservoirs in commercial shellfish harvesting areas within North Wales, UK.**
- 2. Determination of the relative contribution of previously identified bacterial reservoirs in shellfish tissues under different mitigation strategies.**
- 3. Examination of the relationship between bacterial and viral reservoirs within shellfish tissues.**

Molluscan bivalve shellfish are a nutritious food source and an important component of the human diet (Potasman et al. 2002). However, as selective filter feeders, bivalve shellfish are capable of accumulating pathogenic micro-organisms that are present within the wider environment; these pathogenic organisms may then be transferred into the human food chain, vectoring illness amongst the human population (Burkhardt and Calci 2000). Current EU standards, which are in place to reduce the risk to consumers, focus only on the bacterial quality of the shellfish flesh, using faecal indicator bacteria (*E. coli*) as a proxy for pathogenic micro-organisms. Extensive research efforts have largely focused on determining the origins of microbial pathogens present within shellfish (Crowther et al. 2003, Kay et al. 2008, Stapleton et al. 2008, Kay 2009) and on quantifying the environmental and anthropological influences on the bacterial concentrations within shellfish tissues such as rainfall (Oliver et al. 2008), climate change (Laws et al. 2008), topography (Crowther et al. 2003), (DePaola et al. 2010), land use (Kay et al. 2005), and pollution events (Lessard and Beck 1990). A thorough understanding of the factors determining the bacterial accumulation within shellfish tissues can enhance both remediation efforts and consumer safety. However, alternative bacterial reservoirs in shellfish harvesting areas and the interactions between the bacterial communities present within the shellfish flesh and within the wider environment represent an area which is currently under-researched. The bacterial reservoir present within shellfish tissues is subjected to continual flux, accumulation is driven largely by anthropogenic and environmental factors but what remains largely unknown is the role of other environmental bacterial reservoirs within shellfish

harvesting areas, and whether these alternative reservoirs are acting as a source or sink for bacterial concentrations within shellfish tissues. Improving our understanding of the interactions between different bacterial reservoirs within shellfish harvesting areas will further enhance both remediation efforts and consumer safety.

This thesis investigated alternative bacterial reservoirs present within shellfish harvesting areas in North Wales, UK, and attempted to determine the interaction between these bacterial reservoirs and the bacterial content of commercial shellfish, the common or blue mussel (*Mytilus edulis*).

7.2 Identification and quantification of bacterial reservoirs in commercial shellfish harvesting areas within North Wales, UK

The uniqueness of each individual shellfish harvesting area makes it difficult to produce a generalised assessment of the bacterial reservoirs present and their relative influence on the bacterial content of the shellfish. Rather, a site-specific approach is needed to initially identify any potential bacterial reservoirs and then to examine the potential interaction between the external reservoir and the reservoir present within the shellfish tissues. This thesis focuses on one geographical region (North Wales) and attempts to identify and subsequently quantify environmental bacterial reservoirs present within *Mytilus edulis* harvesting areas.

7.2.1 To investigate the spatial and temporal changes in the bacterial reservoirs contained within shellfish tissues

In chapter 2 we conducted an intensive *in situ* survey of a single intertidal, commercial mussel (*Mytilus edulis*) bed to assess for both spatial and temporal variability of the bacterial reservoir present within the mussel flesh. The selected mussel bed is subjected to routine bacteriological monitoring in accordance with EU standards (Table 1.4), based on a small number of sampling points. We aimed to determine whether the classification assigned to the shellfish bed was representative of the level of contamination affecting the entire mussel bed and whether the bacterial concentrations of the mussel flesh were correlated with the position

of the shellfish on the bed. Secondly, we aimed to determine whether the bacterial reservoir within the mussels was influenced by seasonality or tidal state.

The findings from this study demonstrated that the *E. coli* concentrations within the mussel flesh did not exceed the upper limit (4,600 *E. coli*/ 100 g) of the EU standards in any of the samples analysed, however the distribution of *E. coli* over the entire mussel bed was 'patchy' and displayed clear 'hotspots' of contamination which were not correlated with the position of the mussels on the bed. Although none of the samples analysed exceeded the upper limit of the assigned classification, several samples returned results which were below the classification limit (230 *E. coli* / 100 g). Therefore, the location of the sampling points for routine bacteriological monitoring may not be representative of the overall level of bacterial contamination affecting the shellfish bed and the placement of routine monitoring points must, therefore, be carefully considered.

In accordance with previously published findings (Van Donsel et al. 1967, Wilson and Moore 1996, Formiga-Cruz et al. 2002), the concentrations of both *E. coli* and total coliforms displayed a clear seasonal pattern where concentrations of *E. coli* and total coliforms were five to ten times higher in the summer months than in the winter. In contrast to seasonality, the effect of the tidal state on the bacterial concentrations of shellfish flesh is under-researched. However this study demonstrated that whilst the bacterial concentrations of mussel flesh did not differ over a single tidal cycle i.e. 12 hours, the tidal state (spring / neap tide) did influence bacterial concentrations within the mussel tissues. This is most likely attributed to increased immersion times providing a greater opportunity for accumulation and / or depuration of bacteria from within shellfish tissues.

The findings from this study support the current monitoring protocols used to monitor bacterial contamination levels in commercial shellfish. However, the findings from this study are limited to a single intertidal shellfish bed only; further research needs to be conducted to determine whether the results obtained here are applicable to sub-littoral shellfish beds and whether they are also applicable in alternative geographical areas.

7.2.2 To investigate the relationship between the bacterial reservoirs present in the shellfish flesh and in the underlying sediments

In chapter 2 we investigated the role of sediments as a potential bacterial reservoir by determining the concentrations of *E. coli* and total coliforms from both mussel and underlying sediment samples across a single intertidal mussel bed. Sediments have previously been identified as a potential reservoir for bacteria (Martinez-Manzanares et al. 1992, Fries et al. 2008); however, the relationship between the bacteria present in the sediment and mussel flesh is poorly understood. The findings from this study indicated that no correlation existed between the bacterial reservoirs present in the sediment and within the mussel flesh. Bacterial reservoirs present in both the sediment and mussel flesh displayed ‘patchy’ distributions across the mussel bed, however the ‘hotspots’ were inconsistent. Areas of sediment that displayed higher bacterial concentrations did not correlate with bacterial ‘hotspots’ in the mussel flesh. The findings from this study did show that the underlying sediment acted as a much larger bacterial reservoir than the corresponding mussel flesh. The concentrations of *E. coli* were up to five times greater in the underlying sediments than in the corresponding mussel flesh, demonstrating the significance of the sediment as a bacterial reservoir. Molecular typing is required to see if these *E. coli* populations are related.

Although the findings from this study do not conclusively prove there is an interaction between the bacterial reservoirs present in the underlying sediment and corresponding mussels further work is needed in this area as this study has shown the bacterial reservoir contained within the sediment is significantly greater than that contained within the mussels. Therefore, it is important to ascertain the interaction (if any) between the bacterial reservoir in the sediment and within the mussels. Sediments may act as a source of bacterial contamination via sediment re-suspension (Lund-Hansen et al. 1997) or as a sink for bacteria produced from the mussels via the production of pseudo-faeces (Kooijman 2006). The environmental persistence of bacteria within the sediment is also an area which is currently under-researched; sediments may be capable of acting as an ‘historical’ reservoir for bacteria. Bacteria present in the environment may be accumulated into the sediment directly from the overlying waters following pollution events over a large time scale, these bacteria may then be re-suspended into the water column during periods of high hydro dynamical activity, effectively acting as a potential source of bacterial shellfish contamination.

Sediments have been documented as a potential contaminant for shellfish *ex situ* (CEFAS 2008); further work is urgently needed to determine their potential for shellfish contamination *in situ*. In addition, the survival and persistence of bacteria within sediments needs to be fully understood, as does the potential re-suspension rates of bacteria from different sediment types. Specifically, the distribution and persistence of bacteria and viruses within the oxic and anoxic sediment zones is required (i.e. is it just the surface contamination layer that is of significance for future monitoring campaigns?). The relative rates of bacterial turnover in sediments and the biotic and abiotic factors (e.g. climate, grazers) that control this also remain poorly characterised. This knowledge would help to extend the interpretation of current ‘point-in-time’ measurements (i.e. prediction of how populations will change over time).

7.2.3 To investigate the relationship between the bacterial reservoirs present in the shellfish tissues and in the surrounding water

In chapter 5 we directly compared the total coliform concentrations from mussel samples (suspended at 1 m depth, at different offshore locations, over selected time intervals) with the total coliform concentrations observed from the surrounding water. Mussels are filter-feeders capable of filtering up to 10 litres of water per hour (Teplitski et al. 2009). Therefore they are capable of bio-accumulating bacteria present within the surrounding water (Martinez et al. 2009). The findings from chapter 5 show that initially, the bacterial concentrations in the water were slightly higher (~230 coliform CFU per 100 ml) than the initial bacterial concentrations present within the mussel flesh (~100 coliform CFU per 100 g). Despite this, the results from this study concluded that in all cases (over both distance and time) the bacterial content of the mussels decreased as they were able to effectively ‘depurate’ their bacterial content and that this did not correlate with the observed bacterial concentrations present in the surrounding water.

It is possible that the water surrounding shellfish is capable of acting as both a source and also a sink for bacteria dependent upon the bacterial concentrations. Although the data is not presented in this thesis, in chapter 6 water samples were also taken in parallel to the mussel samples collected from around a known sewage outfall. Bacterial concentrations in the water were significantly higher than those observed in chapter 5, ranging from 200 - 1000 coliform CFU per 100 ml (dependent on the distance from the sewage outfall). Despite the significantly

elevated bacterial concentrations observed in the water, there was also no observed correlation with the coliform concentrations determined from mussel samples collected from the same locations.

The water surrounding shellfish can be considered a potential bacterial reservoir, however the interactions between the bacterial reservoirs in the water and within the shellfish needs further research. It is likely that the ability of water bodies to act as either as source or sink for bacteria respective to shellfish will be determined by a series of complex environmental factors including, but not limited to, the initial bacterial concentrations, flow rate, salinity, turbidity, turbulence and localised currents.

7.2.4 To investigate the potential for epizoic barnacles to act as a bacterial reservoir

In chapter 3 we investigated the potential of epizoic barnacles (barnacles attached to shellfish) to act as potential bacterial reservoir. In this study we examined the bacterial content of mussel and barnacle samples taken from three separate intertidal beds. The findings from this study indicated that the epizoic barnacles contained between 83.4 and 97% of the total coliform population present and as such, represent a more significant bacterial reservoir than the corresponding mussels. More interestingly, the results from this study also suggest that non-native barnacle species represent a larger bacterial reservoir than native species.

The findings from this study have important implications with regard to consumer safety. Although the barnacle species studied (acorn barnacles) are not directly consumed by humans, there is an increasing market for more ‘natural looking’ shellfish i.e. shellfish sold complete with attached barnacles (and other encrusting organisms). As this study has shown that barnacles represent a significant bacterial reservoir, there is the potential for barnacles to act as secondary contaminants to commercially harvested shellfish post-harvest (Sagoo et al. 2007). However, further work needs to be conducted in this area to examine the findings from this study on a commercial scale. This should also extend to include an investigation of consumer behaviour within kitchens and the likelihood for cross contamination within domestic homes and restaurants.

The idea that barnacles may serve to contaminate harvested shellfish is not new, it was first noted by Clegg and Sherwood in 1947; (Clegg and Sherwood 1947), however, this original

notion was never followed up, possibly as at the time there was no real necessity (as the convention was to remove the barnacles prior to retail). This study represents the first attempt to quantify the bacterial content of the epizoic barnacles and to demonstrate their potential as a significant bacterial reservoir on commercial shellfish beds.

Extensive research is urgently required in this area to determine the interaction between the bacterial reservoirs in both shellfish and barnacles. It is hypothesised that due to the different modes of feeding (suspension feeding in barnacles, versus selective filtration feeding in shellfish) barnacles may preferentially accumulate certain bacterial species, which may be pathogenic to humans. In addition the findings from this study indicate that a certain degree of site-specificity exists, therefore this study would need to be repeated over a larger number of sample sites in order to gain a better understanding of the significance of epizoic barnacles as a bacterial reservoir.

7.2.5 To investigate the relationship between different bacterial species present within shellfish tissues

Although current EU standards utilise faecal indicator bacteria (FIB) as a proxy for pathogenic organisms, there are other naturally occurring bacteria present within the shellfish harvesting areas which may be pathogenic to humans. In chapters 2, 3 and 4 we examine the relationship between faecal indicator bacteria (*E. coli* and total coliforms) and naturally occurring bacteria (*Vibrio spp.* and marine heterotrophs). In chapter 2 we examined the relationship between FIB and naturally occurring bacteria within mussel flesh *in situ* over a single mussel bed. No significant correlations were observed between the FIB concentrations and concentrations of presumptive *Vibrio spp.* and marine heterotrophs within mussel flesh. All bacterial species showed ‘patchy’ distributions, but these were not consistent across the mussel bed.

In chapter 3 we examined the differential uptake of FIB and naturally occurring bacteria in both mussels and barnacles. The findings from this study show that *Vibrio spp.* represents the majority of the total culturable bacteria, demonstrating their significance as part of the measurable bacterial community. It should be noted, however, that most marine organisms are not cultureable using standard plate technology and further metagenomic work is required to investigate other non-cultureable components of the microbial biomass within

shellfish, seawater and sediments. Interestingly, in both mussels and barnacles, those sites which showed the highest concentrations of FIB also showed the lowest concentrations of *Vibrio spp.* indicating that there may be an element of either bacterial competition between the bacterial species, or preferential accumulation by the host species.

In chapter 4 we examined the differential elimination of both FIB and naturally occurring bacteria (*Vibrio spp.* and marine heterotrophs) from both mussel and barnacle tissues. The results from this study show that after 48 hrs, FIB was successfully eliminated from mussel flesh and significantly reduced within the barnacles; however the concentrations of the naturally occurring bacteria were not successfully eliminated or reduced.

The findings from all of these studies suggest that a differential relationship exists between FIB and naturally occurring bacterial species, a finding which is supported by many studies (Murphree and Tamplin 1995, Canesi et al. 2005, Marino et al. 2005). Many pathogenic species of *Vibrio* exist (*Vibrio vulnificus*, *Vibrio parahaemolyticus*, and *Vibrio cholerae*) and the current EU standards applied in the monitoring of commercial shellfish beds (using FIB as a proxy) may be insufficient to safeguard consumers against the risk of vibrio related illness.

7.2.6 To investigate the relationship between selected nutrient and physico-chemical parameters and concentrations of faecal indicator bacteria in both shellfish tissues and sediments

In chapter 2 we investigated the relationship between the concentrations of *E. coli* and total coliforms within mussel flesh and sediment and selected nutrient and physico-chemical parameters. The findings from this study demonstrated that the selected nutrient parameters (total organic carbon, total nitrogen, nitrate, nitrite, ammonium and phosphorous) were positively correlated with the concentrations of FIB within the sediment, whereas, the concentrations of FIB within the mussel flesh demonstrated a negative relationship with increasing nutrient levels. FIB concentrations within the sediment demonstrated a significant positive association with salinity, but a significant negative association with pH levels. FIB concentrations in the mussels displayed the opposite trend, showing a significantly negative association with salinity and a positive association with pH levels.

The results from this study have important implications for the management of shellfish harvesting areas, as bacterial cells are capable of entering a dormant state, termed “viable but non-culturable or (VBNC)” when environmental conditions are not favourable (Oliver 2000). Contamination events affecting shellfish harvesting areas may not only increase the bacterial loading into shellfish harvesting areas, but may also increase nutrient levels (Lessard and Beck 1990) providing more optimum conditions for bacterial growth and ‘reactivating’ dormant bacterial cells. The findings from this study suggest that the bacteria present in the sediment reservoir are more susceptible to changes in nutrient concentrations than the bacterial reservoir within mussel flesh.

Bacterial cells present in the VBNC state are not detected by routine screening procedures and therefore an influx of nutrients from contamination events may provide a trigger for the ‘reactivation’ of bacterial cells present in the VBNC state. The VBNC state of bacterial cells is poorly understood and extensive work needs to be conducted in this area, likewise it is hypothesised that certain nutrient ratios may limit bacterial population growth and this represents another area that requires extensive research.

It is also important to fully understand the association between bacterial concentrations and physico-chemical changes in the environment. Hibbing et al. (2010) suggested that physico-chemical changes may provide the driver for differential bacterial accumulation. However, this also represents an area which is currently under-researched.

7.3 Determination of the relative contribution of previously identified bacterial reservoirs in shellfish tissues under different mitigation strategies

Current EU standards regarding the sale of live bivalve molluscs and the respective mitigation strategies employed to reduce the risk of shellfish vectored illness are outlined in Table 1.4. Dependent upon the concentrations of *E. coli* present within shellfish flesh, shellfish are required to undergo either depuration (for a minimum of 42 hours) or relaying (for a minimum of 2 months) to reduce the bacterial content of the shellfish. Both strategies have been shown to be effective in the reduction of *E. coli* concentrations within mussel flesh (Marino et al. 2005, Buck 2007, Barile et al. 2009). However, the effectiveness of these treatments on other bacterial and viral reservoirs remains an area which is poorly understood.

This study aims to investigate the effectiveness of both depuration and offshore relaying in the reduction of both bacterial and viral contamination of commercial shellfish.

7.3.1 To investigate the effects of depuration on the concentrations of different bacterial species (E. coli, total coliforms, vibrio spp. and marine heterotrophs) within shellfish tissues

In chapter 4 we examine the effectiveness of a standard depuration treatment in reducing the concentrations of both total coliforms and naturally occurring bacteria such as *Vibrio spp.* and marine heterotrophs from within mussel flesh. The results from this study show that a standard 48 hour depuration treatment was sufficient to reduce the concentration of total coliform bacteria to negligible levels, but was insufficient in reducing the concentrations of naturally occurring bacteria which may pose a threat to human health.

As discussed above, naturally occurring bacterial species such as *Vibrio spp.* contain potentially pathogenic species which are missed by routine monitoring procedures which utilise FIB as a proxy for human pathogens. The interaction between FIB and naturally occurring bacteria is currently poorly understood and requires further research both *in situ* and *ex situ* in order to adequately protect human health from the risks posed by other bacterial species. In addition, the behaviour of both naturally occurring bacteria and other pathogenic bacterial species i.e. salmonella, undergoing depuration, needs further research to ascertain whether the current depuration time frame is sufficient to mitigate against the risk posed by alternative bacterial species.

7.3.2 To investigate the effects of depuration on the concentrations of different bacterial species (E. coli, total coliforms, vibrio spp. and marine heterotrophs) within epizoic barnacles

Building on the work presented in chapter 3 where we established that epizoic barnacles were a significant bacterial reservoir, we then investigated the behaviour of both total coliforms and naturally occurring bacteria within the barnacles whilst undergoing a standard depuration treatment. This work is presented in chapter 4.

Whilst a standard depuration treatment was successful in the elimination of total coliforms from within mussel tissue, it was ineffective at eliminating total coliforms as well as naturally occurring bacteria from within the epizoic barnacle reservoir, despite the fact that barnacles demonstrated a higher rate of coliform elimination than the corresponding mussel samples. This has important implications for the current practices employed by the shellfish industry. However, some caution must be taken when using indicator bacteria. In the case of coliforms, many hundred strains are known to be potentially human pathogenic, however, it is unclear whether the coliforms not capable of removal from the mussel flesh are pathogenic or not. As noted above, typing of *E. coli* is urgently required to evaluate this.

As previously discussed, there is a growing trend for shellfish to be marketed complete with epizoic organisms. Shellfish that subsequently undergo depuration treatment and are deemed 'safe' for human consumption based on the bacterial concentrations present within the shellfish flesh may be subjected to secondary contamination post-depuration due to the ineffectiveness of the depuration procedure in reducing the bacterial concentration present within the barnacle tissues, which may then re-contaminate the shellfish during transit, processing, or at the point of retail. In the interests of human health, it could then be argued, that the depuration treatment time should be extended for shellfish intended to be depurated complete with epizoic barnacles. Further work in this area is needed to determine the appropriate time frame required for successful elimination of bacteria from epizoic barnacles.

7.3.3 To investigate the effects of offshore relaying on the bacterial and viral concentrations of shellfish tissues

In chapter 5 we investigated the potential for offshore relaying as a mitigation measure in the reduction of both bacterial (*E. coli* and total coliforms) and viral (norovirus) contamination of experimentally deployed mussels over varying offshore distances. Whilst FIB have been repeatedly shown to be poor surrogates for viral contamination of shellfish both *in situ* and *ex situ* (Power and Collins 1989, Doré et al. 2000, DePaola et al. 2010), this study was only able to draw preliminary conclusions regarding the relationship between bacterial and viral contamination of mussels and the mitigating effect of offshore relaying as the initial concentrations of *E. coli*, total coliforms and norovirus were significantly lower than anticipated (< 230 *E. coli* CFU / 100 g) and all both norovirus GI and GII were below the limit of

quantification for the assay used, despite mussels being collected from an area classified as ‘class B’ (230 - 4,600 *E. coli* / 100 g). It was hypothesised that offshore relaying would be sufficient in mitigating the risk as shown by utilising *E. coli* as a proxy but would be insufficient in the reduction of viral contamination of mussels due largely to the increased environmental persistence of norovirus relative to *E. coli* (Seitz et al. 2011).

7.4 Examination of the relationship between bacterial and viral reservoirs within shellfish tissues

Whilst *E. coli* remains the current indicator utilised by EU legislation there are increasing numbers of studies which demonstrate its unsuitability as a proxy for norovirus (Loisy et al. 2005, Ueki et al. 2007, Lowther et al. 2008). Norovirus is increasingly being cited as the leading cause of shellfish vectored illness (Le Guyader et al. 2009); however until recently norovirus could not be detected directly from shellfish tissues (Lees 2010). Now that a methodology exists to directly quantify the norovirus content of shellfish, we decided to evaluate the relationship between FIB and norovirus within mussel tissues.

7.4.1 To investigate the spatial contamination patterns of both faecal indicator bacteria and norovirus within shellfish tissues

Chapter 6 investigates the spatial contamination patterns of both FIB and norovirus within mussel tissues, from experimentally caged mussels deployed at specific distance intervals from a sewage outfall and compares the results to a model simulation of predicted *E. coli* concentrations in water, over the same area.

The findings from this study strongly support the findings from previous studies demonstrating the inadequacy of *E. coli* as a proxy for norovirus contamination within mussel tissues. The model simulation of predicted *E. coli* concentrations in water was almost perfectly correlated with determined norovirus concentrations within mussel tissues. However, there was only a very weak relationship between the modelled *E. coli* concentrations and the actual determined concentrations of *E. coli* within mussel flesh.

This may be attributed to the origins of both *E. coli* and norovirus in the area. Whilst the majority of norovirus particles present in the mussels can be assumed to originate from the sewage outfall itself, *E. coli* concentrations in mussels were shown to be higher in locations outside where the model predicted the sewage effluent would be dispersed. It can be assumed that these elevated *E. coli* concentrations were not therefore the result of uptake of sewage-derived bacteria, rather they were the result of another external source of contamination, potentially a diffuse pollution source originating from the land, as the sites showing the highest *E. coli* concentrations (with the exception of those closest to the sewage outfall) were those closest inshore.

Another alternative to explain the results of this study is that norovirus has been shown to have a greater environmental persistence relative to *E. coli* (Loisy et al. 2005). As the *E. coli* and norovirus concentrations were determined after the mussels had been *in situ* for one month it is possible that the mussels had been able to effectively eliminate the *E. coli* from within their flesh, but were unable to effectively eliminate norovirus at the same rate.

Nevertheless, these findings demonstrate that *E. coli* is a poor surrogate for norovirus contamination in shellfish and a bacteriological standard is inadequate in protecting consumers from the risk of shellfish vectored viral illness. Further work is urgently required to incorporate appropriate virological standards into EU legislation.

7.5 Final conclusions and future management recommendations

The findings from the studies presented in this thesis have successfully identified and quantified numerous bacterial reservoirs present in commercial shellfish harvesting areas in North Wales, UK. Some of these reservoirs had been previously identified, but not fully investigated and quantified. With regard to these bacterial reservoirs, particularly the underlying sediment, overlying water and the bacterial reservoir present within mussel flesh, the studies presented in this thesis serve to fill some of the current ‘knowledge gaps’, and to improve our understanding of the complex interactions between these bacterial reservoirs *in situ*. One of the key findings from this thesis was the identification of a previously over-looked bacterial reservoir. Epizoic barnacles present on commercial shellfish represent a significantly larger bacterial reservoir than the commercial shellfish onto which they are attached. This

study represents the first attempt to both identify and quantify the bacterial reservoir within epizoic barnacles and has important implications for the shellfish industry, challenging the effectiveness of current industry practices.

In light of these findings, we propose the following management recommendations:

- Current EU legislative standards which only quantify FIB present within shellfish flesh, utilising a small number of shellfish samples, may not provide an accurate representation of the levels of bacterial contamination affecting shellfish production areas. It is recommended that a full ecological and microbiological survey of each individual harvesting area be conducted and the relative influences of alternative bacterial reservoirs be incorporated into individual management plans.
- The current microbiological monitoring protocols for shellfish (utilising *E. coli* as a proxy for pathogen contamination) are inadequate to protect consumers from the risk of illness from other bacterial species such as *Vibrio spp.* In addition, the current microbiological monitoring protocols focus only on the enumeration of culturable bacteria. This methodology may significantly under-estimate the risk to the consumer. Therefore monitoring protocols should be considered that encompass not only the culturable bacteria, but also the total viable populations, in order to provide a more accurate assessment of risk to the consumer.
- Current mitigation strategies such as depuration may be insufficient in reducing the risk posed to consumers from alternative bacterial reservoirs e.g. epizoic barnacles. It is recommended that in order to safeguard consumers, either epizoic organisms are removed pre-depuration or that the current depuration time frame be extended for shellfish entering into depuration complete with epizoic organisms.
- Current EU standards which utilise FIB as a proxy for viral contamination of shellfish are insufficient in safeguarding consumers against the risk of shellfish vectored viral illness. It is therefore recommended that a suitable virological standard should be incorporated into current legislation to protect consumers against the risk of shellfish vectored viral illness.

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APPENDIX I

Spatial variation of waterborne *Escherichia coli* – Implications for routine water quality monitoring

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This manuscript was produced as part of a preliminary bacteriological survey of the Conwy Estuary in October 2010. Much of this work represented a training opportunity for Katie Clements (KC). KC assisted in the experimental design, conducted the sample collection and subsequent sample processing and assisted with the data analysis and production of the manuscript.

Spatial variation of waterborne *Escherichia coli* – implications for routine water quality monitoring

Richard S. Quilliam, Katie Clements, Caroline Duce, Simon B. Cottrill, Shelagh K. Malham and Davey L. Jones

ABSTRACT

Escherichia coli are often used as faecal indicator bacteria (FIB) to provide a measure of microbial pollution in recreational and shellfish harvesting waters. However, although model forecasts for predicting the concentrations of FIB in surface waters are becoming more robust, they suffer from an inconsistency in quantification methods and an understanding of the spatial variation of FIB within a water course. The aim of this study was to investigate the transverse spatial variation in *E. coli* numbers (as an indicator of faecal pollution) across the estuary of the River Conwy, UK. Water samples were collected from four transverse transects across the estuary. Spatial variation of *E. coli* was significantly different from one side of the river to the other, although was not correlated with depth or the physiochemical properties of the water. Subsequently, microbial water quality classifications on the two opposite banks suggested very different levels of pollution coming down the river. This work has shown that the side of the river that routine water monitoring samples are taken from can make a significant difference to the classification of microbial water quality. This has important implications for sampling strategies and the use of microbial source tracking (MST) techniques.

Key words | faecal coliforms, faecal indicator bacteria (FIB), microbial pollution, quantitative microbial risk assessment (QMRA), water framework directive

Richard S. Quilliam (corresponding author)
Davey L. Jones
School of Environment,
Natural Resources & Geography,
Bangor University,
Bangor,
Gwynedd LL57 2UW,
UK
E-mail: r.s.quilliam@bangor.ac.uk

Richard S. Quilliam
Katie Clements
Caroline Duce
Shelagh K. Malham
School of Ocean Sciences,
Environment Centre Wales,
Bangor University,
Bangor,
Gwynedd LL57 2UW,
UK

Simon B. Cottrill
Conwy County Borough Council,
Bodlondeb,
Conwy LL32 8DU,
UK

INTRODUCTION

Levels of faecal indicator bacteria (FIB), such as *Escherichia coli*, are often used as a measure of microbial pollution in recreational and shellfish harvesting waters. Although *E. coli* is now considered a poor surrogate for most pathogenic bacteria, viruses and protozoa (Brookes *et al.* 2005; Savichtcheva & Okabe 2006), its presence is still widely accepted as being an important indicator for faecal contamination. Furthermore, compared with quantifying individual waterborne pathogens (Quilliam *et al.* 2011), the measurement of *E. coli* (either as MPN or CFU) is relatively straightforward. Epidemiological studies have established that exposures to FIB in recreational waters is significantly linked to a decrease in public

health (Wade *et al.* 2003; Wiedenmann *et al.* 2006), and maintaining and improving the microbial quality of freshwaters has resulted in legislative pressures through implementation of the Drinking Water (98/83/EC) and Water Framework (2000/60/EC) Directives (E.C. 1998, 2000). As a consequence, model forecasts for predicting the concentrations of FIB in surface waters are becoming an increasingly important management decision tool (Hellweger & Masopust 2008; Gronewold *et al.* 2009). However, despite two-dimensional models combining data on the rate of mixing and die-off (Smith & Putz 1993; Vandenberg *et al.* 2005) together with depth and temporal variability (Kashefipour *et al.* 2002; Li *et al.* 2008;

Parks & van Briesen 2009; Pote *et al.* 2009), an inconsistency in quantification methods and a lack of understanding of the spatial variation of FIB within a water course can hinder the robustness of such models. Ultimately this could lead to the unnecessary closure of public beaches or the restricted harvesting of shellfish.

The aim of this study therefore, was to determine the spatial transverse variation in *E. coli* numbers across the estuary of the River Conwy in North Wales, UK. This area is important for the commercial harvesting of shellfish, and has several public beaches with designated EC bathing waters (Bathing Water Directive, 76/1160/EEC) (E.C. 1976). There is a dynamic deposition of sediments within this estuary that results in heterogeneously dispersed banks of mud and sand. This provides contrasting habitats for coliforms (Howell *et al.* 1996) and although progress is being made to incorporate sediment reservoirs into mathematical models (Jamieson *et al.* 2005; Badgley *et al.* 2011), the spatial re-suspension of sediment-associated *E. coli* due to tidal movements and storm events is still poorly understood. We envisage that our results will have a significant impact on future sampling strategies for routine water quality monitoring. In addition, this work will facilitate further developments in microbial source tracking (MST) techniques and

contribute to the improvement of hydrodynamic and water quality models.

MATERIALS AND METHODS

Boat sampling was carried out in the estuary of the River Conwy during the first week of October, 2010, in an area that did not contain any large point sources. Four transverse transects were conducted on the same day (Figure 1), with four replicate water samples collected from each point in the transect. Samples were taken approximately 1 m below the surface with sterile 1 L plastic bottles. Following EU guidelines, all samples were stored at 4 °C and processed within 6 h of collection. Each water sample was briefly shaken and 25 mL was vacuum-filtrated through a 0.2 µm cellulose acetate membrane (Sartorius Stedim Biotech., Gottingen, Germany). The membrane was aseptically transferred to the surface of a plate containing M-endo agar LES (Oxoid Ltd., Basingstoke, UK); the plate was inverted and incubated at 37 °C and enumerated 24 h later. Turbidity was measured with a T-100 Turbidimeter, and electrical conductivity (EC), salinity and pH were measured directly using standard electrodes.

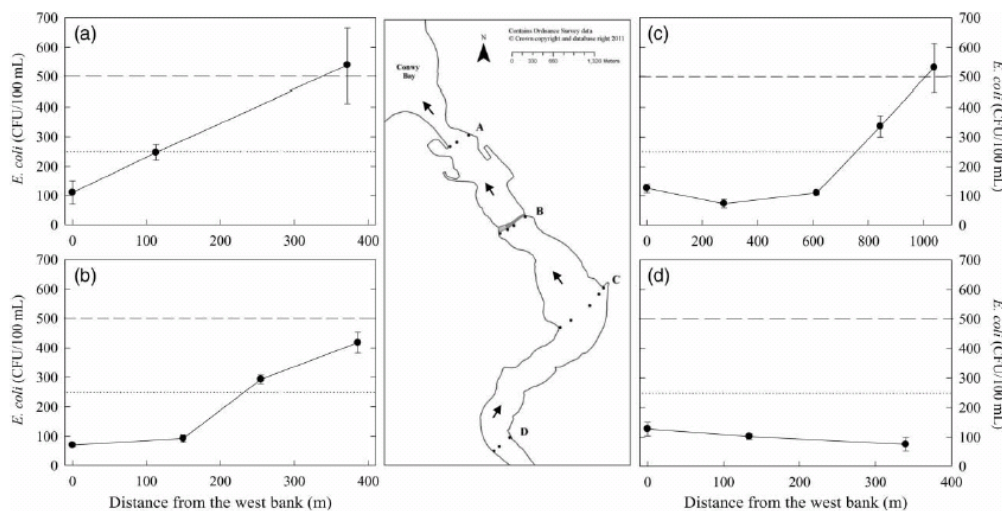


Figure 1 | Transverse transects across the River Conwy. The locations of the four transects and approximate sampling points are shown on the map and all have been presented as distance from the west bank. Arrows show direction of river flow. The Revised Bathing Water Directive (2006/7/EC) classifications of 'excellent quality' (250 *E. coli* CFU/100 mL) and 'good' or 'sufficient' quality (500 CFU/100 mL) are marked on graphs a–d as a dotted and dashed line respectively. Data points represent the mean of 4 replicates ± SEM.

RESULTS AND DISCUSSION

Although there were no significant differences in pH, EC, turbidity, temperature or salinity across each transect ($P > 0.05$), there was significant spatial variation in *E. coli* numbers in three of the transects ($P < 0.001$), with approximately five times more CFU on the east side of the river compared to the west side (Figure 1(a)–(c)). We believe that this result has important implications for sampling strategies, for example interpreting the Revised Bathing Water Directive (2006/7/EC) (E.C. 2006), which classifies a concentration of 250 *E. coli* CFU/100 mL as ‘excellent quality’ while 500 CFU/100 mL is only classified as ‘good’ or ‘sufficient’ depending upon the percentile evaluation. Although this stretch of water is not specifically designated as ‘bathing water’, it does have a direct impact on several public beaches within the catchment, and drains directly into EC bathing waters and commercial shellfish harvesting areas. It is clear that the side of the river that water samples are taken from does make a significant difference to the concentration of indicator bacteria. Classifications on the two opposite banks suggest very different levels of pollution coming down the river, which will have important implications for public health and the management of bathing waters. Although the water on the west side of the transverse transects was deeper, this was not significantly correlated with the concentration of *E. coli* ($P = 0.254$). Sampling bias may be introduced however, as the shallower east side of the river has much easier access for sampling whilst fulfilling the minimum 1 m depth requirement for sampling water for microbial monitoring.

Over the last two decades a number of MST methods have been developed, with the aim of pinpointing exact sources of microbial pollution (Simpson et al. 2002). One of the major limitations associated with MST methods is the complexity associated with the persistence and survival of indicator species within the environment, together with the spatial and temporal heterogeneity within these different environmental matrices. Such spatial heterogeneity can confuse attempts at identifying the cause of microbial pollution, particularly when water bodies are not well mixed. Although the physiochemical variables measured here implied that the water was well mixed across the

transect, the variation in *E. coli* numbers suggests that localised re-suspension from the sediment may significantly affect spatial concentrations of FIB. Our results have demonstrated the importance of the sampling location within a watercourse for routine water quality monitoring and the effect this can have on interpreting data used for MST.

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APPENDIX II

Using PCR-DGGE to track the flux of *Escherichia coli* communities through an estuarine nature reserve

Quilliam R. S^a, Gertler C^b, **Clements K^c**, Duce C^c, Cotttrill S. B^d, Malham S. K^e and Jones D. L.^f (2013) Using PCR-DGGE to track the flux of *Escherichia coli* communities through an estuarine nature reserve. *In prep.*

^aBiological and Environmental Sciences, School of Natural Sciences, University of Stirling, Stirling, FK9 4LA. ^bSchool of Biological Sciences, Bangor University, Bangor, Gwynedd, LL57 2UW. ^cSchool of Ocean Sciences, Bangor University, Menai Bridge, Anglesey, LL59 5AB. ^dConwy County Borough Council, Bodlondeb, Conwy, LL32 8DU. ^eCentre for Applied Marine Sciences, Bangor University, Menai Bridge, LL59 5AB. ^fSchool of Environment Natural Resources and Geography, Bangor University, Bangor, Gwynedd, LL57 2UW.

This research was undertaken as a direct result of the First Conwy Estuary Stakeholders meeting (2010) in which the mussel producers argued that the Conwy RSPB reserve was responsible for increased microbial loading into the estuary and was negatively impacting on the microbial quality of the mussel beds. The Conwy RSPB reserve was artificially created as a mitigation measure for the construction of the A55 expressway and approximately 10,000 birds over winter in this area, increasing the faecal loading into the estuary. This study was intended to provide training to KC on Microbial Source Tracking (MST). KC assisted with the experimental design, sampling, subsequent sample processing and data analysis.

Abstract

There is growing evidence that direct faecal loading by birds can significantly contribute to the microbial pollution of watercourses. The aim of this study was to assess an *E. coli*-specific culture-dependent PCR-DGGE method to investigate whether a bird reserve adjacent to the estuary of the River Conwy (UK) was making a significant contribution to faecal pollution in the estuary, by comparing the similarity of DGGE fingerprint profiles through nonmetric multidimensional scaling (nMDS). Differences in *E. coli* community structure was likely a consequence of localised faecal input from birds, although these initial results provide no evidence for the bird reserve directly contributing to *E. coli* pollution in the estuary. This pilot study demonstrates how this approach could be used as a microbial community tracking method. Although the water in the lagoons of the bird reserve does represent a point-source reservoir for FIB, with the concentration of *E. coli* at all five sites greater than 10^3 CFU 100 ml⁻¹, the potential impact on microbial pollution in the estuary is relatively low due to the significant dilution effect caused by the large volume of water passing through the estuary.

Keywords: Birds; Faecal Coliforms; Faecal Indicator Bacteria (FIB); Microbial Pollution; Microbial Source Tracking (MST); Water Quality.

Introduction

Levels of faecal indicator bacteria (FIB), such as *Escherichia coli*, are often used as a measure of microbial pollution in recreational and shellfish harvesting waters. Although *E. coli* is now considered a poor surrogate for most pathogenic bacteria, viruses and protozoa (Brookes *et al.*, 2005; Savichtcheva and Okabe, 2006), its presence is still widely accepted as an important indicator for faecal contamination. Additionally, compared with quantifying individual waterborne pathogens (Quilliam *et al.* 2011a), enumerating *E. coli* is relatively straightforward. Epidemiological studies have established that exposures to FIB in recreational waters is significantly linked to a decrease in public health (Wade *et al.*, 2003; Wiedenmann *et al.*, 2006), and maintaining and improving the microbial quality of freshwaters has resulted in legislative pressures being implemented by the Drinking Water (98/83/EC) and Water Framework (2000/60/EC) directives.

The environmental pathways for contamination of watercourses by FIB can include both diffuse and direct inputs, e.g. sewage discharges and effluent from leaking septic tanks, or livestock defecation when rivers are used for drinking or as crossing points. Non-point source inputs can occur following high precipitation, particularly flooding events, which can lead to agricultural run-off and result in the indirect contamination of rivers (Williams *et al.*, 2008). The surface application of livestock faeces or contaminated irrigation water to either pasture or arable fields can further add to the dispersal and FIB loading of watercourses (Deeks *et al.*, 2005). However, there is growing evidence that faecal loading by wild animals, and in particular gulls, waterfowl and birds roosting on bridges and piers, can significantly contribute to the microbial pollution of recreational waters and beaches (Edge and Hill, 2007; Wither *et al.*, 2003; Lévesque *et al.* 2000; Alderisio and DeLuca, 1999). It has been calculated that gull faeces can contain between 10^5 - 10^9 *E. coli* CFU g⁻¹ and between 10^4 - 10^8 enterococci CFU g⁻¹ (Fogarty *et al.*, 2003), with an average wet weight of faeces excreted by gulls ranging from 11.2 to 24.9 g day⁻¹ (Gould and Fletcher, 1978). For geese, *E. coli* CFU can range from 0- 10^7 g⁻¹ wet faeces and between 10^2 - 10^7 g⁻¹ for enterococcus (Middleton and Ambrose, 2005). Studies from watercourses in North America have estimated that the relative contribution to the annual input of faecal coliforms from waterfowl can range from between 34% and 67% (Yan *et al.*, 2007; Weiskel *et al.*, 1996). Furthermore, Haack *et al.* (2003) have estimated that the daily loading from 50 birds (pigeons, geese, ducks and gulls) in a swimming area on Lake Michigan (100 m long, 10 m offshore, and 1 m deep) could result in between 775 and 1720 *E. coli* CFU per 100 ml and 2810-6250 enterococci per 100 ml. Consequently, there is growing concern that

increased FIB levels due to faecal loading by birds can affect water quality and lead to the unnecessary closure of public beaches or restrict the harvesting of shellfish.

There has been much research effort devoted to developing microbial source tracking (MST) methods for tracing faecal indicator organisms through aquatic environments, and broadly these technologies can be classed into either library-dependent or library-independent methods, although both approaches have their limitations (Field and Samadpour, 2007). Denaturing Gradient Gel Electrophoresis (DGGE) is a technique that can discriminate between species, or strains of a species, based on the melting property of target DNA derived from a PCR product. Although this approach is most commonly utilised for fingerprinting microbial communities (e.g. Esseili et al. 2008), it has also been successfully used as an MST method (Sigler and Pasutti, 2006). The aim of this study was to use a DGGE fingerprinting approach to assess whether a small nature reserve, specifically created as a high tide refuge for wading birds, significantly contributed to faecal contamination in the Conwy estuary, which discharges into shellfish waters and surrounding beaches. To address this we have used an *E. coli*-specific culture-dependent PCR-DGGE method to track communities of *E. coli* through the nature reserve and into the Conwy estuary. Recent work in the Conwy estuary has shown that land-use within the catchment is important for the survival of faecal pathogens and water quality further downstream (Thorn et al. 2011), and that the spatial dynamics of FIB within the estuary can have important implications for sampling strategies and the use of MST techniques (Quilliam et al. 2011b). However, whether the nature reserve is significantly contributing to faecal loading within the catchment has not yet been determined.

Materials and Methods

Study area and sampling strategy

The estuary of the River Conwy in North Wales, UK is an important area for the commercial harvesting of shellfish, and has several public beaches with designated EC bathing waters. Following the construction of a road tunnel under the estuary between 1986 and 1991, a nature reserve was constructed, which is leased from the Crown Estate by the Royal Society for the Protection of Birds (RSPB) and covers 47 hectares of grassland, scrubland, reedbeds, salt marsh and mudflats (Fig. 1). Of the 230 species of bird that have been recorded on the reserve, 83 species occur regularly including 43 species of waterbird. Over the last decade the average monthly counts have stayed fairly constant, with about 400 wildfowl, 200 waders, 200 gulls and 40 other waterbirds. Additionally the reserve provides roosting habitat for a large number of other birds, e.g. starlings.

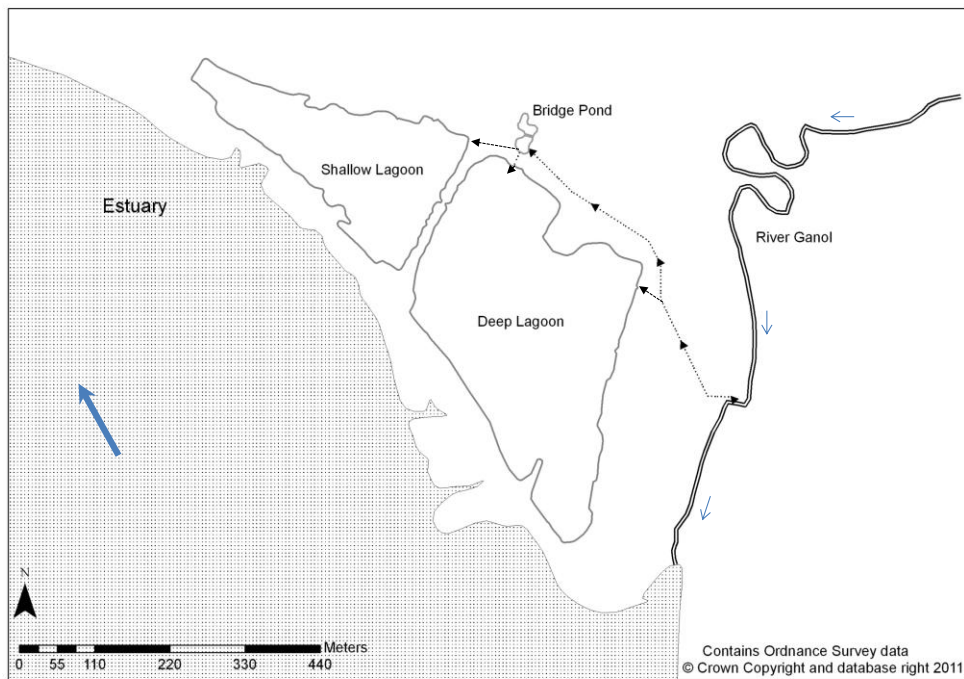


Fig 1. Map of the RSPB Conwy Nature Reserve. The underground pipe that pumps water from the River Ganol to the Bridge Pond is represented by a dashed line. Arrows indicate the direction of water flow in the River Ganol and during the ebb tide in the estuary.

All water samples were collected from the estuary area of the River Conwy during the ebb tide and from three sites within the Conwy RSPB nature reserve (Bridge Pond, the Deep Lagoon and the Shallow Lagoon) during July 2010. This date was chosen to reflect a time when shellfish harvesting and recreational activity in the coastal zone was maximal. When water levels in the lagoons become too low, the RSPB are licensed to pump water in from a tributary of the River Conwy (the River Ganol), either directly to the Deep Lagoon, or to a holding pond (the Bridge Pond), from where it can be pumped into either the Deep Lagoon or the Shallow Lagoon (Fig. 1). During the period of April 2010 to March 2011 36,000 m³ was pumped from the River Ganol into the lagoons. Therefore, water samples were also collected from the River Ganol and from the Conwy Estuary downstream of the nature reserve. Four replicate water samples from each of the five locations were collected in sterile 1 L plastic bottles. Just prior to collection each sample bottle was rinsed three times in the water to be sampled. All samples were stored at 4 °C and processed within six hours of collection. Turbidity was measured with a T-100 Turbidimeter (Oaklon Instruments, Illinois, USA) and expressed as nephelometric turbidity units (NTU), and electrical conductivity (EC) and pH were measured directly using standard electrodes.

Each water sample was briefly shaken and aliquots were serially diluted with sterile Ringers solution before vacuum-filtration through a 0.2 µm cellulose acetate membrane (Sartorius Stedim Biotech., Göttingen, Germany). The membrane was then aseptically transferred to the surface of a plate containing MacConkey agar (Oxoid Ltd., Basingstoke, UK); the plate was inverted and incubated at 37 °C for 24 h. Following enumeration, the membrane filters were frozen at -20 °C for further analysis. Heterotrophic bacterial cells from each water sample were simultaneously quantified (in duplicate) by directly plating out 10-fold dilutions, in quarter strength Ringer's solution, on plates of R2A agar (20 °C for 48 h). Statistical analysis of CFU and water chemistry were performed by analysis of variance (ANOVA), Tukey multiple comparison tests, and regression analysis (Minitab 12.0 software, Minitab Inc., PA, USA).

DNA extraction and DGGE fingerprinting

The frozen membrane filters containing the *E. coli* colonies were sliced into ca. ten pieces with a sterile razor blade and DNA was extracted by using the PowerSoil DNA isolation kit (Cambio Ltd., Cambridge, UK), following the manufacturer's instructions. PCR amplifications used 2 µl of template DNA in a 50 µl reaction, with the *E. coli*-specific primer pair *phoE-f* and *phoE-r* (Spierings *et al.*, 1993), with a GC-clamp added to the forward primer for subsequent DGGE analysis. Amplifications were carried out under the following conditions: initial denaturing step, 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and a final extension step of 72 °C for 5 min. Electrophoresis in an ethidium bromide stained 1 % agarose gel was used to confirm the presence of the 348 bp product. Denaturing gradient gel electrophoresis was carried out according to the method of Muyzer *et al.* (1993) using an Ingeny phorU electrophoresis system (Ingeny, Netherlands). Gels contained 6% (w/v) polyacrylamide with a linear gradient of 15-55% (where 100% denaturant is 7 M urea and 40% (v/v) formamide) and were run in 1X TAE buffer at 100 V, for 16 h at 60°C. Gels were stained with a 1X SYBR-Gold (Invitrogen) solution in 1X TAE and visualised with a gel documentation system (BioRad, Hercules, USA).

DGGE fingerprinting analysis and non-metric Multivariate Statistics Analysis

Gel images of DGGE fingerprints were normalized according to the Roboklon 1kb plus Standard (Roboklon, Berlin, Germany) and analyzed using Quantity One Software (BioRad, Hercules, CA, USA). A total of 34 band classes were assigned to DGGE bands. Densitometric values for each DGGE band were included in the analysis, resulting in a band-matching table.

Square root transformation was used to calculate Bray-Curtis similarity of DGGE fingerprints for each sample and the whole data set. Ordination of similarity matrices were conducted by non-metric multidimensional scaling (nMDS) using PAST software (Hammer *et al.*, 2001).

Results

Following vacuum-filtration of water samples, pink colonies (presumptive *E. coli*) on each membrane were enumerated and expressed as CFU 100 ml⁻¹. The concentration of *E. coli* at all five sampling sites was greater than 10³ CFU 100 ml⁻¹ of water, with significantly higher numbers in the River Ganol, the Bridge Pond and the Deep Lagoon (one-way ANOVA $P < 0.01$) (Fig. 2). There was a similar trend in the numbers of culturable heterotrophic bacteria, which resulted in a significant relationship between the concentration of *E. coli* and heterotrophic bacteria ($R^2 = 0.48$; $P < 0.05$). Turbidity significantly decreased after it had left the River Ganol (Table 1) and was significantly correlated with *E. coli* CFU ($R^2 = 0.46$; $P < 0.05$). The pH of the water increased from 6.62 in the River Ganol to 8.56 in the Shallow Lagoon. A total of 34 different bands were identified with DGGE, and non-metric multidimensional (nMSD) scaling analysis was used to determine the relative spatial separation between the DGGE fingerprints from each site. There was significant overlap between the *E. coli* communities from the River Ganol, Bridge Pond, Deep Lagoon and the estuary, providing evidence for a shared water source (Fig. 3A). However, there was no overlap between the *E. coli* communities from the Shallow Lagoon and any of the other sites. In addition, PCA was used to visualise the relationships between the DGGE banding patterns (Fig. 3B), with the two principal components explaining about 40% of the variance (PC1 = 20.32% and PC2 = 18.92%). The *E. coli* communities from four of the sites showed similar clustering, with no overlap with the Shallow Lagoon (Fig. 3B).

Table 1: Physico-chemical properties of water samples collected at each site; values followed by different letter codes in each column are significantly different from each other (one-way ANOVA $P < 0.001$ and Tukey multiple comparison test). Data points represent the mean of 4 replicates (SEM)

	pH	EC (mS)	Turbidity (NTU)
River Ganol	6.62 (0.03) ^a	0.72 (0.001) ^a	37.7 (0.1) ^a
Bridge Pond	6.53 (0.05) ^a	16.3 (0.07) ^b	22.9 (0.2) ^b
Deep Lagoon	7.79 (0.02) ^b	3.93 (0.04) ^c	18.2 (2.1) ^b
Shallow Lagoon	8.56 (0.02) ^c	3.26 (0.06) ^c	19.3 (4.6) ^b
Estuary	7.08 (0.01) ^d	35.1 (0.58) ^d	13.2 (3.1) ^b

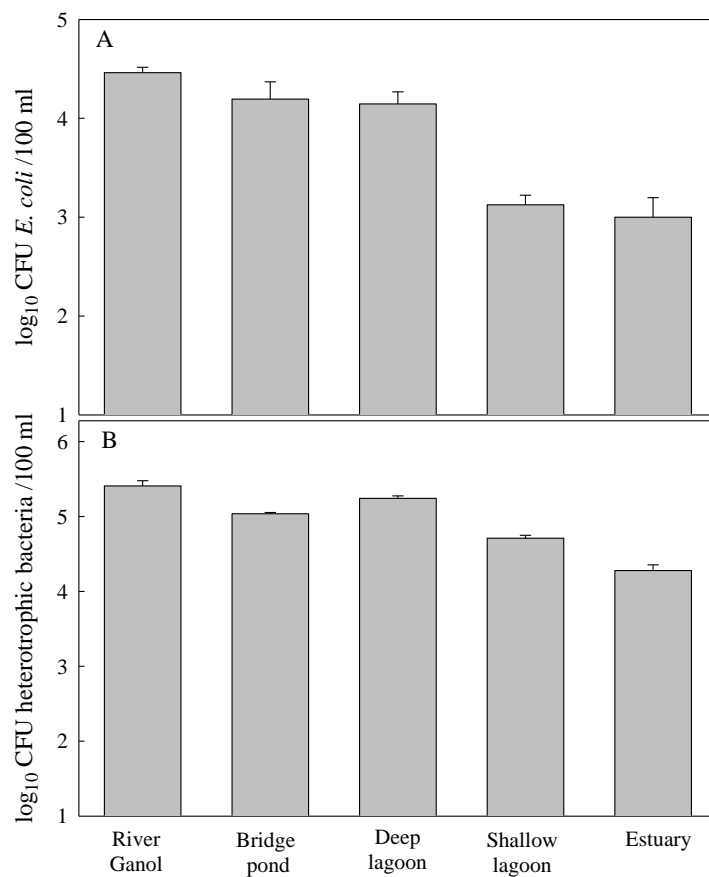


Fig 2. CFU of waterborne *E. coli* (A) and background heterotrophic bacteria (B). Data points represent the mean of 4 replicates \pm SEM

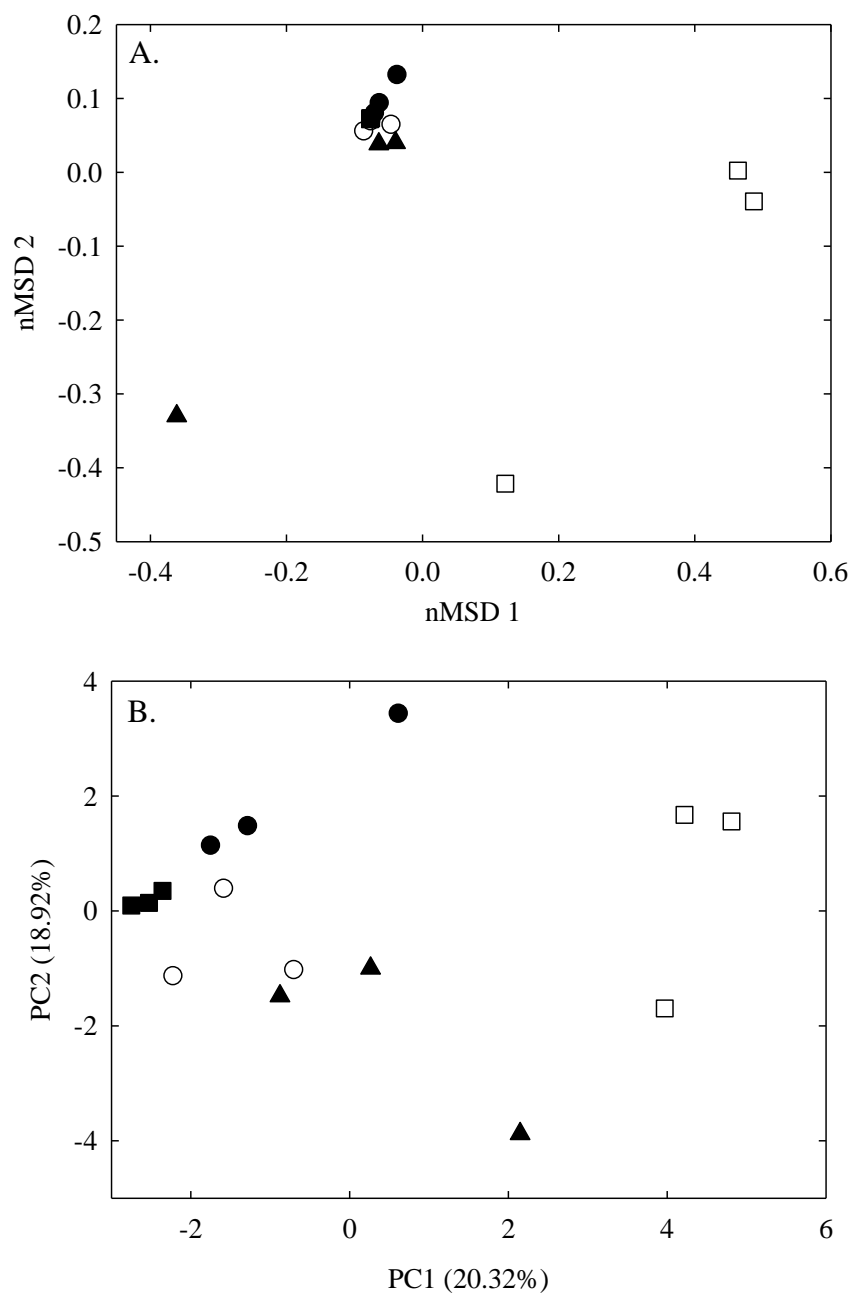


Fig 3. Non-metric multidimensional scaling analysis (A) showing the relative similarity between *E. coli* communities, and Principle component analysis (B) of the DGGE fingerprint. River Ganol -open circles; Bridge Pond - closed squares; Deep lagoon - closed triangles; Shallow lagoon - open squares; Estuary - closed circles. Co-ordinate 1 = 20.32% and coordinate 2 = 18.92%

Discussion

Over the last decade a number of MST methods have been developed, with the aim of pinpointing exact sources of microbial pollution. However, there are serious limitations to many of these approaches, and to date there is not a single MST method that is widely accepted as being accurate, reproducible and unambiguous. A major limitation associated with MST is the complexity of the persistence and survival of indicator species within the environment (Field and Samadpour, 2007). Additionally, waterborne *E. coli* are often associated with suspended particulate matter and sediments, which not only influences their survival, e.g. by providing protection from UV, but also affects their transport dynamics.

The culture-dependent PCR-DGGE approach that we have used in this study negates some of these limitations by allowing the selective enrichment of bacteria including those in biofilms tightly bound to particulate matter, and the extraction of DNA without the hindrance of large quantities of non-target background nucleic acid. By selecting a single indicator species we have been able to track and fingerprint the whole *E. coli* community flux through the nature reserve, which has revealed that significant similarities in community structure is evidence for a shared water source. Non-metric multidimensional scaling analysis suggested that three of the sites (River Ganol, Bridge Pond and the Estuary) were very similar in their *E. coli* community composition. Due to their temporal-spatial connection, *E. coli* communities in the River Ganol and the Estuary should be almost identical, with any differences resulting from either faecal loading into the estuary further up the catchment or through seepage from the nature reserve. Likewise, the relative similarity in the community composition between the River Ganol and the Bridge Pond is a consequence of a shared water source, i.e. water being directly pumped from the River Ganol to the Bridge Pond.

We have tested whether the two lagoons in the nature reserve are contributing to FIB loading in the Estuary by comparing the similarity of their DGGE fingerprint profiles with those from sites 'upstream' and 'downstream' of the lagoons. The DGGE fingerprints of the *E. coli* communities from the Shallow Lagoon were very different to all of the other sites and while two of the three replicate Deep Lagoon DGGE fingerprints clustered with the River Ganol, Bridge Pond and the Estuary, one replicate was clearly dissimilar to the others suggesting a degree of dissimilarity in community structure between the Deep Lagoon and the Estuary (and hence providing support for a lack of continuity between the two water bodies). Both of the lagoons are temporally disconnected from the other sites and water is only pumped in from the Bridge Pond several times a year. This is supported by PCA analysis, where there were clear differences on PC1 between the Shallow Lagoon and the other sites, and between

one of the Deep Lagoon replicates and the other sites. Differences in *E. coli* community structure between the lagoons and the other sites is likely a consequence of localised faecal input from birds, while differences in the *E. coli* composition between the two lagoons may reflect the dichotomy in niche preference of bird species for either deep or shallow water. Furthermore, feeding behaviour is likely to be a contributing factor, with higher numbers of *E. coli* found in the faeces of carnivorous birds compared to granivorous birds (Steele et al. 2005).

Suspended particulate matter within water provides a beneficial habitat for bacteria and can significantly contribute to the total number of FIB within a watercourse. Previous studies have demonstrated that the survival of *E. coli* cells in the environment is enhanced by associating with suspended particulate matter (Muirhead *et al.*, 2006); and as FIB loading due to agricultural run-off is often associated with soil particles it is unsurprising that *E. coli* numbers are significantly correlated with turbidity. Birds are almost exclusively responsible for the *de novo* loading of FIB into the two lagoons, although a significant proportion of bacteria pumped in from the River Ganol will survive in the sediment. Far fewer birds use the Bridge Pond due to regular human disturbance, and the FIB loading in this pool will be mainly due to the contaminated water from the River Ganol. During periods of disturbance (e.g. storm events or windy conditions), *E. coli* in sediments are likely to get re-suspended and cycle back through the water column. Dissimilarity in *E. coli* communities could also be generated by differences in the environmental variables between the sites, including differences in sediment structure. Site-specific variables, including temperature, salinity, grazing and competition, can dictate the survival of particular strains within each environment (Anderson *et al.*, 2005), and are likely to explain slight differences in community structure between replicates e.g. with one of the Deep Lagoon replicates. Similarly, differential survival of *E. coli* strains over time could explain differences in community structure, with the dominance of particular strains being reflected by their culturability over time. However, the significantly different composition of the *E. coli* communities in the Shallow Lagoon and the estuary suggest that FIB are not seeping through the lagoon wall and contributing to the faecal loading in the Estuary.

A related route for faecal contamination of the estuary is via direct deposition by gulls, waders and geese that have crossed over from the nature reserve to feed on the mudflats at low tide. Although the diversity of these birds changes over the seasons, the numbers of total birds has stayed fairly constant since the creation of the reserve. To understand whether direct faecal loading by wild birds significantly affects downstream concentrations of FIB would require further investigations. However, the environmental management of wild bird populations is

very different to managing an artificial nature reserve. The aim of this pilot study was to determine whether the nature reserve was a contributing factor to *E. coli* contamination of the Conwy estuary. Our results have demonstrated that although the two lagoons within the nature reserve represent a potential reservoir for FIB contamination, PCR-DGGE provides no evidence that they directly contribute to *E. coli* pollution in the estuary.

Although there is no gauging station in the Conwy estuary, Oliver et al. (2008) have reported mean daily flow rates of between $7 \text{ m}^3 \text{ s}^{-1}$ and $72 \text{ m}^3 \text{ s}^{-1}$, while the estimated volumes of the Shallow and the Deep Lagoon are 30000 m^3 and 100000 m^3 , which when scaled up from our measurements contain 40×10^{10} CFU and 14×10^{12} CFU respectively. Importantly, this calculation does not take into account seasonal fluctuations, the *E. coli* persisting in the sediment or viable but non-culturable cells, and also presumes that waterborne *E. coli* are homogeneously distributed throughout the lagoons. Previous studies have detected an average of 400-500 CFU *E. coli* 100 ml^{-1} in the Conwy estuary (Quilliam et al., 2011b; Thorn et al., 2011); therefore, although the water in the lagoons does represent a point-source reservoir for FIB, the potential impact on microbial pollution in the estuary would be relatively low due to the significant dilution effect caused by the large volume of water passing through the estuary.

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APPENDIX III

Epizoic barnacles act as pathogen reservoirs on commercial shellfish beds: Implications for the shellfish industry

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

Note: This work was presented orally by Katie Clements at the 9th ICMSS conference.

Epizoic barnacles act as a reservoir for pathogenic bacteria on commercial shellfish beds: Implications for the shellfish industry.

Katie Clements¹, Luis Giménez¹, Davey L. Jones³, James Wilson⁴, Shelagh K. Malham².

¹School of Ocean Sciences, Environment Centre Wales, Bangor University, Bangor, Gwynedd, LL57 2UW. Email: ospc02@bangor.ac.uk. ²Centre for Applied Marine Sciences, Environment Centre Wales, Bangor University, Bangor, Gwynedd, LL57 2UW. ³School of Environment, Natural Resources and Geography, Bangor University, Bangor, Gwynedd, LL57 2UW. ⁴Deepdock Ltd, Bwthyn Y Mor, Llanfaethlu, Holyhead, Anglesey, LL65 4HD.

Abstract

Routine monitoring of commercial shellfish beds in the European Union currently focuses on quantifying the bacterial content within shellfish flesh as an indicator of faecal contamination. Previous studies have documented the presence of other significant bacterial reservoirs within commercial shellfish beds e.g. sediments. This study examined the importance of epizoic barnacles as a potential bacterial reservoir across three intertidal mussel *Mytilus edulis* beds in North Wales, UK. Results demonstrated that over 80% of the total coliform reservoir was held within the epizoic barnacles in comparison to the mussel flesh, concluding that epizoic barnacles represent a significant bacterial reservoir within commercial shellfisheries. The implications for the shellfish industry are discussed.

Keywords: Indicator organisms, Faecal Coliforms, Bacteria, Reservoirs, Human Pathogen

Introduction

The consumption of bivalve shellfish has been cited as the causative agent in several cases of foodborne illness (Potasman *et al.* 2002) primarily due to their ability to bio-accumulate pathogenic micro-organisms (Roslev *et al.* 2009). To protect consumers, and to preserve the quality of the shellfish products, the industry is closely regulated. In the European Union (EU), by law, all commercial shellfish beds must be routinely monitored for potential microbial contamination (EU 2004 a,b).

Indicator species are often used as a proxy for pathogenic species (Field and Samadpour 2007). EU legislation (EU 2004a) uses *Escherichia coli* as a generic indicator organism for both pathogenic bacteria and viruses, assigning each commercial shellfish bed a classification based on routinely monitored *E. coli* concentrations within the shellfish flesh (Table 1). The assigned

classification minimises the risk to consumers and also helps to promote the economy of the shellfish industry by providing reassurance to consumers that the product they are purchasing is considered safe for consumption.

Table 1: Summary of the European Microbial standards based on *E. coli* per 100g by 5 tube, 3 dilution MPN method (EU, 2004a)

Classification	<i>E. coli</i>	Information
A	< 230	Live shellfish may be collected and sold directly for human consumption
B	< 4,600	Live shellfish may be collected and placed on the market for human consumption only after purification treatment or relaying as to meet the standards for class A.
C	< 46,000	Live shellfish may be collected but placed on the market for human consumption only after relaying to meet the standards for class A.

However, current legislation focuses only on the bacterial levels contained within the shellfish flesh itself potentially ignoring other sources of contamination. Recent shifts in consumer preference for food stuffs viewed as more “natural” (Acebron and Dopico 1999) combined with an increasing global pressure to supply the human population with cheap protein (Naylor *et al.* 2000) have led to the increase in sales of shellfish sold complete with their epizoic (associated) organisms e.g. barnacles.

Whilst other potential bacterial reservoirs on commercial shellfish beds have been identified e.g. sediments (Martinez-Manzanares *et al.* 1992), epizoic organisms have yet to be fully assessed as a reservoir for potentially pathogenic micro-organisms.

Research on the bacterial content of shellfish flesh is abundant (Oliveira *et al.* 2011), however research on other potential pathogenic reservoirs found *in situ* on commercial mussel beds is currently lacking. Therefore the primary aim of this work was to determine if epizoic barnacles associated with the common mussel *Mytilus edulis* represented a significant *in situ* bacterial reservoir for pathogenic bacteria and the potential implications this may have for the global shellfish industry.

Materials and Methods

Sampling Location

Three intertidal, commercial mussel (*Mytilus edulis* L.) beds were sampled between 1st April and 10th April 2011. Located in the Conwy region (North Wales, UK), Conwy Bridge (53.280279N, -3.838767W), Llanfairfechan (53.259132N, -3.980289W) and Conwy Morfa (53.298015N -3.854535W) represented three commercially harvested shellfish beds that are routinely monitored for bacterial contamination. Mussels and their associated barnacles were collected by hand from 15 random sample points per mussel bed and

subsequently pooled prior to laboratory analysis for bacterial determination. All samples were transported and stored at 4°C and processed within 6 h of collection.

Determination of Bacterial Load

Only live shellfish were chosen for evaluation. Shellfish samples were washed with sterile seawater to remove any residual sediment and debris before surface swabbing with 100% methanol to eliminate the surface biofilm. Samples were left to dry for 30 min at room temperature to allow the methanol to fully evaporate before aseptically removing 50 g of the encrusting barnacles and adding them to 50 mL of 25% strength Ringer’s solution. The associated mussels were then opened aseptically and 50 g of flesh and extra cellular fluid was obtained. Barnacle and 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 was plated onto Brilliance® selective agar (#CM1046; Oxoid Ltd, Basingstoke, UK) to determine total coliform counts. All plates were inverted and incubated at 37°C and bacterial colony forming units (CFU) enumerated after 24 h.

Statistical Analysis

Data was analysed using PASW statistics v18 (IBM Corp., Armonk, NY). Normality was assessed using a one sample Kolmogorov-Smirnov test ($P \geq 0.05$). Bacterial count data was analysed using the Independent Samples Kruskal-Wallis test and any significant differences ($P \leq 0.05$) were investigated further using independent samples Mann-Whitney U test, with fixed factors of either site or bacterial species and three replicate units per analysis.

Results

Total coliform concentrations were significantly higher in barnacles compared to mussels across all three sample sites ($P < 0.05$;

Fig. 1). There was also a significant difference in coliform concentration observed in both barnacles ($P = 0.001$) and mussels ($P = 0.033$) between the three sampling sites. Total coliform levels in mussel tissues were all below the upper threshold for European Union “Class B” classification (4,600 *E. coli* CFU 100 g⁻¹). In contrast, barnacles at all sites showed total coliform concentrations in exceedance of the upper threshold for this critical classification.

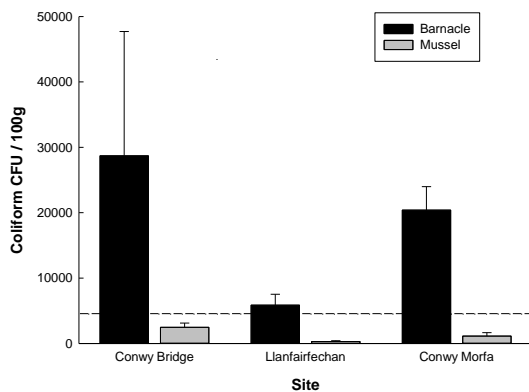


Figure 1: Total coliform population observed in mussels and barnacles across three commercial shellfish beds. For reference the dashed line represents the upper EU threshold for ‘class B’ grading of mussels (4,600 *E. coli* CFU 100 g⁻¹) (EU 2004a). In all cases $n = 15$ where data points represent the mean \pm SEM.

Coliform concentrations corrected by weight per unit area (data not shown) and expressed as a percentage of the total (Table 2), show that across all sites the epizoic barnacles are a much larger reservoir for coliform bacteria than their associated mussels. The bacterial reservoir contained within the mussel tissues is less than 20% of the reservoir contained within the barnacles attached to the shell of the mussel. Llanfairfechan showed the lowest coliform concentrations (Fig. 1) and also the lowest relative coliform reservoir present within the mussel tissues.

Table 2: Proportion of the coliform reservoir associated with mussels and their epizoic barnacles in three commercial shellfisheries.

Shellfishery	Coliform reservoir (% of total)	
	Mussel	Barnacle
Conwy Bridge	13.6	86.4
Llanfairfechan	3.0	97.0
Conwy Morfa	16.6	83.4

Discussion

The results of this study demonstrate that epizoic barnacles are a significant bacterial reservoir on commercial shellfish beds, containing over 80% of the total coliform concentration per unit area. Little data exists on the *in situ* bacterial flux between shellfish and their epizoic organisms. This study highlights the need for further research in this area.

The ability of sediments to act as a reservoir for bacteria has been well documented (Martinez-Manzanares *et al.* 1992) as a direct result of this, shellfish collection protocols for routine monitoring state that shellfish samples must be rinsed to remove sediment and debris to avoid recontamination during transit (CEFAS 2008). Epizoic barnacles have been shown to be a significant bacterial reservoir (Clements *et al.* 2013a) and the capability of epizoic barnacles to recontaminate harvested shellfish was first documented in 1947 (Clegg and Sherwood 1947) however limited research and documentation in this area (Sagoo *et al.* 2007) has failed to quantify the bacterial flux between organisms *ex situ*. Further study is needed to quantify this flux and to determine accurate protocols to minimise the impact of secondary contamination of commercially harvested shellfish during transit and storage.

It has also been inferred that different barnacle species may show differential accumulation of bacteria (Clements *et al.* 2013a). Further research is also required to quantify the accumulation of bacteria between different barnacle species and assess the impact of this to the shellfish industry.

Recent research on commercial shellfish entering depuration or purification facilities complete with epizoic barnacles have shown that the barnacles eliminate bacteria at a slower rate than the corresponding shellfish (Clements *et al.* 2013b). Bacterial indicator concentrations within the epizoic barnacles indicated that a longer depuration time may be necessary for shellfish entering depuration complete with epizoic barnacles. The same research also tentatively suggested that depuration times could be halved for shellfish entering the system without epizoic organisms. This would have huge economic implications to the shellfish industry.

The shellfish industry has a responsibility to provide consumers with a product that is of good quality and is safe to eat. Compliance with current EU legislation (based on indicator organisms) minimises the risk to the consumer. However, current legislation and protocols should 'factor in' new research to not only protect the consumer, but to safeguard the shellfish industry.

Acknowledgements

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This work represents a short synopsis, for a full account of the work undertaken please refer to Clements *et al.* (2013a).

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APPENDIX IV

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

Manuscript submitted to the proceedings of the 9th International Conference on Molluscan Shellfish Safety (ICMSS). Based on the work presented in Chapter 6.

Note: this work was presented orally by James B Winterbourn at the 9th ICMSS conference.

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 Clements², James Lowther⁴, Shelagh K. Malham², James E. McDonald³ and Davey L. Jones¹

¹School of the Environment, Natural Resources & Geography, Bangor University, Bangor, Gwynedd, LL57 2UW, UK *afpc58@bangor.ac.uk, ²School of Ocean Sciences, Bangor University, Bangor, Gwynedd, LL59 5AB, UK, ³School of Biological Sciences, Bangor University, Bangor, Gwynedd, LL57 2UW, UK, ⁴Cefas, 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 virus (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).

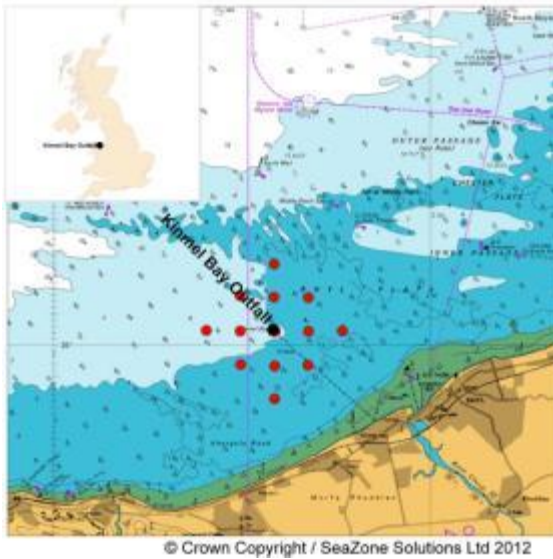
Materials and Methods

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 m³ d⁻¹ with a dry weather flow not exceeding 15,941 m³ d⁻¹. Sewage released from the outfall receives only secondary

treatment (activated sludge). No tertiary treatment is applied. 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. 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*. Aliquots 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) T_0 $n=10$. *In situ* $n=3$ per site/month.

Statistical and geostatistical analysis

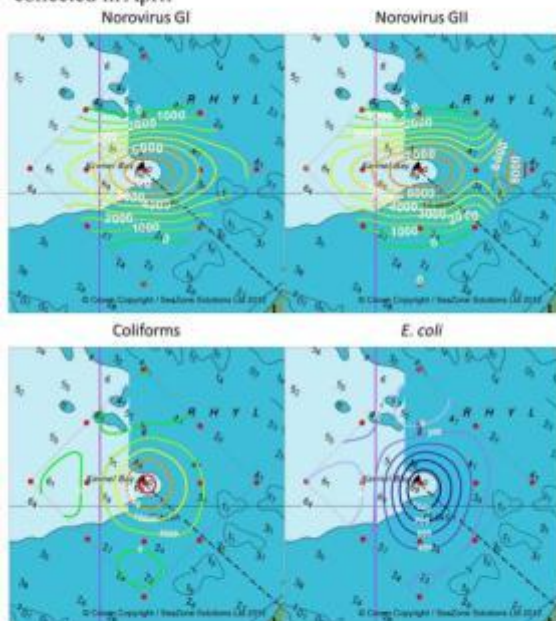
To ensure our data are comparable with UK survey data generated by the National Reference Laboratory (Lowther *et al.* 2012a), samples returning “not detected” results for a particular NoV genogroup were assigned a score of 20 copies/g for that genogroup (half the limit of detection (LOD)). Samples giving 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, however were 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 detected 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 correlative 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

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APPENDIX V

Sediment composition influences spatial variation in the abundance of human pathogen indicator bacteria in the Conwy Estuary, North Wales

Tracy L. Perkins¹, **Katie Clements**², Jaco H. Baas², Colin F. Jago², Davey L. Jones³, Shelagh K. Malham^{2,4} and James E. McDonald^{1*}.

School of Biological Sciences¹, School of Ocean Sciences², School of Environment, Natural Resources and Geography³, Centre for Applied Marine Sciences⁴, Bangor University, UK

Running title: Faecal indicator bacteria in estuarine sediments

*For correspondence E mail: j.mcdonald@bangor.ac.uk, Tel: +44(0)1248 383077

Manuscript prepared for submission to Environmental Microbiology. Due to potential IP issues only the abstract is presented in this thesis. KC assisted with the experimental design, processed the water samples, conducted the data analysis for the water samples and contributed to the final manuscript.

Abstract

Faecal bacteria that may comprise human pathogenic strains enter estuarine environments via several point and diffuse sources (e.g. wastewater, agricultural runoff). In order to determine the relationship between sediment composition (grain size and organic matter) and the abundance of pathogen indicator bacteria (PIB), sediment and water samples were collected from four transverse transects of the Conwy estuary, UK. The abundance of culturable *Escherichia coli*, coliforms, enterococci, heterotrophic bacteria, *Campylobacter*, *Salmonella* and *Vibrio* spp. in sediments was determined in relation to sediment grain size, organic content, salinity, depth and temperature. Sediments that comprised of higher proportions of silt and/or clay, and therefore organic content, showed significant positive correlations with the abundance of coliforms, enterococci, heterotrophs and *Vibrio* spp. In addition, the abundance of each bacterial group detected (with the exception of *E. coli* with heterotrophs) was positively correlated with the presence of all other groups enumerated. *Campylobacter* and *Salmonella* spp. were not isolated from estuarine sediments. Enumeration and comparisons of culturable *E. coli*, coliforms and *Vibrio* spp. revealed that their abundance was 282, 426 and 58-fold greater in sediments when compared with the water column, respectively. Faecal bacterial abundance in water and sediment showed no correlation with depth, salinity or temperature. However, the abundance of ubiquitous *Vibrio* spp. residing within the water column displayed a positive correlation with salinity and a negative correlation with temperature. These data provide important insights into sediment conditions that favour 'hotspots' of potential pathogen contamination, with implications for the modelling and prediction of public health risk based on sediment re-suspension and transport.

APPENDIX VI

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

JAMES B. WINTERBOURN,¹ KATIE CLEMENTS,² DAVID L. JONES,¹ AND
SHELAGH K. MALHAM³

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;*

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

Manuscript currently in preparation for submission to the Journal of Shellfish Research. Due to potential IP issues, only the abstract is presented in this thesis. Bacteriological work was conducted by KC and virological work was conducted by JBW. All authors discussed results and contributed to the preparation of the manuscript.

Abstract

Bivalve shellfish have the capacity to accumulate human pathogens from growing waters contaminated with human sewage. Depuration in clean seawater is the principle control measure applied in the E.U. to reduce health risks associated with contaminated shellfish. Subsequent suitability for sale is determined by compliance with bacterial end-product standards. This approach was historically successful in reducing the occurrence of shellfish-vectoring illness of bacterial aetiology. However, noroviruses (NoV) are now considered the principle agent of shellfish associated gastroenteric illness. Recently developed quantitative methods for NoV have demonstrated that NoV contamination is difficult to eliminate from oysters under depuration and may persist after bacterial standards are met. Such studies have mostly been applied to oysters because they are often eaten raw or lightly cooked, accentuating the risk to consumers and occurrence of outbreaks. Less information relating to other bivalve shellfish sold for consumption is available. This experiment aimed to determine the efficacy of a commercially operated depuration system to eliminate NoV from commercially harvested mussels (*Mytilus edulis*), which have also been implicated in illness following raw consumption and to which virological standards may apply in future. Two commercially harvested batches of mussels were tested for NoV at harvest and immediately pre- and post-depuration, using a quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). A sub sample of the first batch was also depurated under laboratory replicated 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 system was able to successfully reduce NoV contamination in live *Mytilus edulis*.

Keywords; Purification, shellfish, norovirus, depuration, *E. coli*.

APPENDIX VII

Evaluation of the use of brilliance[®] selective media for the enumeration of *E. coli* and total coliforms from food and environmental samples.

Introduction

Current routine bacteriological monitoring of shellfish utilises *E. coli* as a proxy for pathogenic micro-organisms. The current standard of enumerating *E. coli* from shellfish samples utilises a standardised national protocol for sample collection and enumeration, consisting of a 5 tube, 3 dilution Most Probable Number (MPN) method (ISO/TS 16649-3:2005). However, this technique returns a value after a 48hr incubation period. Brilliance™ *E. coli* / total coliform selective agar (#CM1046, Oxoid, Basingstoke, UK) represents an alternative method of enumerating *E. coli* from shellfish samples, via direct plating, with only a 24 hour incubation period. The aim of this study was to compare the accuracy of the two methods.

Materials and Methods

Shellfish samples were collected from three commercial mussel (*Mytilus edulis*) beds in North Wales, UK in accordance with national sampling guidelines. Three replicate samples from each site were processed at the National Reference Laboratory (NRL) in accordance with ISO/TS 16649-3:2005. Three samples (from the same batch) were also processed using the direct plating method as outlined in Chapter 2. Post-incubation the results from the two methodologies were compared.

Results

The results from the methodological comparison can be seen in Fig. 1. The *E. coli* concentrations determined from both methodologies appears to be similar. No statistical analysis could be performed due to the absence of raw data values from the MPN results.

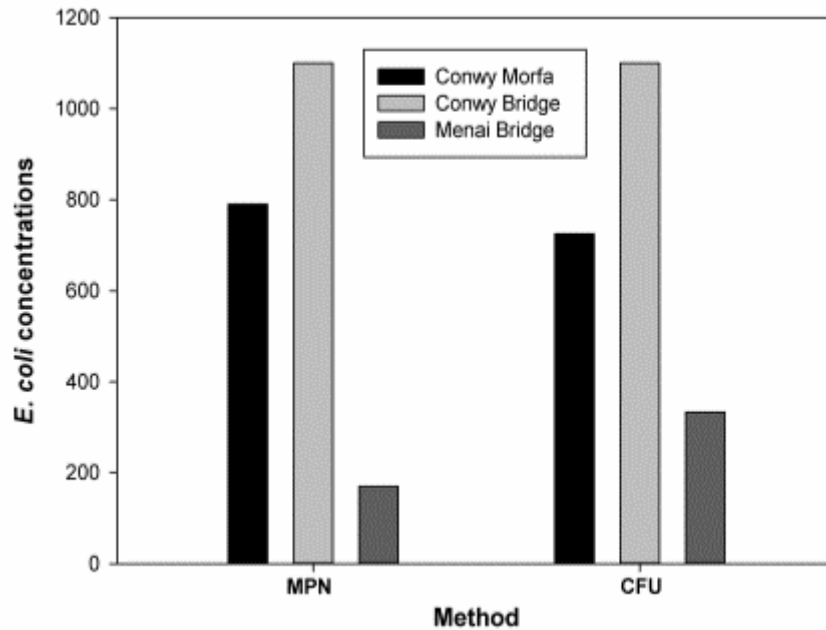


Fig. 1. Comparison of the MPN and direct plating methods for the enumeration of *E. coli* from shellfish samples.

Discussion

The results from this study suggest that direct plating using Brilliance™ selective media is a suitable alternative to the MPN method utilised under ISO/TS 16649-3:2005. The advantages of the direct plating method are its simplicity and the faster analysis of shellfish samples (24 hours as opposed to 48 hours). However, it must also be noted that the results of this study are severely limited due to the small number of sample sites utilised. Further extensive work needs to be conducted in order to appropriately validate the use of direct plating using selective agar with the current methodology utilised in European legislation, including inter-laboratory ring trials, comparisons with different shellfish species and geographical areas.

References

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APPENDIX VIII

Industry directed research

Introduction: Funding body and project objectives

The Knowledge Economy and Skills Scholarship (KESS) is a joint EU and Welsh Government scholarship established in Wales in 2009. Funded through the European Social Fund (ESF), KESS scholarships are designed to build collaborations between academic institutions and external industrial partners based in the convergence area of Wales. This PhD project was completed in collaboration with researchers at Bangor University and the local shellfish industry, represented through the consortium of 'Bangor Mussel Producers' which incorporated three local mussel production companies in North Wales, UK. As part of the KESS program participants are obliged to spend four weeks per annum working with their industrial co-sponsor in order to gain experience working within an industrial capacity. In this case, due to the nature of the mussel fishing industry, the industrial placement was carried out in smaller work packages over the three year duration of this PhD and included a week long internationally accredited course (STCW 95), which is a requirement for all boat crew and includes modules on sea survival, first aid at sea and radio operation. Other work packages included boat and shore based mussel surveys to monitor the ecology of the local commercially harvested mussel beds. Boat based surveys were also undertaken to monitor by-catch species within commercial trawling operations. In addition, a limited amount of industry directed research was conducted (Appendix II and VIII) which focused exclusively on addressing research questions or concerns from the shellfish industry. Research outcomes were disseminated verbally to representatives from the shellfish industry during regular scheduled meetings and also disseminated to a wider audience at bi-annual "Conwy estuary stakeholder meetings". These meetings were 'open invitation' events and were attended by a diverse stakeholder group including academic representatives from Bangor University, industrial representatives from the shellfish industry, Welsh Water, representatives from the local councils and regulatory bodies such as Natural Resources Wales (formally known as the Environment Agency and Countryside Council for Wales), also in regular attendance were representatives from many non-government organisations such as 'surfers against sewage' and the RSPB (Royal Society for the Protection of Birds) as well as interested members of the public. These meetings proved to be very useful for research dissemination as well as discussion of the research findings, directing future research efforts and also for promoting the collaboration between academia and industry.

Case Study: The Menai Bridge mussel (*Mytilus edulis*) fishery.

Introduction

Mussels (*Mytilus edulis*) collected from sub-littoral beds off the North Wales coast for commercial purposes on the 3rd March 2011 were then shipped via Holland and onto France. Despite passing quality control checks in Holland the entire consignment of mussels, consisting of 60,000 tonnes was condemned due to a single case of shellfish vectored illness in France, traced back to the original mussel shipment from North Wales. As a result of this the local mussel fishing industry enlisted the assistance of researchers at Bangor University to test a selection of sample points across the commercial bed in question to determine if the mussels had been contaminated *in situ* or during transit to the final point of retail.

Materials and methods

The local shellfish producers identified seven potential sites across one commercially harvested mussel bed, located in the Menai Straits in North Wales (Fig. 1). At each sample site samples of mussels, water and sediments were collected, in triplicate, and subsequently transported back to the laboratory for analysis (following standard protocols).

Mussel samples were collected via short trawl and were subsequently processed to determine the total *E. coli* content as described in Chapter 2. Water samples were collected in sterile containers approximately 1m below the water surface and processed as described in Chapter 5 and Appendix I. Sediment samples were taken using a small 30 cm x 30 cm van veen grab and subsequently processed as described in Chapter 2.

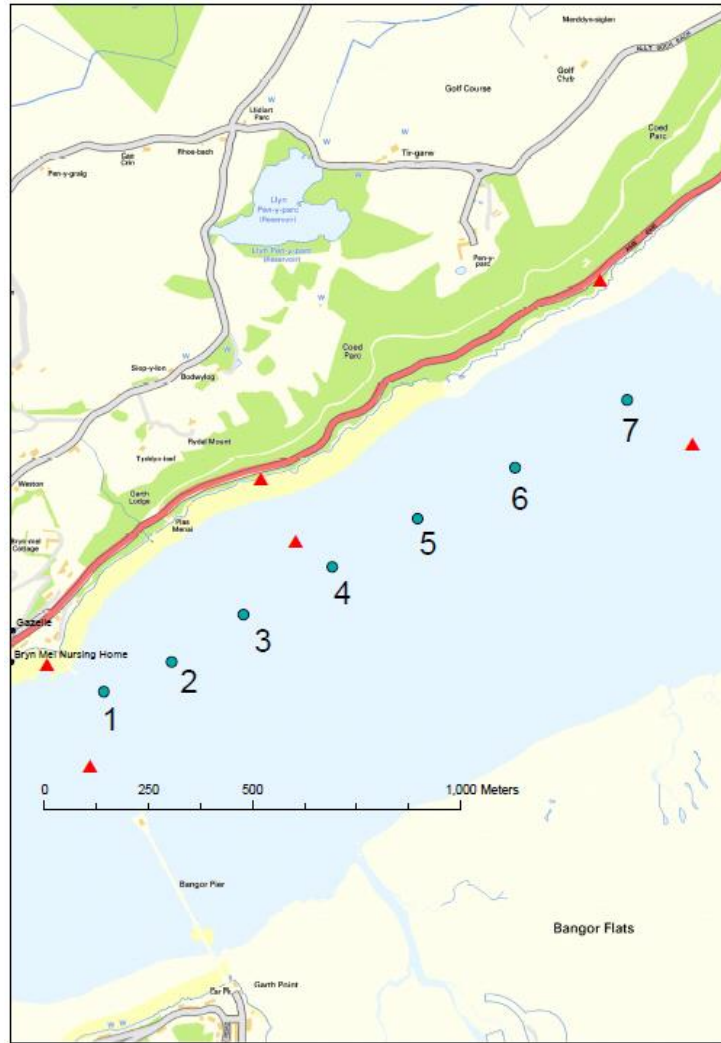


Fig.1 Map showing the location of the sample sites within the Menai Strait, North Wales (circles). Triangles mark the location of known sewage discharges.

Results

During sampling the sediment results became contaminated with seawater, subsequent laboratory processing did not determine any *E. coli* from any of the collected samples (Data not presented). 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).

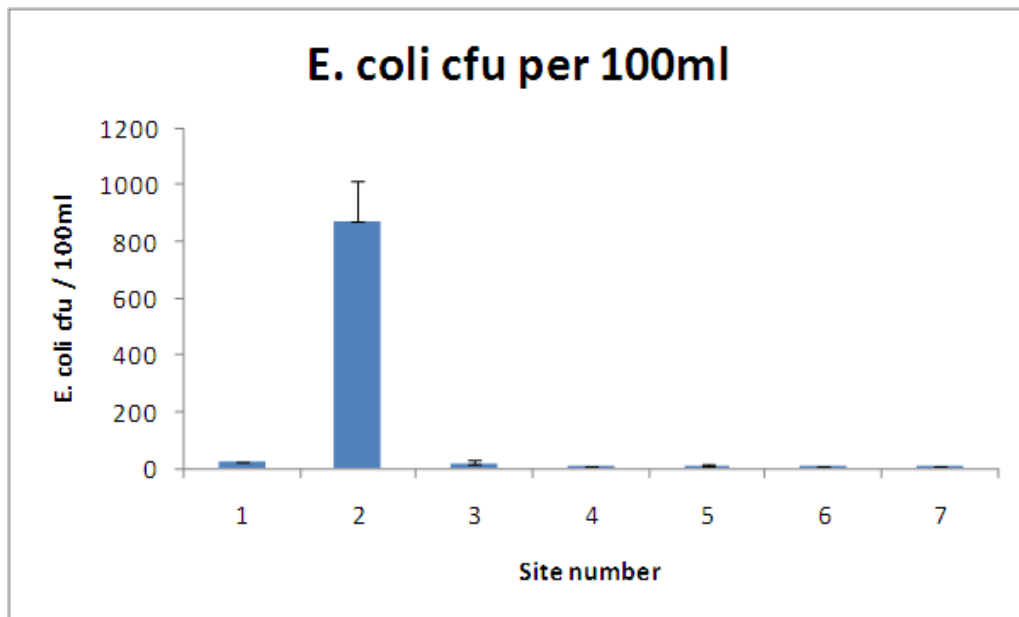


Fig. 2 Determined *E. coli* concentrations from water samples. Expressed as *E. coli* CFU / 100 ml. Error bars represent the SE and n = 3 in all cases.

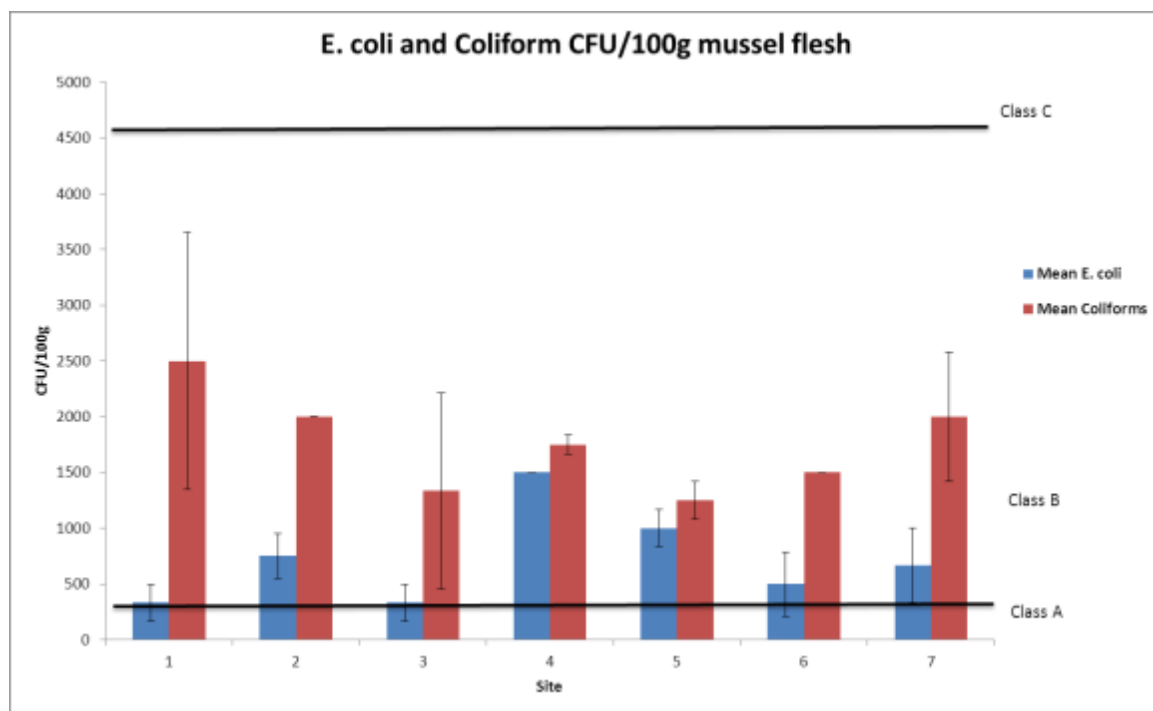


Fig. 3 Determined *E. coli* and total coliform concentrations from mussels, expressed as CFU / 100 g flesh and intervalvular fluid. Error bars represent the SE. n = 3 in all cases.

Discussion

The results from this study conclude that there is no major source of contamination affecting the mussels in this region and that these mussels should be considered safe for commercial harvesting. The anomalous result of the water at site 2 could be explained by contamination during collection or laboratory processing. Environmental contamination should be considered as a factor in explaining this result, however further research would need to be conducted in this area.

A follow up study was planned for one week after the initial sample collection in order to compare the levels of *E. coli* over time. It was proposed that this site would be sampled weekly and the results disseminated to the local shellfish industry. However, following the first sample collection and results dissemination to the shellfish producers the area was trawled, therefore no comparative study could be made.

APPENDIX IX

Other investigations undertaken

1. Intensive survey of the Conwy Estuary, North Wales.

In October 2010 an intensive boat based survey of the Conwy Estuary, North Wales was undertaken to assess for sources of faecal contamination. Results from this survey are presented in Appendix I and II. Additional work included a longitudinal survey of the Conwy river, from the estuary to the tidal limit on both the flood and the ebb tide, collecting water samples for the enumeration of *E. coli* (Fig.1).

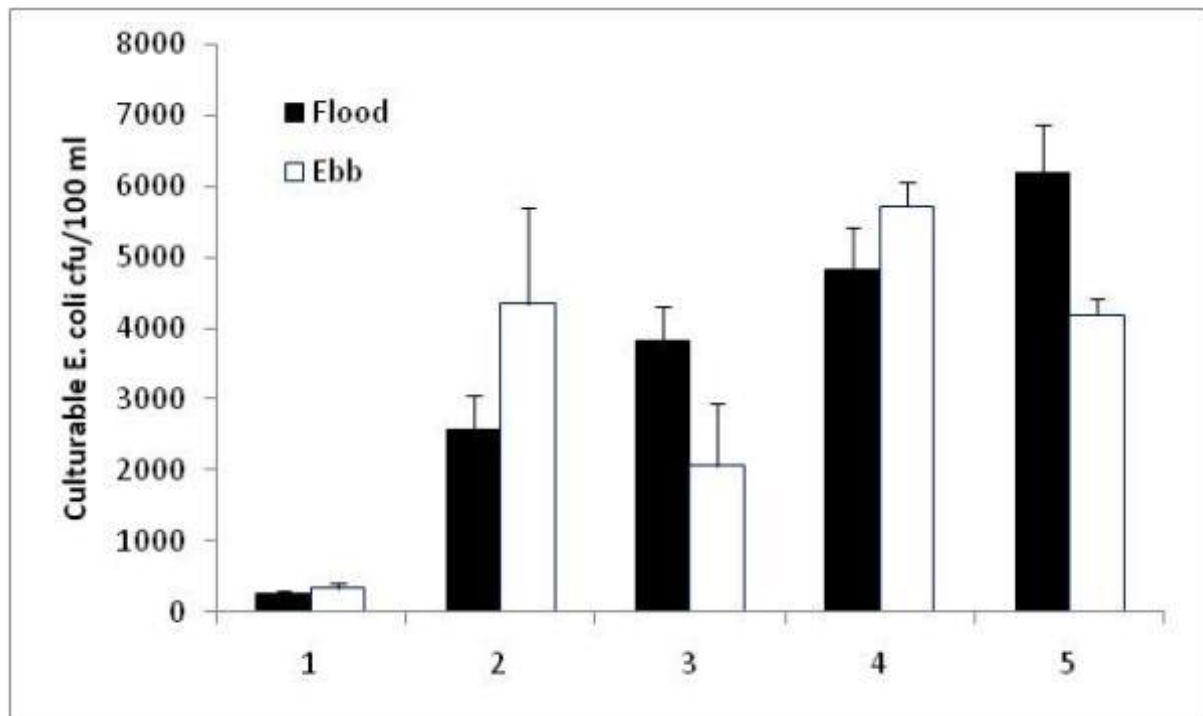


Fig.1. Concentrations of *E. coli* present in water samples at selected sampling points along the Conwy river. Site 1 represents the estuary and site 5 the tidal limit. $n = 4$ in all cases.

Water samples were processed as described in Appendix I. On the flood tide the *E. coli* concentrations increased with increasing distance upstream. However on the ebb tide the *E. coli* concentrations appeared to be affected by the dynamics of the river flow.

On the basis of both these results and the results described in Appendix I, further investigation into the sources of *E. coli* contamination was conducted. This consisted of both boat and land based surveys to determine possible point and non-point sources of contamination. This data was then used to focus subsequent spatially intensive studies into microbial pollution in the Conwy Estuary (Data not presented). Finally, the temporal fluctuations of *E. coli* within water samples was investigated at the tidal limit of the Conwy river over a 24 hour period (Fig. 2).

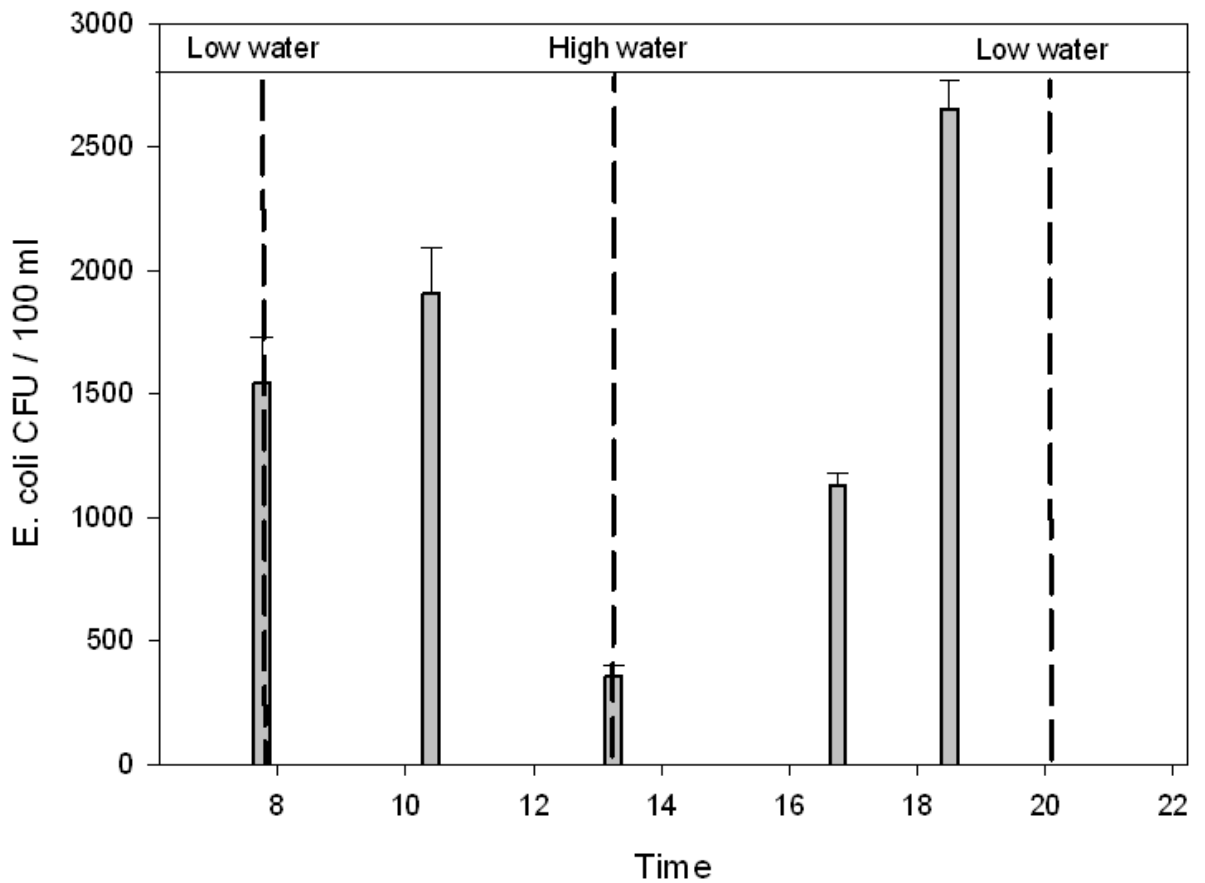


Fig.2. Temporal changes in *E. coli* concentrations in water at the tidal limit of the Conwy river. $n = 4$ in all cases + SE.

The findings from this study concluded that there was a significant effect of the tidal state on the determined concentrations of *E. coli*. This data was used to inform subsequent sampling events.

2. Potential application of Petrifilm™ plates to enumerate *E. coli* and coliforms from seawater samples.



Fig.3. Petrifilm plates for the enumeration of *E. coli* and coliforms.

Seawater samples collected from various locations around the North Wales coast were simultaneously analysed to determine the *E. coli* concentrations, by means of Petrifilm plates and the standard vacuum-filtration method as described in Appendix I. Detection of *E. coli* CFU from seawater was significantly lower when using the Petrifilm plates compared to the vacuum filtration method (Data not presented). In conclusion, petrifilm plates are unsuitable for accurate *E. coli* determination from seawater samples.

3. Potential of pea crabs (*Pinnotheres pisum*) to act as a pathogen reservoir on shellfish beds.

Pea crabs (*Pinnotheres pisum*) are a known parasite of commercial shellfish, this study aimed to investigate their potential as a bacterial reservoir on commercial shellfish beds. This investigation was carried out over the summer of 2012 when it was anticipated that there would be high numbers of pea crabs present within mussels (*Mytilus edulis*), based on observations from the previous year. Despite opening in excess of 2,000 mussels, only 1 pea crab was located in 2012.

4. Does intervalvular fluid act as a bacterial reservoir?

Intervalvular fluid (the fluid contained within the shell cavity of shellfish) has been proposed to act as either a bacterial reservoir, or a dilutant of bacterial concentrations in shellfish samples taken for routine monitoring procedures. This investigation aimed to quantify the concentrations of *E. coli* and total coliforms within the intervalvular (IV) fluid of shellfish samples and to compare them to the bacterial reservoir contained within the shellfish flesh. Samples of IV fluid were taken alongside samples of mussel flesh and subsequently processed by both direct plating and vacuum filtration to determine the *E. coli* concentrations respective to the concentrations determined directly from the shellfish flesh. In all cases, no *E. coli* was detected from the IV fluid, despite the shellfish flesh containing greater than 230 *E. coli* CFU / 100 g. We can therefore conclude that IV fluid does not represent a significant bacterial reservoir within commercial shellfish beds.

5. What's in a mussel? Comparison of the bacterial content of small v large mussels and male v female mussels.

It was hypothesised that there would be a differential uptake of bacteria between small (<47 mm) and large (>47 mm) mussels due to the relative size differences of their inhalant siphons. This study tested three batches of small and large mussels and determined the concentrations of *E. coli*, coliforms, *Vibrio spp.*, presumptive salmonella, enterococcus,

campylobacter and total marine heterotrophs using direct plating onto selective agar. The results from this study show that 99% of the total culturable bacteria were marine heterotrophs and the other bacterial species combined, represented less than 1% of the total culturable bacteria. There was no apparent difference in the proportion of different bacterial species present in either small or large mussels.

Male and female mussels were distinguished from one another visually, by flesh colour. Three replicate batches of male and female mussels were processed to determine if the sex of the mussel has an effect on bacterial accumulation or retention. Mussel samples were processed to determine *E. coli* concentrations only. No significant difference was observed in *E. coli* concentrations between mussels of different sex.

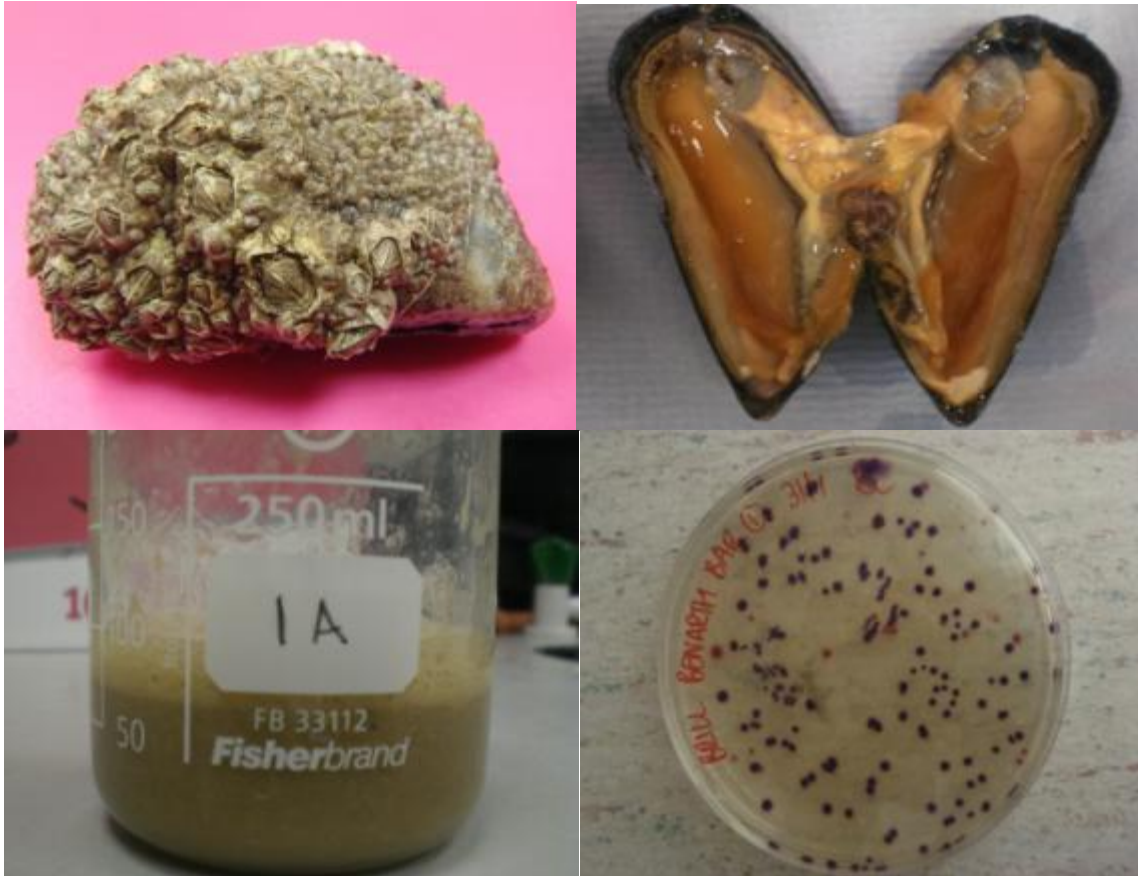
6. Bacterial competition experiment.

Homogenised mussel samples were processed to determine the concentrations of *E. coli*, coliforms, *Vibrio spp.* and marine heterotrophic bacteria from six mussel samples. After the initial measurement, three of the homogenates were incubated at 37°C and three homogenates at 4°C. Bacterial concentrations were monitored every 24 hours for 3 days. At day 3, four of the homogenates (2 from each treatment) were swapped to the opposing treatment, leaving two of the homogenates as controls. Bacterial concentrations were subsequently determined for a further 3 days. The results from this investigation show that *E. coli* and coliform concentrations rapidly increase when held at their optimum temperature (37°C) to concentrations where they are Too Numerous To Count (TNTC). Likewise, *Vibrio spp.* and marine heterotrophs were also TNTC when held at 4°C. All bacterial species displayed limited population growth when held at sub-optimum temperatures. Post-switch all species demonstrated a lag in population growth, however regardless of the original temperature, all bacterial species were TNTC when held for 3 days at optimum temperatures.

The findings from this study have subsequently led to further investigation into the bacterial competition between native and non-native bacterial species and to the investigation of bacteria in the VBNC state.

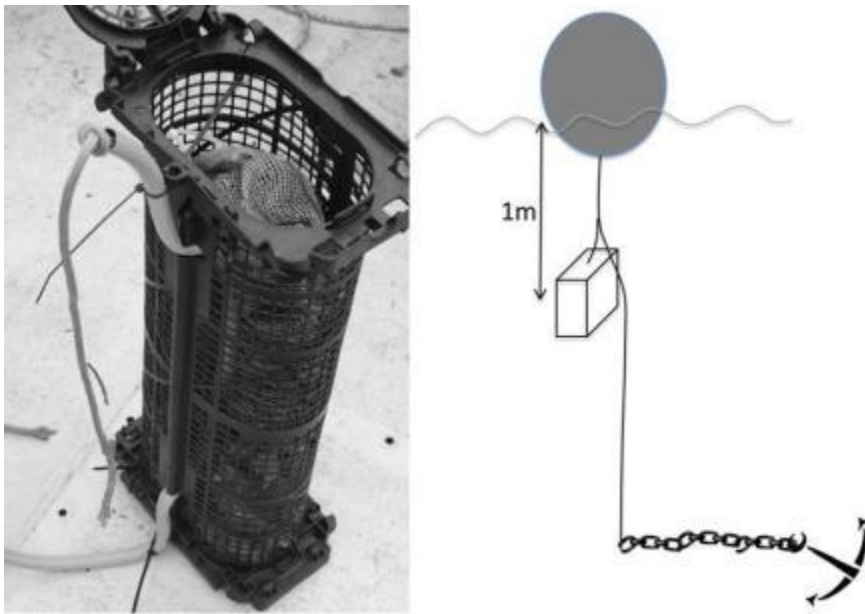
APPENDIX X

Photographs depicting the experimental methodologies



Photographs showing the process of enumerating bacteria from mussel (*Mytilus edulis*) samples. Top left: Cleaned mussel sample, complete with epizoic barnacles. Top right: opened mussel sample, showing internal organs. Bottom left: Homogenised mussel sample.

Bottom right: Bacterial enumeration from homogenised mussel samples, on Brilliance selective agar, pink colonies represent total coliform CFU and purple colonies represent *E. coli* CFU.



Photograph and schematic of the experimental apparatus used in Chapters 5 and 6.