Final Report: Evaluating the effectiveness of depuration in removing norovirus from oysters

FS101068

February 2017

Catherine McLeod[1], David Polo[2], Jean-Claude Le Saux[2] and Françoise S. Le Guyader[2]

[1]Seafood Safety Assessment Ltd. and the [2]French Research Institute for Exploitation of the Sea

Disclaimer

This report has been produced by Seafood Safety Assessment Ltd. under a contract placed by the Food Standards Agency (the Agency). The views expressed herein are not necessarily those of the Agency. Seafood Safety Assessment Ltd. warrants that all reasonable skill and care has been used in preparing this report. Notwithstanding this warranty, Seafood Safety Assessment Ltd. shall not be under any liability for loss of profit, business, revenues or any special indirect or consequential damage of any nature whatsoever or loss of anticipated saving or for any increased costs sustained by the client or his or her servants or agents arising in any way whether directly or indirectly as a result of reliance on this report or of any error or defect in this report.

Table of Contents

Executive Summary

Pollution of coastal waters with non-treated, or partially treated, human effluent can result in contamination of bivalve molluscan shellfish with microbial pathogens, including human enteric viruses. Norovirus (NoV) is now considered to be the main cause of human gastroenteritis worldwide, and is the most common viral pathogen associated with illness from shellfish consumption. Oysters are the most common shellfish species implicated in NoV outbreaks and recent studies in the UK have suggested that a significant proportion of oyster production areas contain NoV RNA.

The reduction or elimination of microbial contaminants from oysters via the process of filter feeding can occur in either the natural environment in unpolluted seawater (relaying) or in land based facilities (depuration). Depuration is an effective post-harvest treatment for the removal of most bacterial species from oysters (with the notable exception of *Vibrio* spp.) and has successfully reduced illness outbreaks of typhoid and cholera. However, the efficiency of depuration for the removal of NoV is questionable. This study was therefore commissioned to evaluate the efficacy of depuration in removing NoV from oysters through a review of the literature (Part 1), and to undertake pilot laboratory experiments to investigate a novel depuration approach (Part 2).

The literature review has identified at least 17 published articles which report illness outbreaks of NoV and hepatitis A virus (HAV) from the consumption of oysters that were subjected to depuration. Reported concentrations of NoV in oysters post depuration were between 10^2 and 10^3 genome copies/g oyster tissue, far in excess of the infectious dose which is estimated to be as low as 10 viral genome copies.

Optimising environmental conditions such as temperature and salinity to suit the physiology of oysters results in increased depuration rates for bacteria and some viral indicator species. This relates to maximising oyster clearance, filtration and digestion rates. However, these changes only result in small or no improvements in viral depuration rates for NoV and HAV, suggesting a special relationship between NoV and oysters. Indeed, genogroup I (GI) and II (GII) NoV bind to histo-blood group (HBGA) A-like ligands in oyster digestive tissues and GII NoV binds to sialic acid (SA) residues in the gills; NoV binding to these ligands is hypothesised to facilitate accumulation. Additionally, NoV and HAV show high acid resistance which may also contribute to prolonged persistence within the acidic environment of oyster haemocytes.

The differences in the way in which NoV interacts with oysters compared to other surrogate viruses which can be cultured (e.g. feline calicivirus, murine norovirus) is highlighted by the findings of this review, which shows that NoV and HAV are retained for much longer periods in oysters than surrogate viruses. For NoV, 50% of published reduction experiments (n=16) showed no reduction in levels during depuration, and for studies in which reductions did occur it took between 9 and 45.5 days for a 1 log reduction. For HAV, the estimated days to achieve 1 log reduction was between 7 and 16.1 days, with two of the five experiments showing no loss of HAV. These timeframes are clearly much longer than those used routinely for depuration in the UK (around 42 h). The culturable surrogate viruses are more rapidly depurated than NoV and HAV under a variety of depuration conditions; a comparison of the days to achieve 1 log reduction shows that the mean number of days to reduce NoV and HAV is 19 and 12 respectively, whereas for surrogate viruses the mean time to achieve 1 log reduction is 7.5 days. Given the special relationship between NoV and oysters, it is unlikely that optimising the physiology of oysters through parameters such as salinity, temperature, and presence/absence of food will result in significant reductions, as demonstrated by studies to date.

Given that depuration does not reduce NoV to levels below an infectious dose and illness outbreaks have occurred from consumption of depurated oysters, depuration is not an effective post-harvest control for NoV in oysters at this time. On the other hand, relaying has been more successful, with NoV reduced to around the LoD of the test method when oysters are placed in clean open seawater for around four weeks, and no illnesses have been reported to be associated with relayed products.

This literature review has identified a number of data gaps and uncertainties, including:

- There is no direct information on the infectivity of NoV following depuration and relaying, however some information on infectivity can be inferred from studies with HAV for which a culture method exists, and from illness outbreaks that have occurred following depuration;
- The quantitative test methods for NoV used in some historical depuration studies may not have been appropriate, due to a lack of quantitative viral standards and lack of rigour in the sampling plans used;
- The biological basis of why HAV persists in oysters for long periods is unknown, further work on specific interactions between HAV and oysters may assist in elucidating this;
- For most illness outbreaks involving depurated oysters, the depuration conditions used are not known/stated;
- Few depuration studies have sought to optimise a combination of conditions;
- The relative rates of depuration of NoV from Pacific and Native oysters is unknown; and
- The depuration rates of naturally contaminated oysters vs. lab contaminated oysters has not been investigated.

Thus, it is suggested that the following topics be given high priority when considering further research and work in this area:

- A major focus should be placed on improvements in coastal water quality. Further collaboration between UK water companies, Local Authorities and industry should be prioritised in order to develop cohesive and practical strategies to achieve this goal.
- Collaborative research to investigate the infectivity of NoV in oysters during depuration and relaying.
- Improving understanding of the virus-oyster relationship and specifically the binding interactions.
- Investigations into post-harvest interventions that aim to disrupt the specific binding of NoV to oysters.

Depuration approaches which include a step to exploit/disrupt the specific linkage between NoV and HBGAs in oysters may enhance the reduction of NoV. As part of this project a pilot study (Part 2) was undertaken to investigate if such an approach holds promise. The idea was to test different compounds which were selected on the basis of their activity against HBGAs, with the hypothesis that the treatment would destroy the ligands and lead to the release of NoV particles inside the oyster tissues during depuration.

The efficacy of eight different compounds in reducing levels of NoV in oysters were evaluated using two experimental approaches. Firstly, ten depuration trials were conducted in which oysters contaminated with NoV were dipped in one of the selected compounds and then subjected to depuration; and secondly an *in vitro* approach was used to evaluate compound efficacy, this involved treating the digestive tissue and gills of oysters that had accumulated NoV with different compounds in cell culture plates.

The results of the dipping and *in vitro* experiments showed that two compounds, proteinase K and papain, have promise in further reducing NoV concentrations during depuration. The development of the *in vitro* test was valuable and allowed more rapid screening of potentially effective compounds.

While the pilot studies show promise, further experiments are recommended to confirm the potential of these compounds to enhance depuration:

1. Quantitation by real time RT-PCR was not precise enough to discriminate levels within a log; further work using a more precise method such as digital PCR would assist in further evaluating efficacy of the compounds.
2. The effect of proteinase K and papain were evaluated using grossly contaminated oysters; the work should be repeated using oysters contaminated at a lower level (such as those naturally contaminated in the environment), and for accidental contamination events in which oyster exposure to NoV is short (i.e. for less than an hour).
3. Lastly, the reduction effect may be enhanced further by trialling options to deliver the compounds more directly to the sites of interest within oysters. In this regard, microencapsulation of the compounds of interest may be an interesting avenue to pursue.

Glossary

CEFAS	Centre for Environment, Fisheries and Aquaculture Science
dPCR	Digital Polymerase Chain Reaction
DALYs	Disability Adjusted Life Years
DT	Digestive tissue
EU	European Union
EC	European Commission
FCV	Feline calicivirus
FRNA	F-specific RNA
FSA	Food Standards Agency
GI	Genogroup I
GII	Genogroup II
GIV	Genogroup IV
GMT	Geometric mean titre
HAV	Hepatitis A virus
HBGA	Histo blood group antigen
ID_{50}	Infectious dose
ISO	International Organisation for Standardisation
LA	Local authority
LoD	Limit of detection
LoQ	Limit of quantitation
MgV	Mengo virus
MNV	Murine norovirus
NoV	Norovirus
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PFU	Plaque forming units
PK	Proteinase k
PV	Poliovirus
RASFF	EU Rapid Alert System for Food and Feed
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RT-qPCR	Real time quantitative reverse transcriptase polymerase chain reaction
SA	Sialic acid
ST	Supernatant component
TV	Tulane virus
UK	United Kingdom
UV	Ultra violet
VLP	Virus-like-particles
WHO	World Health Organization

Acknowledgements

We thank David Jarrad and the Shellfish Association of Great Britain for facilitating the participation of the UK oyster industry in the project. We are grateful to members of the UK oyster industry for providing advice and feedback on their current depuration practices. We thank Mandy Pyke for reading the draft review and providing useful feedback. We also thank regulatory and industry representatives from China, Netherlands, United States of America, Portugal, Norway, Ireland, New Zealand, Australia and Spain for providing advice on depuration practices in their countries. We are grateful to Dr Andreas Kiermeier, Statistical Process Improvement Consulting and Training Pty Ltd., for advice on estimating the reduction of viruses during depuration.

Part One: Literature Review of the Effectiveness of Depuration in Removing Norovirus from Oysters

Authors: Catherine McLeod, David Polo, Jean-Claude Le Saux and Françoise S. Le Guyader

1 Introduction

1.1 Background

Pollution of coastal waters with non-treated, or partially treated, human effluent can result in contamination of bivalve molluscan shellfish with a variety of microbial pathogens, including human enteric viruses. Human enteric viruses replicate in the human alimentary tract with large quantities of virus shed in the faeces (Atmar et al., 2008; EFSA, 2011). An important enteric virus, norovirus (NoV), is the predominant cause of human gastroenteritis. Recent estimates from the WHO on the global burden of foodborne diseases suggest that the most frequent causes of foodborne illness were diarrhoeal disease agents, particularly NoV and *Campylobacter*; NoV gastroenteritis was estimated to contribute 7.6% of the total DALYs (Disability Adjusted Life Years) (Anon, 2015a).

Globally, NoV is the most common viral pathogen associated with illness from shellfish consumption. Bellou et al. (2013) found that 83.7% of shellfish borne viral illness outbreaks identified (n=359) involved NoV, and 12.8% involved HAV. Bivalve shellfish may become contaminated with NoV and HAV through the process of filter feeding, in which large quantities of seawater and associated particulate matter, including any enteric viruses present, are ingested and localised in the digestive tract. While all bivalve shellfish are susceptible to contamination, oysters are more frequently implicated in illness outbreaks than other shellfish species: Bellou et al. (2013) conducted a systematic review to investigate shellfish borne viral outbreaks and found that the most common type of shellfish involved in outbreaks were oysters (58.4% of outbreaks). Clams were responsible for a reported 22.6% and mussels for 0.5% of outbreaks (Bellou et al., 2013).

There are several possible reasons why oysters appear to play a more dominant role in the transmission of NoV compared to other shellfish species, including:

1. The mode of consumption (primarily raw);
2. The close proximity of inter-tidal oyster production areas to sources of human effluent;
3. The specific retention of NoV in oysters via binding to ligands that are present within the oyster tissues (Le Guyader et al., 2006a; Le Guyader et al., 2012); and
4. Relatively slow inactivation and elimination of NoV from oyster tissues (McLeod et al., 2009a, 2009b; Richards et al., 2010).

Oysters are an important commodity in the UK, with approximately 1300 tonnes produced annually and considerable potential for industry expansion. Two species of oyster are commercially produced, the Pacific oyster (*Crassostrea gigas*) (91% of oyster production) and the Native oyster (*Ostrea edulis*). Figure 1.1 provides an overview of oyster production in the UK between 2010 and 2013 (the latest available production statistics). In accordance with EU law, bivalve shellfish production areas in the UK are classified as A, B or C, based on the presence and levels of the faecal indicator bacteria, *E. coli*. Shellfish harvested from areas that are classified as B must be subjected to depuration, cooking or relaying. In England and Wales, in 2014/15 there were 111 areas classified for the production of oysters, of which 101 were class B. Over the same period in Scotland, 33 areas were classified for the production of oysters, of which 20 were class A/B, and 13 were class A. Northern Ireland had 12 areas classified for oyster production, of which 7 were class B. Given (1) the prevalence of class B areas in the UK (78% are class B in particular seasons), (2) the requirement to either depurate, cook or relay shellfish from class B areas, and (3) the fact that cooked oysters are not normally considered a

desirable product by consumers, there is a heavy reliance by the UK oyster industry on depuration as a process control (as opposed to cooking).

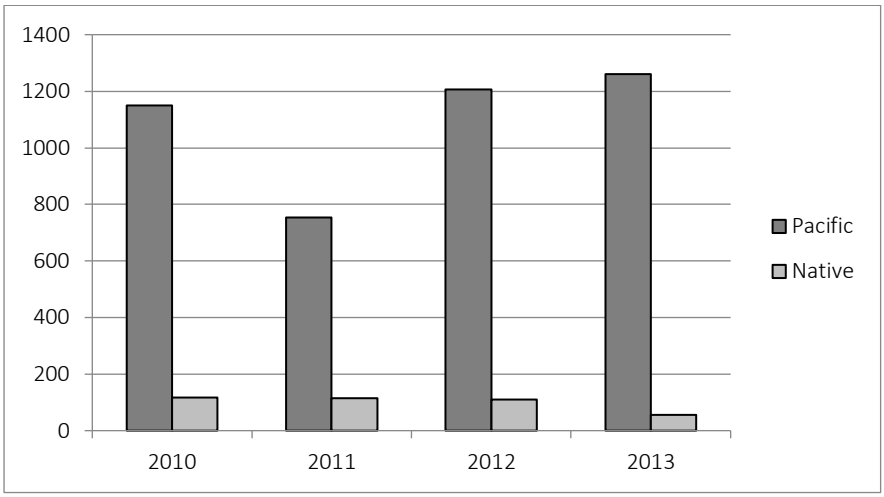

Figure 1.1. Oyster production (tonnes) in the United Kingdom (England, Wales, Scotland and Northern Ireland) between 2010 and 2013.

While it is clear that depuration is a highly effective post-harvest treatment process for removing *E. coli*, the efficiency of depuration for the removal of NoV is questionable, with several studies suggesting low reduction rates (McLeod et al., 2009a; Schwab et al., 1998; Ueki et al., 2007), and some cases of NoV related illness from oysters that had been depurated (Gallimore et al., 2005; Grohmann et al., 1981; Le Guyader et al., 2010; Stafford et al., 1997).

The illness outbreaks have prompted a variety of scientific investigations into NoV contamination of oysters, including a study to evaluate the prevalence of NoV in UK oyster production areas. The study suggested that a significant proportion of the oyster production areas contained NoV RNA, with each of the 39 production areas tested giving at least one NoV positive result (using real time Polymerase Chain Reaction (PCR) to detect the viral RNA) during the study (Lowther et al., 2012). The recent findings of high prevalence of NoV RNA, the reported illness outbreaks, and the high reliance by industry on depuration as an end product control, have together led to the prioritisation of research to evaluate the effectiveness of depuration for NoV reduction in oysters (Anon, 2012) (Food Standards Agency Conference, 2013). Elimination of NoV from oysters via depuration is thus the subject of this review.

The reduction or elimination of microbial contaminants from oysters via the process of filter feeding can occur in either the natural environment in unpolluted seawater or in land based facilities. For the purposes of this review, elimination in a natural setting is referred to as relaying, and elimination in tanks is referred to as depuration (also frequently described as 'purification' in the literature).

1.2 Aims of the review

The overall objective of this review is to evaluate the effectiveness of depuration in removing NoV from oysters. Minor additional aims of the review include:

1. To evaluate the effectiveness of relaying in removing NoV from oysters;
2. To summarise current depuration practices in the UK and overseas;
3. To review the mechanism by which NoV is bound and retained in oysters; and
4. To identify new potential approaches for viral depuration.

1.3 Scope of the review

The review involves the appraisal of literature with respect to the efficiency of depuration in removing NoV from oysters, the following study types were considered in the review:

- Depuration studies of commercially produced oyster species (including Pacific and Native oysters - *Crassostrea gigas* and *Ostrea edulis*);
- Depuration of both GI and GII NoV strains and HAV;
- Depuration of viral surrogates for NoV e.g. F+ bacteriophage;
- NoV illness outbreaks from oysters which had been depurated; and
- The binding mechanism of NoV to oysters.

The following subjects were not considered in the review:

- Depuration studies of non-oyster shellfish species;
- Depuration studies of non-related viruses and bacteria (e.g. poliovirus, *E. coli*); and
- The binding mechanism of non-related viruses and bacteria to oysters or other shellfish.

2 Approach to the Review

2.1 Literature review

Literature searches were undertaken to collate information on the following subjects:

- Illness outbreaks caused by NoV in depurated oysters;
- Persistence of NoV in oysters following depuration and/or relaying; and
- The mechanism by which NoV is bound and retained by oysters.

Depuration studies of non-oyster shellfish species and bacteria were not considered in this review. Information on the depuration of other viruses, particularly hepatitis A virus (HAV) and so called indicator or surrogate viruses were also collated.

Literature searches began with a structured electronic search using the Google Scholar and PubMed search engines. Electronic literature searches commenced with the following key words:

1. oyster AND outbreaks AND depuration AND (norovirus OR Norwalk virus)
2. (norovirus OR Norwalk virus) AND oyster AND (persistence OR relaying OR depuration)
3. (norovirus OR Norwalk virus) AND oyster AND (localisation OR binding)

For the first literature search above, the titles and abstracts of the first 300 citations identified and sorted by Google Scholar (n=1660) were reviewed for relevance. All abstracts of the citations identified using PubMed (n=119) were reviewed. Articles were included in the review if it was clear that oysters implicated in outbreaks had been subjected to depuration; only 15 articles met this criterion. The outbreaks identified spanned the period 1979 to 2012. A further 30 articles were identified that described outbreaks attributed to oyster consumption in which the oysters either had not been depurated, or it was not stated if depuration occurred, and were thus excluded. The following information was collated and tabulated from the identified publications:

- The number of cases in the outbreak
- Year of the outbreak
- Country of the outbreak
- Country of the oyster origin
- Information regarding an epidemiological link between outbreak and oysters
- Analytical confirmation of NoV in human faeces and oysters
- Levels of NoV present in oysters post depuration.

For the second literature search noted above, the titles and abstracts of the first 300 citations identified and sorted by Google Scholar (n=1270) were reviewed for relevance. All abstracts of the citations identified using PubMed (n=29) were reviewed. Of the identified publications, 14 peer reviewed research articles contained quantitative data on the reduction of NoV, HAV and viral surrogates in oysters during depuration and relaying, and thus, the data were included in the review. To compare the reduction rate of NoV, HAV and surrogate viruses across different studies, viral levels in oysters were determined for each study from either raw data reported in publications, or estimated from graphs using WebPlotDigitizer (http://arohatgi.info/WebPlotDigitizer/). Similar to other authors (Choi and Kingsley, 2016; Loisy et al., 2005; Love et al., 2010), it was assumed that viral loss was exponential and a linear regression model was fitted to the estimated $\log_{10}$ transformed viral concentrations to assess the reduction in viruses over time (Excel, 2016, Microsoft®). The models were then used to predict an estimated average single log reduction time for viruses within oyster tissues for each published study (Tables 1.4, 1.5 and 1.6).

For the third literature search on localisation of NoV, the titles and abstracts of the first 300 citations identified and sorted by Google Scholar (n=1280) were reviewed for relevance. All abstracts of the citations identified using PubMed (n=12) were reviewed.

Non-English language studies were not included. Additional papers were accessed using the reference list of reviewed publications.

2.2 Critical appraisal process

A critical appraisal of the most relevant papers included in the review was undertaken. The appraisal process used was similar to that undertaken in a recent literature review by McLeod (2014). This involved the following steps:

1. Papers were initially filtered to identify those that are most relevant to the objectives of this study. The criteria used to identify papers of highest relevance included:
    - Studies in which Pacific and/or Native oysters were contaminated with NoV (either naturally or through bioaccumulation) and subjected to depuration or relaying and the concentrations of NoV measured before and after the cleansing process.
    - Reports of illness outbreaks related to NoV in oysters that had been subjected to depuration and/or relaying.
    - Studies that significantly influenced the outcomes of the literature review conclusions.

2. Studies of high relevance (as identified in 1 above) were evaluated using the following questions:
    - Were appropriate analytical test methodologies used for NoV (i.e. the ISO standard method or equivalent)?
    - Were depuration parameters noted in the study (e.g. temperature, salinity, time, disinfection approach, flow rate, loading density)?
    - Did the study design, data and statistical treatment support the conclusions?
    - For uptake and depuration studies:
        - Were oysters maintained in appropriate conditions to ensure they were alive and functioning optimally?
        - Was the number of sampling occasions and/or oyster samples analysed sufficient to support conclusions regarding relative elimination efficiency for NoV?
    - For reports on illness outbreaks related to NoV in oysters:
        - Does the epidemiology evidence presented strongly implicate oysters as the vector (i.e. were the epidemiology investigations analytical and have statistics presented)?
        - Were oysters tested for the presence of NoV?

3. The questions above were evaluated for each of the high relevance papers, and a score of 0 (no), 1 (acceptable/generally) or 2 (yes) was allocated for each question. A total score was calculated for each paper, thus high scoring papers are suggestive of robust results and conclusions (a maximum score of 10 is possible). The results of the critical appraisal for each relevant paper are presented in Appendix 1.

2.3 International depuration practices: survey approach

A key objective of this project is to evaluate the efficacy of the depuration process in reducing the levels of NoV in oysters. To fulfil this objective it is ideal to have robust information on two different subjects: (a) the way in which depuration is performed currently by the oyster industry (what the current process entails), and (b) the results of NoV reduction studies on oysters that have been depurated. If robust depuration studies on NoV have been conducted using a depuration process that is broadly similar to commercial processes currently used in the UK, this should enable a desktop evaluation of the efficacy of the process.

Thus, to gain insight into the way in which oysters are currently depurated in the UK and overseas, two questionnaires (one for depuration operators in the UK, and the other for overseas industry members) were prepared to seek information, particularly focused on the process parameters used during depuration, such as seawater temperature, salinity, disinfection approach, stocking density etc. The questionnaires were distributed to: (a) all approved depuration establishments in the UK; and (b) industry, scientific and regulatory contacts in a variety of shellfish producing countries. The questionnaires were dispatched in 2015, and are included in Appendix 2.

3 The Depuration Process

3.1 Overview of depuration

The depuration process involves placing shellfish into tanks (custom made, or off the shelf designs), which are filled with clean seawater. During the process, shellfish should be able to filter feed normally and open and shut their valves without encumbrance, and by doing so the shellfish purge the contents of their digestive tract, including contaminants that may be associated with digested food and faecal matter. The shellfish faeces settle to the bottom of the tank and are removed following the depuration process. This natural purging process is considered to reduce the amount of most pathogenic bacteria that are present in shellfish to 'acceptable' levels.

Depuration was first investigated in the late 1800s as a method to reduce the levels of pathogenic bacteria in shellfish, in response to shellfish-associated outbreaks of cholera and typhoid fever in both the UK and USA (reviewed in Richards (1988); Richards (1991)). Some of the early systems were shown to be highly effective in reducing bacteria; for mussels and oysters, depuration reduced lactose-fermenting bacteria by 3 logs in several days (Dodgson, 1936; Richards, 1988). Since this time, depuration has been used as a post-harvest control for pathogenic bacteria in many parts of the world (including a variety of European countries, USA, Japan, China and Australia) and for a variety of bivalve species (including different species of oysters, mussels and clams). When depuration is applied using appropriate parameters and process controls, most bacteria are efficiently eliminated in relatively short timeframes, for example:

- *Salmonella enterica* serovar Typhimurium was eliminated from Pacific oysters in 12 h using a UV and chlorine based depuration system (de Abreu Corrêa et al., 2007).
- *Escherichia coli* in mussels (*Perna viridis)* was reduced to 0.2% of the starting contamination level following 15-20 h depuration (Ho and Tam, 2000).
- The bacteria *E. coli* and *Enterococcus faecalis* showed higher depuration rates in oysters and clams compared to HAV and poliovirus (Love et al, 2010).

While depuration has been found to be effective for most pathogenic bacteria, not all bacteria are depurated efficiently, with the notable exception of bacteria from the genus *Vibrio*. Richards notes in his review entitled *'Shellfish Depuration'* that "shellfish moderately contaminated with most bacterial indicators and pathogens can be adequately depurated within 72 hours". However, he further points out that bacteria of the genus *Vibrio* are indigenous in the marine environment and persist following standard depuration conditions (Richards, 1991). *Salmonella enterica* serovar Newport has also been found to persist in oysters following depuration (Morrison et al, 2012).

A variety of factors impact on the efficacy of shellfish depuration and the process must be controlled to ensure that shellfish are healthy and resume filter feeding when placed in the depuration tanks and that they are not re-contaminated during the process (Anon, 2009). The seawater used can either be from the natural environment or artificial seawater. Most depuration set-ups are based on the use of either 'flow-through' or 're-circulating' seawater, the former uses seawater that flows through the tank once and is constantly replenished with new water (hence these systems tend to be close to shore and have ready access to clean seawater), whereas re-circulating systems (common in the UK) use seawater that is re-circulated through the tank. Some bacteria that are present in the shellfish faeces (which settle to the bottom of the tank) are released into the overlying seawater; the extent of the release depends on contact time and temperature (Rowse and Fleet, 1982). The release of bacteria from the faeces can cause recontamination of shellfish, particularly those animals that are lower in the tank. Thus, depuration also involves disinfection of the seawater to prevent the build-up of bacteria and recontamination of shellfish.

A variety of different approaches for the disinfection of seawater have been used, including the use of chlorine, iodophores, ozone and ultraviolet (Richards, 1988; Richards, 1991). With respect to the use of chlorine, iodophores and ozone, shellfish are very sensitive to these compounds, which are reported to impact on shellfish pumping. Early experiments were undertaken to investigate the impact of using chlorine to disinfect the water, with results suggesting that chlorine interferes with the normal functioning of mussels and impaired purification (Dodgson, 1936). Chlorine has also been suggested to cause organoleptic changes in shellfish meat and could give rise to chlorinated by-products that are possible carcinogenic agents (Lee et al, 2008). Depuration operators can overcome these issues by de-chlorinating the seawater before adding it back into the tanks and it is more commonly used in larger flow-through depuration facilities than in closed systems. With respect to iodophors, Richards (1988) notes that systems using 0.1 to 0.4 mg/L result in bacterial reductions without impacting on shellfish activity. Ultraviolet (UV) and ozone disinfection have increased in popularity, but they have relatively high set up and running costs. Ozone can also form by-products in shellfish (e.g. bromates) and, similar to chlorine, requires operators to ensure that residual levels do not directly contact the shellfish, as this can reduce activity and depuration effectiveness (Lees et al., 2010a). UV disinfection is highly effective for bacterial reduction and has the advantage of not leaving residual concentrations and so does not interfere with the physiological processes of shellfish. Thus, UV is widely used for disinfection purposes in the USA, Australia and the UK. Key factors to ensure the efficacy of UV disinfection are the need for low turbidity, appropriate flow rate and that UV lamps are operating effectively. Thus, while all the disinfection methods can result in an effective depuration process (for bacterial contaminants), it is necessary to ensure that each technique has the appropriate controls in place.

To ensure that shellfish filter feed normally during the depuration process, a variety of parameters relating to water quality need to be considered and controlled, including dissolved oxygen levels, tank loading, water flow rate, salinity, temperature, turbidity and pH. The parameters that are used are specific to the species of shellfish to be depurated; in the UK, specific guidance and criteria have been developed.

3.2 Depuration processes in the UK

3.2.1 Regulatory requirements and guidelines for depuration in the UK

Regulation (EC) No 854/2004[1] contains the production area classification criteria and the post-harvest treatment that is required for bivalve shellfish produced in the European Union (Table 1.1). Shellfish from Class B areas must be depurated, relayed or cooked by an approved method.

[1]Criteria for classification are given in Regulation (EC) 854/2004 and Regulation (EC) 2073/2005. The criteria for Class B areas were amended by Regulation (EC) 1021/2008. The criteria for Class A areas were amended by **Regulation (EU) 2015/2285, which will be implemented in the UK on the 1st of January 2017.**

Table 1.1. Criteria for the classification of bivalve shellfish production areas.

Class	Microbiological standard	Post-harvest treatment required
A	Samples of live bivalve molluscs from these areas must not exceed, in 80% of samples collected during the review period, 230 *E. coli* per 100g of flesh and intra valvular liquid. The remaining 20% of samples must not exceed 700 *E. coli* per 100g of flesh and intra valvular liquid.	None
B	Live bivalve molluscs from these areas must not exceed, in 90% of samples, 4,600 MPN *E. coli* per 100g of flesh and intra valvular fluid. In the remaining 10% of samples, live bivalve molluscs must not exceed 46,000 MPN *E. coli* per 100g of flesh and intra valvular fluid	Depuration (purification), relaying or cooking by an approved method
C	Live bivalve molluscs from these areas must not exceed the limits of a five tube, three dilution MPN test of 46, 000 *E. coli* per 100g of flesh and intra valvular fluid	Relaying or cooking by an approved method

If shellfish harvested from Class B areas are subjected to relaying, the placement of shellfish into non-polluted seawater at the relaying site is required under the EU legislation to be for a minimum period of two months, which is considered to be enough time for elimination and inactivation of all microbial pathogens, including viruses. Regulation (EC) 853/2004 also contains other requirements for relaying. There are currently six classified relaying areas in England and Wales and none in Scotland or Northern Ireland, thus while the regulation permits relaying as a control option, it is a relatively uncommon practice in the UK and oysters harvested from Class B areas in the UK are nearly always depurated. Cooking is not generally used as an end product control option for oysters, as the consumer preference is for a raw product. Anecdotal evidence suggests that oysters harvested from Class A areas are also commonly depurated in the UK.

Regulation (EC) 853/2004 contains depuration requirements that must be adhered to, e.g.:

- Structural requirements for depuration centres i.e. tank location and structure;
- Shellfish must be free of mud and debris prior to depuration process;
- The shellfish must be able to filter feed and shell opening should not be impeded;
- The quantity of shellfish must not exceed the tank capacity;
- The depuration period is not specified in legislation but must enable compliance with the microbiological criteria contained in Regulation (EC) 2073/2005; and
- Shellfish within a depuration tank must be the same species, and crustaceans, fish or other marine species should not be kept in the depuration tank.

In the UK a variety of systems may be used for depuration, including Seafish 'standard' design systems, for which operating manuals can be consulted (http://www.seafish.org/), custom-built 'non-standard' tanks, and commercially available systems that are bought 'ready to use'. Under Regulation (EC) 853/2004 approval for purification centres must be given by the Local Authority. A series of specific requirements for purification centres has been developed in the UK by CEFAS and the FSA (Anon, 2009; Lee, 2010), these include requirements for: dissolved oxygen, tank loading, water flow rate, salinity, temperature, UV treatment, turbidity, purification period, and drain down procedure (to avoid re-suspension of sediment/faeces). A brief summary of the key water quality requirements is given below (and further summarised in Table 1.2), for full details however, the guidelines referenced above should be consulted.

Table 1.2. Summary of recommended depuration water quality criteria in the UK (summarised from Anon (2009); Lee (2010))

	Pacific oysters	Native oysters
Minimum temperature	8°C	5°C
Minimum salinity	20.5 ppt	25 ppt
Loading arrangement	Double layer	Single layer
Loading density	530 animals/m^2	
Minimum depuration period	42 hours	
Minimum UV dose	10mJ/cm^2	
UV lamps	2 x 25 watt lamps	
Dissolved oxygen	50% (5 mg/L)	

Dissolved oxygen must be adequate for shellfish to be able to function and undertake respiration normally. In the UK, dissolved oxygen levels are generally recommended to be > 5 mg/L (50%).

The way in which a tank is loaded with shellfish is important because water needs to be able to flow throughout the tank in a uniform manner ('dead spots' should be avoided), with minimum disturbance to the shellfish (i.e. from turbulence). Additionally, when shellfish filter feed they open their valves, this can be inhibited if too many shellfish are stacked on top of each other. It is recommended in the UK that the loading density of oysters in tanks not exceed 530 animals/m^2. For Pacific oysters the recommended loading arrangement is a double layer, whereas for Native oysters a single overlapping layer is recommended. The flow rate of systems varies depending on the type of system used, the type of shellfish depurated, the loading density and other factors. Seafish provide minimum recommended flow rates for standard depuration systems in the UK, all exceed 15L/minute. The specific recommended flow rate for a particular system type is specified in the approval document for each purification plant.

With respect to temperature, the metabolic rate of the shellfish is affected by the temperature of the seawater in which they are held. Generally, as temperatures decrease shellfish become less active, given this, particular temperature ranges are recommended for each shellfish species in the UK. Similarly, minimum allowable salinities are also provided. The oxygen carrying capacity of the seawater can be impacted by temperature and salinity, thus it is also important for these two parameters to be controlled to ensure shellfish can respire normally. For Pacific oysters and Native oysters respectively, minimum depuration temperatures of 8°C and 5°C are recommended in the UK, and minimum salinities of 20.5 ppt and 25 ppt are recommended.

In the UK, seawater is disinfected using UV treatment. There are several recommendations regarding the use of UV in the UK:

- The minimum applied UV dose is required to be not less than 10 mJ/cm^2 (it is recommended that higher doses or longer residence times be considered to assist in viral inactivation);
- UV lamps should be free of slime and other substances;
- The system should have 2 x 25 watt or greater UV lamps; and
- Lamps should be changed after 2500 hours of use.

In order to ensure that contaminants do not build up in the seawater, it is necessary to ensure that the seawater is of adequate transparency so that the UV can penetrate the water and inactivate microbes that may be present. If turbidity is too high, UV irradiation may be absorbed by suspended organic particles, thereby reducing the efficacy of disinfection. When turbidity is an issue, settlement or filtration of seawater may be required.

Lastly, historically a minimum depuration period of 42 hours has been recommended in the UK. The CEFAS guidelines note that "a properly functioning system should be able to reduce *E. coli* levels from ≤ 4,600 *E. coli*/100 g, to less than 80 *E. coli*/100 g in 42 hours". However, it is noted that the Hygiene Regulations do not specify a minimum period, thus from November 2016 food businesses in the UK will be able to apply alternative purification times provided they can demonstrate that the period used is appropriate.

3.2.2 Snapshot of actual industry practice in the UK

Questionnaires on oyster depuration practices and operational parameters were distributed electronically and in hard copy, where appropriate, to depuration operators identified by the FSA (information from Local Authorities). A total of 46 operators were identified across the UK (32 in England, nine in Scotland, two in Wales and three in Northern Ireland). One questionnaire 'bounced' with no alternative hard copy address available, while two respondents replied that they no longer depurated oysters. A total of 11 completed questionnaires were received out of the effective distribution of 43 forms (four in England, five in Scotland, one in Wales and one in Northern Ireland), an overall response rate of 25.6%.

The majority of systems (73%) were commissioned since 2000, with self-construction the preferred means of provision for 36% of operators. The purchased systems were predominantly 'Martin Laity' (71%), with 'Tropical Marine' and 'Depur' making up the balance (14% each). In terms of species, 36% processed only Pacific oysters, while 64% depurated both Pacific oysters and Native oysters.

Although there were a variety of systems, as detailed above, every operator used oysters loose on trays for depuration with the favoured densities ranging from 10 - 20 kg/tray for self-constructed facilities to 10 kg/tray for Depur and 15 kg/tray for the Martin Laity systems, with all of the systems falling in the range of 10 - 20 kg/tray. The manufactured systems were all limited in scale with maximum loads of 60 - 90 kg/cycle. The self-constructed systems generally exceeded these volumes, ranging from 200 kg/cycle to 1500 kg/cycle.

All respondents met or exceeded the recommended minimum depuration period of 42 hours. Elapsed time for a complete cycle was generally in the range of 42 - 48 hours (82% in summer, 73% in winter), including all manufactured systems. Self-constructed systems exhibited longer hours, typically 72 hours in summer and 96 hours in winter with one extreme example claiming a range of 42 - 120 hours in both seasons.

The majority of operators (64%) used local water sources for their supply, while 27% used artificial water and 9% relied on tanker deliveries. Filters were not installed in any of the systems, while water reuse was generally in the range of 1 - 3 times (45%), although 27% did not reuse water at all.

With respect to water disinfection, all respondents used UV disinfection with lamps exceeding 25 watts (the recommended strength for UV lamps in the UK). There were no reports of testing for turbidity, apart from a visual assessment, with several respondents noting that they followed a settlement process if turbidity was observed. Only 18% of respondents tested for microbiological contamination following the depuration process. Regarding the use of ozone for water disinfection, 91% of respondents reported never using ozone, with the balance aiming for a concentration in the range of 320 - 340 redox.

Salinity of process water ranged from 20.5 ppt (9%) through 27 - 30 ppt (27%) and 31 - 35 ppt (55%) to 40 ppt (9%). In all cases, operational temperatures were reported as ranges, with summer values ranging from minima of 8°C to 16°C and a maxima of 14°C to 22°C; winter values were minima of 2°C

to 14°C and maxima of 10°C to 18°C. Notably, some operators recorded temperatures below the UK recommended minimum values (8°C and 5°C for Pacific and Native oysters respectively) in the winter months.

The majority of respondents failed to test for, or report on, levels of dissolved oxygen in the systems (55%) and those who noted levels indicated an expectation of no change between start and end of each cycle. All respondents who reported on dissolved oxygen met, or exceeded, the recommended level of 50% (5 mg/L).

The only problems that were noted by respondents with respect to depuration operations, were difficulties in keeping the system cool in summer (with resulting spawning) (27% of respondents) and claims that depuration shortened the shelf life of the product (9%).

Depuration was generally not expected to remove NoV from oysters (55%), although 27% were willing to consider it might be possible; only 18% considered that the process offered significant potential for NoV removal. With respect to the one operator who uses ozone for disinfection, it was noted that viral testing results using PCR did not show a decrease in NoV level following the depuration process, however the company did note a decrease in customer complaints regarding illness.

3.3 Depuration processes used in other countries

Questionnaires were designed to elicit information about depuration parameters and characteristics used in a number of oyster producing countries around the world, with recipients including industry operators, regulators and research scientists. The results are therefore more 'impressionistic' than a tightly focused operators survey, however this was considered to be sufficient to highlight any major differences between UK and international practices. Sixteen responses were received, from nine countries, including: China, Netherlands, United States of America, Portugal, Norway, Ireland, New Zealand, Australia and Spain.

The first question aimed to establish the prevalence of depuration in each country. Spain, Netherlands and Portugal noted heavy reliance on depuration as a process control for oysters, with estimates of 75 - 100% of production being purified by depuration prior to placing oysters on the market. Australia (NSW), Ireland, USA and China noted that around 25 - 50% of production was subjected to depuration, Norway noted that a very small proportion of oysters were depurated (too small to quantify), and oysters from New Zealand were not subjected to depuration at all.

In terms of oyster species, seven of the eight countries in which depuration occurs noted that Pacific oysters were depurated, with the USA as the only apparent exception, with a focus on the Eastern oyster (*Crassostrea virginica*). In Portugal, a related species, *Crassostrea angulata* is also depurated in addition to Pacific oysters. Australia also depurates *Saccostrea glomerata*, the 'Sydney Rock Oyster', while local variations of 'native' oysters (*Ostrea edulis*) are also depurated in Spain and Australia.

The use of both manufactured and self-constructed depuration systems appears common in Spain, Netherlands and Ireland, while in Portugal, all systems are reported to be purchased manufactured facilities. A preference for self-constructed systems is reported for Australia (NSW), USA and China. Although the capacity of systems was generally reported as 'variable', typical capacities were identified in a number of countries, ranging from 240 kg in Portugal, to 500 kg in Netherlands and USA, to 800 kg in Australia. The implication is that the scale of depuration facilities overseas tends to be larger than in the UK, possibly due to the greater reliance on self-constructed systems (in the UK self-constructs also tend to have greater capacities than purchased systems).

Depuration cycle times varied between countries, from 24 hours in Portugal (*C. gigas* and *C. angulata*), 24 - 36 hours in Ireland (*C. gigas*), 36 hours in Australia (*C. gigas*, *S. glomerata*) and China (*C . gigas*), 36 - 48 hours in Netherlands (*C. gigas*), 44 hours in the USA (*C. virginica*), and 44 - 48 hours in Spain. These cycle times are generally shorter than UK times, with the typical time for the favoured manufactured systems being 42 - 48 hours and self-constructs reporting longer times of 72 (summer) and 96 hours (winter).

Operational temperatures varied, but within a relatively narrow band and without any species differentiation in each country. Reported temperatures were as follows: >10°C in USA, 13°C in Portugal, 13 - 15°C in Spain, 15 - 25°C in China, and 18 - 25°C in Australia (NSW). While the range of temperatures reported for the UK (from 2°C to 22°C from winter through summer) overlaps with the temperatures reported in other countries, it is notable that the winter temperatures appear to be substantively lower in the UK than those reported by the overseas respondents. This may contribute to the relatively longer depuration periods used in the UK. Salinities also varied between countries, but not between species nationally, from 15 – 31 ppt in the USA, >18 ppt in Australia, 30 ppt in the Netherlands and China, 32 – 35 ppt in Spain and 35 ppt in Portugal. These are broadly similar to those reported in the UK.

With respect to seawater disinfection approaches, similar to the UK, USA and Australia use UV disinfection routinely. The use of ozone was not widespread, with only one country (Portugal) reporting that it is consistently used in depuration facilities, and Spain and China reporting occasional use of ozone. Ozone is not used in Australia and the USA. 'Unknown' responses were recorded from Ireland and the Netherlands.

NoV illness outbreaks from oysters, which were depurated before being placed on the market, were reported by questionnaire respondents from Spain, Portugal, Australia and China, although there were no indications of the scale of the outbreaks. Several of these outbreaks have been reported in the scientific literature and are discussed in the subsequent section.

3.4 Norovirus illnesses related to depurated oysters

A recent review involved a systematic approach to identify shellfish borne viral outbreaks globally (Bellou et al., 2013). The authors interrogated a variety of different literature search engines/databases (Scopus, Pubmed etc), as well as the global electronic reporting system ProMED. The literature search yielded 61 research articles that met the inclusion criteria, and these reported on around 360 outbreaks of shellfish borne viral illness between 1980 and July 2012. The majority of outbreaks were identified in East Asia, followed by Europe. NoV was the most common virus implicated (300 outbreaks in the scientific literature), and oysters were the most frequent type of shellfish implicated (215 outbreaks reported in literature). While it is clear that there are a significant number of oyster-borne NoV outbreaks that occur globally, the review does not comment on what proportion of outbreaks resulted from oysters that had been depurated.

A major objective of this review is to evaluate the efficacy of depuration in removing NoV from oysters. Studies have been undertaken to investigate the reduction of NoV in oysters during the depuration process (see Section 5). However, one limitation of reduction studies is that they use PCR based methods for viral detection. PCR methods detect a small fragment of the viral RNA and do not provide information as to whether the virus is intact or capable of inducing infection in humans. Therefore, to provide some information on the potential infectivity of NoV in oysters after the depuration process, a literature search was undertaken to identify outbreaks that were attributed to oysters that had been depurated.

The literature search (process and keywords noted in Section 2.1) identified 1660 citations using Google Scholar and 119 citations using PubMed (but omitting the word 'depuration' from the search in PubMed to broaden the number of results). Only 15 articles met the inclusion criteria (noted in Section 2.1) for outbreaks in which oysters had been depurated. The outbreaks identified spanned the period 1979 to 2012. A further 30 articles were identified that described outbreaks attributed to oyster consumption in which the oysters either had not been depurated, or it was not stated if depuration occurred, and were thus excluded.

A summary of the oyster-borne NoV outbreaks caused by oysters that were depurated is shown in Table 1.3. Epidemiological data were presented for 12 of the 15 outbreaks. Laboratory investigations were conducted on clinical specimens (faeces) for 14 of the 15 outbreaks, and on oysters for 10 of the 15 outbreaks. In the majority of outbreaks (57%), NoV was detected in both human faecal samples and oysters.

The reported outbreaks occurred in four countries: UK, Ireland, Australia and France. In comparison, Bellou et al. (2013) notes that the reported shellfish-borne viral outbreaks (all shellfish types and viruses) occurred in 17 different countries. The smaller geographical distribution found in this review likely relates to the literature search being narrowed to only outbreaks relating to oysters that had been depurated and perhaps reflects the relatively higher implementation of depuration in the UK, France, Ireland and Australia, as compared to other countries, particularly those in East Asia.

The articles were lacking details regarding the depuration conditions used; two articles noted the seawater temperature ('ambient' and 12 - 13°C), salinity was not mentioned in any article, three articles noted the use of UV disinfection, and only one article mentioned flow rate. However, information presented in Section 3.3 on depuration conditions in different countries, suggests that the water quality parameters used to depurate oysters in the four countries implicated in these outbreaks (UK, France, Ireland and Australia) differ. For example, the temperature range reported to be used in the UK was between 2 and 22°C. For the 2012 outbreak in Australia, seawater temperature was reported to be 18°C (Anthony Zammit, Personal Communication, 2016), and the seawater temperature in the 2006 and 2008 French outbreaks was reported to be between 4 and 6°C, and 8 and 10°C respectively (Jean-Claude Le Saux, Personal Communication, 2016). Salinities used in depurating the implicated oysters were also likely to differ, with salinity in Australia reported to be quite low at >18 ppt (Section 3.3), whereas 55% of respondents to the survey undertaken as part of this review noted that salinity was between 31 and 35 ppt in the UK (Section 3.2). UV disinfection is ubiquitously used in the UK and Australia (57% of the outbreaks came from these countries). For the 2006 French outbreak, UV was also used, but in the 2008 outbreak a recirculating depuration system was used with aeration only (Jean-Claude Le Saux, Personal Communication, 2016). The variety of depuration conditions used in the purification process for oysters responsible for the 15 outbreaks displayed in Table 1.3 may imply that altering water quality parameters such as salinity, temperature, flow rate, and stacking density will not be sufficient to reduce NoV to levels that are below an infectious dose. Further information on viral reduction studies in which the alteration of water quality parameters has been investigated are presented in Section 5.

For six of the outbreaks the concentration of NoV following depuration was reported; concentrations varied between 1.7×10^2 and 8×10^3 NoV genome copies/g. Concentrations of NoV in oysters were not reported pre 2002, largely due to the lack of methods that enable accurate quantification to be carried out. Recent estimates of the infectious dose of NoV suggest that it is very low (Atmar et al., 2014; Teunis et al., 2008) (Section 4). Thus, it is not surprising that oysters containing between 1.7×10^2 and 8×10^3 viral genomes were implicated in gastroenteritis outbreaks, and this demonstrates that a proportion of the viruses present in the oysters were capable of inducing infection and illness despite the depuration process being applied. As discussed above, in at least three of the reported

outbreaks the depuration process involved UV treatment (Gill et al., 1983; Grohmann et al., 1981; Heller et al., 1986), and in one of the outbreaks an extended depuration period was used, consisting of 15 days (Heller et al., 1986). These measures were clearly ineffective in reducing NoV to 'safe' levels.

While undertaking the literature search on outbreaks of NoV related to depurated oysters, two additional articles on outbreaks of Hepatitis A related to the consumption of depurated oysters were identified (Table 1.3) (Conaty et al., 2000; Guillois-Becel et al., 2009). Epidemiological data were provided for both outbreaks and HAV was confirmed in the sera of patients from one outbreak and in the oysters of the second outbreak. Both outbreaks involved the depuration of the oysters for relatively short periods, 36 and 48 hours.

.

Table 1.3. Published outbreaks of NoV illness related to the consumption of depurated oysters

Number of cases	Year	Oyster species	Country of origin	Epidemiological linkage	Virus detected in faeces by PCR	Virus detected in oysters by PCR	Virus copies/g in oysters (post depuration)[4]	Depuration time	Reference
NoV outbreaks									
18	2012	Pacific oysters	Ireland	NS	Y (GI and GII)	Y (GI and GII)	2.38 x 10^3	NS	Rajko-Nenow et al. (2014)
8	2012	NS	Australia	Y	Y (GII)	Y (GII)	NS	36 hours	Fitzgerald et al. (2014)
70	2010	Pacific oysters	Ireland	NS	Y (GII)	Y (GI and GII)	2.35 x 10^3	NS	Dore et al. (2010); Rajko-Nenow et al. (2014)
≥ 240	2009	NS	UK[1]	Y	Y (GI and GII)	Y (GI and GII)	NS	NS	Smith et al. (2012)
23	2008	Pacific oysters	France	Y	Y (GII)	Y (GII)	2.4 x 10^3	1-23 days[5]	Le Guyader et al. (2010)
>93	2007	Pacific oysters	UK	NS	Y (GII)	Y (GII)	8 x 10^3	NS	Lowther et al. (2010)
19	2007	NS	Australia	Y	Y	N	NA	36 hours	Huppatz et al. (2008)
205	2006	Pacific oysters	France	Y	Y (GI and GII)[2]	Y (GI and GII)[2]	2.4 x10^3	1-12 days[5]	Le Guyader et al. (2008)
15	2004	NS	UK	Y	Y (GI and GII)	NT	NA	42 hours	Gallimore et al. (2005)
329	2002	Pacific oysters	France	Y	Y (GI and GII)	Y (GI and GII)	1.7 x 10^2	48 hours	Le Guyader et al. (2006b)
9	1997	Pacific oysters	UK	Y	Y[3]	NT	NA	NS	Ang (1998)
97	1996	NS	Australia	Y	Y (GII)	N	NA	NS	Stafford et al. (1997)
16	1985	Pacific oysters	UK	Y	N	NT	NA	15 days	Heller et al. (1986)
181	1983	Pacific oysters	UK[1]	Y	Y[3]	NT	NA	72 hours	Gill et al. (1983)
52	1979	*Saccostrea glomerata*	Australia	Y	Y	NT	NA	48 hours	Grohmann et al. (1981)
HAV outbreaks									
111	2007	Pacific oysters	France	Y	Y (sera)	N	NA	48 hours	Guillois-Becel et al. (2009)
467	1997	*Saccostrea glomerata*	Australia	Y	NT	Y	NS	36 hours	Conaty et al. (2000)

Y = yes; N = No; NS = Not Stated; NA = Not Applicable; NT = Not tested

[1]Outbreak in UK, location of supplier not noted.

[2]Other enteric viruses were also detected in the oysters and faeces, including Aichi virus, Astrovirus, Enterovirus and Rotavirus

[3]Small round structured viruses (SRSV) were visualised in faecal samples.

[4]Where multiple values reported, the maximum value is recorded in the table. Where possible, the values reported are from samples collected from either the restaurant or the consumer (not harvesting area).

[5]Depuration time for oysters consumed and implicated in the outbreak were not stated in the publication. However, information obtained from the authors provided a range of days that oysters implicated in the outbreak were depurated for

.

4 Norovirus – Oyster Interactions

When considering the efficiency of depuration and relaying in reducing NoV in oysters, it is necessary to understand how NoV is ingested, retained and excreted by oysters, because this may provide some explanation for the observed illnesses relating to oysters that have been depurated (Section 3), and for the slow elimination rates reported for NoV in oysters (Section 5). Thus, the following section presents basic biological information on NoV, uptake of NoV by oysters, and the state-of-the-art with respect to specific ligands that are present in oysters that act to selectively concentrate and retain NoV in the oyster digestive tract and other tissues.

4.1 Norovirus

NoVs are a group of highly diverse viruses that belong to the *Caliciviridae* family. They have a single stranded RNA genome, which is around 7500 bp long, and they are non-enveloped and icosahedral viruses (Le Guyader et al., 2012). NoV causes gastroenteritis, the symptoms often include vomiting, abdominal cramps, fever, watery diarrhoea, headaches, chills and myalgia, and illness normally lasts two to three days (Glass et al., 2009). NoVs infect humans by binding to histo-blood group antigens (HBGAs), these are highly conserved glycans (carbohydrates) which are present on a wide variety of gastrointestinal and epithelial cell types and are used as receptors by many viruses and bacteria (Le Pendu et al, 2014).

There are currently seven genogroups, of which three infect humans (GI, GII and GIV) (Zheng et al., 2006). NoV is the main cause of non-bacterial gastroenteritis outbreaks worldwide, with GII.4 strains responsible for the majority of outbreaks (Glass et al., 2009; Koopmans, 2008; Siebenga et al., 2010). In a recent review by Le Guyader et al. (2012), information on NoV genotypes detected in stools and shellfish implicated in illness outbreaks was collated; this demonstrated that the frequency of detection of genogroups in shellfish related outbreaks is different, with GI strains more frequently detected in shellfish outbreaks compared to other NoV outbreaks. Similarly, Yu et al. (2014) found that there are more GI sequences reported (NCBI GenBank and the NoroNet outbreak database) from oyster outbreaks (34%), than from non-oyster outbreaks for which 90% are GII strains.

The infectious dose of NoV is considered to be very low, a human trial involving a GI.1 strain determined that the average probability of infection for a single viral genome was 0.5 and the median infectious dose (ID_{50}) was between 18 and 1015 genome copies (Teunis et al., 2008). A separate human trial was recently conducted in USA also involving a GI.1 strain, which reports an ID_{50} of approximately 1320 genome copies for secretor positive persons who were blood type O or A (Atmar et al., 2014). Thebault et al. (2013) statistically analysed data from five published outbreaks resulting from NoV in oysters in France. Median ID_{50} estimates ranged between 1.6 and 7.51 genome copies per oyster consumed and the probability of infection of a single NoV genome copy was close to 0.5 for both GI and GII NoV, suggesting that there is no difference in the infectivity between GI and GII NoVs (Thebault et al., 2013).

4.2 Contamination of oyster production areas and oyster feeding

There are two major routes by which food contamination occurs, through infected food handlers and during the primary production process i.e. through contact of the food with sewage contaminated water. In the case of oysters, contamination primarily occurs in the production area when the seawater becomes contaminated with sewage. Contamination of oyster production areas with sewage can occur in a number of ways, including:

- Through the release of partially-treated or non-treated sewage from wastewater treatment plants or broken sewerage pipes and pump stations (Doyle et al., 2004; Guillois-Becel et al., 2009; Maalouf et al., 2010a);

- Via leachate from septic tanks (on-site sewage disposal units) (Stafford et al., 1997);
- Following high rainfall or flood events in which contaminated run off water pollutes the production area (Conaty et al., 2000; Doyle et al., 2004; Grodzki et al., 2012; Le Guyader et al., 2008; Murphy and Grohmann, 1980);
- From harvesters and/or other people defecating directly into production areas (Berg et al., 2000; Kohn et al., 1995; McDonnell et al., 1997; McIntyre et al., 2012); or
- Through release of sewage by recreational or commercial vessels (Simmons et al., 2001).

Oysters are filter feeding bivalve molluscs. They grow in both intertidal and subtidal areas, however they prefer estuarine areas close to the shore. The gills of the oyster are involved in the capture, selection and transport of food particles, as well as respiration. The cilia on the gills create water currents, which draw seawater across the gills. Mucus on the gills binds particles that are present in the seawater, which are then carried forward to the labial palps and mouth. Unwanted particles are rejected in the pseudofaeces prior to ingestion (Ward et al., 1997, 1998). Viruses are found in both pseudofaeces and faeces, however a higher proportion of non-culturable HAV and PV were detected in faeces compared to pseudofaeces, indicating that viruses are inactivated as they transit the oyster digestive system (McLeod et al., 2009a). Factors such as nutritional value, size and charge of food particles are thought to influence the selection of food by bivalves (Bedford et al., 1978; Shumway et al., 1985; Ward et al., 1997). Additionally, recent research has demonstrated that there is an interaction between carbohydrates on the algal cell surface and lectins within the mucus that covers the feeding organs of mussels (*Mytilus edulis*) and oysters (*Crassostrea virginica*) (Espinosa et al., 2010a; Espinosa et al., 2009, 2010b), and this is suggested to be a common mechanism for particle selection across bivalve taxa.

Pacific oysters can efficiently capture food particles in the 4 to 10 µm size range (Bell, 2005). However, oysters also retain smaller particles, such as NoV (which is around 23 nm in size). A study undertaken in the late 1970s demonstrated that the bioaccumulation efficiency of poliovirus by clams increased when the virus was added to the seawater in conjunction with clay kaolinite or faeces (Metcalf et al., 1979). More recent research demonstrates the presence of NoV in plankton samples (Gentry et al., 2009). Thus, the adhesion of viruses to solids, including plankton, may enhance bioaccumulation in shellfish. It is also likely that viruses bind to the mucus that flows through the gills and labial palps of the oyster. Early research suggested that binding of viruses to the mucous sheath was ionic in nature (Di Girolamo et al., 1977), however given the recent findings regarding the binding of algae to lectins within oyster mucus it seems feasible that carbohydrates on the surface of the NoV capsid may also bind to lectins within the oyster mucus (though this hypothesis remains to be confirmed). Further research has also shown the direct interaction of NoV with oyster ligands present on/in the gills and other digestive structures, as discussed in the next section.

4.3 Interaction of Norovirus with oyster ligands

The recognition that NoV persists for longer periods than bacteria when oysters are subjected to depuration or relaying (Schwab et al., 1998; Ueki et al., 2007) led to suggestions that NoV may be binding specifically to oyster tissues, thus increasing the amount of time that the virus remains in the oyster and prompting investigations to identify ligands that NoV may be adhering to within the oyster.

Firstly, a GI.1 strain was shown to bind to the midgut and digestive diverticula of Pacific oysters, but not to the other tissue types (Le Guyader et al., 2006a). In contrast GII NoV was shown to bind to a variety of oyster tissue types, including the digestive diverticula, midgut (intestine), gills, mantle, and labial palps (McLeod et al., 2009b; Seamer, 2007; Wang et al., 2008). Collectively these results suggested strain specific variations in binding patterns.

Secondly, Le Guyader et al. (2006a) determined that binding of GI.1 NoV to Pacific oyster tissues was

inhibited by human saliva from type A and O secretors, and that a mutation in the glycan binding site of NoV virus-like-particles (VLPs) prevented them from binding to oyster tissues. This contributed to the conclusion that GI.1 NoV was binding to oyster tissues via an A-like carbohydrate structure, similar to the HBGAs used for NoV attachment to human epithelial cells. Similar results were obtained by a different research group, who showed that binding of GI.1 NoV to *Crassostrea virginica* (the Eastern oyster) was inhibited by anti-blood group A antibodies (Tian et al., 2006), also supporting the hypothesis that binding occurs through an A-like antigen.

The binding of GII NoV to oyster digestive tissues was also shown to occur through an A-like antigen, but binding of GII strains to gills and mantle tissue is facilitated by a sialic acid residue (Maalouf et al., 2011; Maalouf et al., 2010b). This bioaccumulation study also confirmed that GII NoV strains bind to a number of tissues, but GI strains are confined to binding to the digestive tissue (Maalouf et al., 2011). Furthermore, the GII.3 strain showed transient expression in the gills and mantle before being almost exclusively localised in the digestive tract. The authors hypothesised that this observation was due to the binding of GII.3 to a sialic acid ligand in the gills and mantle, which facilitated destruction of the virus (Maalouf et al., 2011).

Further bioaccumulation studies conducted at different times of the year demonstrated that there is a seasonal impact with respect to accumulation of GI NoV in oysters; this is mirrored by the expression of the A-like HBGA ligand in oyster digestive tissue. In contrast, no seasonal effect was observed in bioaccumulation studies of GII NoVs in oysters or of the sialic acid ligand (Maalouf et al., 2010b).

Thus oysters are not just passive filters, but they use specific ligands to selectively accumulate NoV. GI and GII NoV strains are considered to bind to A-like antigens in the digestive tissue, which facilitate their accumulation in oysters. This specific binding may help to explain their prolonged retention, as observed in depuration and relaying studies to date (Section 5) and account for illness outbreaks attributed to depurated oysters (Section 3). In contrast, the binding of GII strains to a sialic acid ligand in the mantle and gills is hypothesised to facilitate their elimination from oysters. For further information, Le Guyader et al. (2012) describe these issues in more detail in a recent review on the transmission of viruses through shellfish.

4.4 Current detection method for Norovirus in oysters

A variety of methods have been published for the detection of NoV in shellfish (Atmar et al., 1995; Boom et al., 1990; Greening and Hewitt, 2008; Henshilwood et al., 1998; Lees, 2010b; Shieh et al., 2000; Shieh et al., 1999b; Sobsey et al., 1985). The main challenge is to remove inhibitors of the polymerase chain reaction (PCR), which are found in the shellfish, such as glycogen. NoV is primarily concentrated in the oyster digestive tract (McLeod et al., 2009b), which is dissected from the oyster and the entire surrounding white gonad tissue is discarded. Methods for recovering the virus from the oyster digestive tract have included approaches such as alkaline elution using glycine buffer (Traore, 1998), acid adsorption (Shieh et al., 1999a), and protease digestion (Jothikumar et al., 2005). Some methods incorporate polyethylene glycol to concentrate the virus (Lewis and Metcalf, 1988). Following the evaluation of a variety of methods, a standard method has been developed and validated under the auspices of the European Committee on Standardisation (CEN) (Lees, 2010b). Two approaches have been developed: standard method ISO/TS 15216-1:2013, which is quantitative, and ISO/TS 15216-2:2013, which is qualitative. The standard methods were published in 2012 and incorporate protease digestion for virus recovery, followed by guanidine thiocyanate and silica adsorption to purify the RNA. The method uses real-time PCR to detect a small fragment of the viral genome sequence targeting the conserved region at the 5' end of ORF2. The main drawback of the currently available methods, including the ISO standard approaches, is that they do not differentiate between infectious and non-infectious virus particles. While the methods can be used quantitatively

by incorporating nucleic acid standards, there are many sources of variation that affect results and determining small differences in virus concentrations (i.e. within a log) between samples can prove challenging.

5 Persistence of Norovirus in Oysters

5.1 NoV persistence in oysters in the natural environment following relaying

Several studies have investigated the efficiency with which NoV is eliminated from Pacific oysters in the natural environment (open seawater) over an extended period. Table 1.4 presents a summary of NoV levels recorded in oysters prior to and after relaying and depuration.

Le Guyader et al. (2008) investigated the reduction of GI and GII NoV in oysters (*C. gigas*) that were associated with 205 cases of gastroenteritis in France. The illness outbreaks occurred following a flood event in the implicated production area when flood oysters were collected over a four-week period. GI and GII NoV fell from 3 log genome copies/g to around the LoQ over the four-week period, for which seawater temperatures were between 8 and 10°C. Similarly, Dore et al. (2010) studied the combined use of relaying (17 days) and in-tank depuration (six days) to reduce GII NoV in oysters (*C. gigas*) that were implicated in around 70 cases of illness. Levels declined from 2.9 x 10^3 genome copies/g to 492 copies/g following the 17-day relaying period (seawater temperature 15 - 17°C), and fell to below the LoQ after a further six days of depuration. Greening et al. (2003) artificially contaminated Pacific oysters with GII NoV and then suspended the oysters in open clean seawater (18 - 12°C) for a period of six weeks. Initial concentrations were around 4 log PCR units/g and NoV was able to be detected through to week 4, but not after this period of time.

All studies which have evaluated the reduction of NoV in oysters in the natural environment have relied on PCR methods to detect the virus as there is no culture-based method which is amenable to the routine analysis of shellfish for NoV. Thus, there is only indirect information available about the infectivity of NoV following relaying, such as data from studies on the elimination of culturable viruses from oysters over extended periods of time.

Two trials were undertaken on the elimination of FRNA bacteriophage during four weeks of relaying in ponds, followed by in-tank depuration for 48 h. For Pacific oysters, phage was reduced to below the LoD in two weeks in one trial, but low levels remained after four weeks of relaying and depuration in the second trial (Dore et al., 1998). The persistence of HAV in oysters (*C. virginica*) maintained in depuration tanks (recirculating system with UV treatment) was tracked over an extended four-week period. Seawater temperature was 18°C and microalgae were added to the tanks as a food source. HAV was still able to be cultured three weeks after the oysters were contaminated, but not after four, five and six weeks of depuration. In contrast, HAV RNA was detected following six weeks of depuration (Kingsley and Richards, 2003).

Collectively, these studies suggest that a relay period of around four weeks is sufficient to reduce GI and GII NoV and HAV to background levels in Pacific oysters, and that viral infectivity is significantly reduced after this period of time.

Dore et al. (2010) and Le Guyader et al. (2008) both reported that NoV was still detectable in some samples following three to four weeks of purification in open seawater. In both cases oysters were allowed to be sold for consumption following the cleansing process and no further human illnesses were reported. This may imply that risk to consumers is small from low levels of NoV in oysters which have been purified for three to four weeks in clean open seawater. However, the presence of culturable phage (at low levels) following four weeks of purification (Dore et al., 1998) indicates the possibility that a small number of NoV particles may be capable of inducing infection following relaying; it is possible that such low levels of contamination either results in no illness (as it is below the infectious dose), or in such small numbers of cases that they are not identified/reported through epidemiological surveillance systems.

5.2 NoV and HAV persistence in oysters following 'in-tank' depuration

Studies that have investigated the reduction of NoV (GI and GII) in *C. gigas* in depuration tanks have demonstrated that periods of between 23 h and 14 days result in no or negligible reductions of NoV (Table 1.4). McLeod et al. (2009a) demonstrated no reduction of GII NoV over 23 h in Pacific oysters maintained in a re-circulating system at 20°C, likewise Schwab et al. (1998) did not observe any differences in GI NoV level in Eastern oysters before and after 48 h depuration at 22°C. No drop in GII NoV levels were observed over a 10 day period in a flow through system maintained at 10°C (Ueki et al., 2007), or over a 14 day period for Pacific oysters maintained at 8°C in a commercial system with UV disinfection (Neish, 2013). Reductions of around 0.5 log GI NoV were achieved over eight days in a system maintained at 8 - 10°C (Drouaz et al., 2015), likewise a 0.5 log reduction was demonstrated for GII NoV in a system maintained at 16°C (Neish, 2013). Thus, depuration periods of less than two weeks at a variety of temperatures ranging from 8 - 22°C appear to have limited impact on NoV concentrations (Table 5.1).

Several studies investigated the reduction of GI NoV in Pacific (*C. gigas*) and Eastern oysters (*C. virginica*) in depuration tanks over extended periods (five - eight weeks) (Table 1.4). Drouaz et al. (2015) maintained Pacific oysters that were contaminated with 3.8 x 10^4 copies/g of GI NoV for eight weeks in filtered re-circulating seawater at 11°C, levels reached the LoQ by week 8. The reduction of GI NoV was also monitored in Eastern oysters maintained at three temperatures, 7, 15 and 25°C, in depuration tanks with re-circulating UV treated seawater over a six-week period. NoV decreased from 6 log copies/oyster to 4 log copies/oyster over six weeks at 7 and 15°C, but was not detected beyond week 4 in oysters at 25°C, clearly showing that elevated temperatures can enhance depuration efficiency (Choi and Kingsley, 2016).

Table 1.5 presents a summary of HAV levels recorded in oysters prior to and after depuration. Regarding HAV, depuration for 23 h at 20°C in a recirculating system did not result in a significant loss/drop in HAV genomes or plaque forming units (PFU) (McLeod et al., 2009a). Similarly, Love et al. (2010) demonstrated modest reductions of HAV in *C. virginica* depurated for five days at 12 and 18°C, but a significantly higher depuration rate was identified at 25°C, with around 98.5% (<1 log) eliminated in 44 h, again demonstrating that higher temperatures can enhance depuration in Eastern oysters.

To compare the reduction rate of NoV and HAV across different studies, viral levels in oysters were determined for each study from either raw data reported in publications, or estimated from graphs using WebPlotDigitizer (http://arohatgi.info/WebPlotDigitizer/). Similar to other authors (Choi and Kingsley, 2016; Loisy et al., 2005; Love et al., 2010), it was assumed that viral loss was exponential and a linear regression model was fitted to the estimated $\log_{10}$ transformed NoV concentrations to assess the reduction in NoV and HAV over time (Excel, 2016, Microsoft®). The models were then used to predict an estimated average single log reduction time for NoV and HAV within oyster tissues for each published study (Tables 1.4 and 1.5).

Of the 16 published NoV reduction experiments, the observed loss of NoV genomes in eight experiments was too limited to allow a prediction of a log reduction time (i.e. no or very low reductions observed). The estimated days to achieve 1 log reduction of NoV (genomes) in the eight remaining experiments was between nine and 45.5 days (Table 1.4). For HAV, the estimated days to achieve 1 log reduction was between seven and 16.1 days, with two of the five experiments showing no loss of HAV (Table 1.5). A range of factors may influence the rate at which NoV and HAV are purged by oysters, including the oyster species involved, seawater temperature and salinity, the presence or absence of food for the oysters, the length of the contamination period and initial level of contamination. This is discussed further in Section 5.5.

Table 1.4. NoV levels reported for oysters following purification and the number of days of purification estimated to achieve a one log reduction.

NoV Genogroup	Oyster species	Purification period	Seawater temp	Type of purification	Seawater treatment	Feeding during depuration	Virus concentration post accumulation	Virus concentration post depuration	Days to achieve 1 log reduction	Author
Relaying experiments										
GI and GII	*C. gigas*	4 weeks	8 – 10 °C	R	NA	NA	8.2 x 10^3 copies/g	420 copies/g	8.9	Le Guyader et al. (2008)
GII	*C. gigas*	6 weeks	18 - 12 °C	R	NA	NA	1 x 10^4 PCR units/g	Not Detected	10.6	Greening et al. (2003)
GII	*C. gigas*	17 days	15 – 17 °C	R	NA	NA	2.9 x 10^3 copies/g	4.9 x 10^2 copies/g	23.1	Dore et al. (2010)
GII	*C. gigas*	17 days R + 6 days D	15 - 17 °C	R + D	NA NS	NA NS	2.9 x 10^3 copies/g	<100 copies/g	16.6	
Depuration experiments										
GII	*C. gigas*	23 hours	20°C	RC	None	No	2.7 x 10^4 PCR units/g	3.9 x 10^4 PCR units/g	LR	McLeod et al. (2009a)
GI	*C. virginica*	48 hours	20 - 24°C	FT	NA	No	792 PCR units/oyster	734 PCR units/oyster	LR	Schwab et al. (1998)
GI	*C. gigas*	8 days	8 – 10°C	NS	NS	No	1.4 x 10^4 copies/g	4.6 x 10^3 copies/g	LR	Drouaz et al. (2015)
GI	*C. gigas*	8 days	8 – 10°C	NS	NS	Yes	5.9 x 10^4 copies/g	7.6 x 10^3 copies/g	LR	
GII	*C. gigas*	10 days	10°C	FT	Filtration	No	a. 1.7 x 10^3 copies/g b. 5.2 x 10^3 copies/g	a. 1.8 x 10^3 copies/g b. 7.7 x 10^3 copies/g	LR	Ueki et al. (2007)
GII	*C. gigas*	14 days	8°C	RC	UV	No	1.7 x 10^5 copies/g	2.4 x 10^5 copies/g	LR	Neish (2013)
GII	*C. gigas*	14 days	16°C	RC	UV	No	1.7 x 10^5 copies/g	1.1 x 10^5 copies/g	45.5	
GI	*C. gigas*	8 weeks	11°C	RC	Filtration	Yes	3.8 x 10^4 copies/g	<100 copies/g	22.6	Drouaz et al. (2015)
GI	*C. virginica*	5 weeks	25°C	RC	UV	No	1-2 x 10^6 copies/oyster	Not Detected (100 copies at 4 weeks)	10.15	Choi and Kingsley (2016)
GI	*C. virginica*	6 weeks	15°C	RC	UV	No	1-2 x 10^6 copies/oyster	1 x 10^4 copies/oyster	15.85	
GI	*C. virginica*	6 weeks	7°C	RC	UV	No	1-2 x 10^6 copies/oyster	3.8 x 10^4 copies/oyster	LR	

GII = genogroup 2 NoV; GI = genogroup I NoV; R = relaying; D = depuration; RC = recirculating system; FT = flow through system; NA = not applicable; NS = not stated in publication; LR = limited reduction, observed loss of NoV genomes too low to allow a prediction of a log reduction time.

Table 1.5. HAV levels reported for oysters following depuration and the number of days of purification estimated to achieve a one log reduction.

Oyster species	Depuration period	Seawater temperature	Type of depuration	Seawater treatment	Feeding during depuration	Virus concentration post accumulation	Virus concentration post depuration	Days to achieve 1 log reduction	Author
C. gigas	23 h	20°C	RC	None	No	1.2 x 10^5 PCR units/g 8.4 x 10^4 PFU/g	1.1 x 10^5 PCR units/g 4.7 x 10^4 PFU/g	LR	McLeod et al. (2009a)
C. virginica	5 days	25°C	FT	NA	No	NS	NS	13.6	Love et al. (2010)
C. virginica	5 days	18°C	FT	NA	No	NS	NS	16.1	
C. virginica	5 days	12°C	FT	NA	No	NS	NS	LR	
C. virginica	6 weeks	18°C	RC	UV	Yes	3.4 x 10^4 PFU/ml shellfish extract	Not Detected (500 PFU at 3 weeks)	7	Kingsley and Richards (2003)

RC = recirculating system; FT = flow through system; NA = not applicable; NS = not stated in publication; LR = limited reduction, observed loss of HAV too low to allow a prediction of a log reduction time.

5.3 Persistence of surrogate viruses during depuration

As previously noted, NoV is unable to be reliably detected/quantified in shellfish tissues using cell culture methodologies. Thus while it is clear that NoV genomes can persist for prolonged periods, it is not known how long infectivity of NoV is retained within oyster tissues. To overcome this problem, studies have been conducted to evaluate the usefulness of surrogate viruses (which are generally able to be cultured) for assessing the virological safety of depurated oysters. Table 1.6 presents a summary of depuration studies conducted to date using surrogate viruses in oysters. For a surrogate virus to provide useful information on the infectivity of NoV, it is important that the characteristics of NoV and the surrogate virus are similar within oyster tissues, including the way in which they interact with ligands, the stability of the virus capsid and their persistence.

Several studies have been undertaken on the usefulness of FRNA bacteriophage (MS2) as an NoV surrogate during depuration and relaying. Love et al. (2010) followed the depuration of FRNA bacteriophage (MS2), poliovirus and HAV from *C. virginica* over five days at 25°C in a flow through system. Depuration rates, as determined using culture methods for each virus, were the greatest for phage, followed by poliovirus (PV), then HAV; days to achieve a 1 log reduction of each virus were estimated to be 2.1, 6.9 and 13.6 days respectively (Tables 1.6 and 1.5). Because FRNA bacteriophage and PV were removed at faster rates than HAV, it appears they may be poor indicators of the virological status of depurated oysters. Consistent with these results, Loisy et al. (2005) found that MS2 phage was depurated more rapidly than rotavirus VLPs from Pacific oysters, with the time to achieve a 1 log reduction estimated to be 4.1 and 6.6 days respectively (Table 1.6).

Neish et al. (2013) also undertook depuration experiments using Pacific oysters and FRNA bacteriophage. No reduction of NoV was noted at 8°C over 14 days, but phage was reduced by nearly 1 log. Similar results were achieved at the higher temperature of 16°C, with days estimated to achieve a 1 log reduction of 6.5 for phage, and 45.5 for NoV. The difference in depuration rates between phage and NoV appear vast in this study. However, phage was detected using a culture method thus only infectious virus was detected, whereas NoV was detected using PCR which detects infectious and non-infectious particles and may underestimate reductions in infectivity. Nonetheless, the results are indicative of large differences in the behaviour of phage and NoV during depuration, and are consistent with the differences in depuration rates noted between phage and HAV (for which culture methods were used for both viruses).

Ueki et al. (2007) conducted a parallel study to investigate the comparative depuration rate of NoV and feline calicivirus (FCV) – a potential NoV surrogate. Following 72 h contamination, oysters (*C. gigas*) were depurated for 10 days at 10°C in a flow through system with sand filtration. NoV levels did not decrease, whereas FCV was rapidly depurated within three days. This is consistent with the results of Provost et al. (2011), who used RT-PCR to demonstrate that FCV was undetectable one day after contamination of oysters (*C. virginica*), whereas HAV was able to be detected for >21 days. Murine norovirus (MNV) showed an intermediate persistence and was detected for up to 12 days.

McLeod et al. (2009a) compared the elimination rates of HAV, NoV and PV in Pacific oysters over 23 h of depuration in a re-circulating system. While there was no decline in HAV and NoV genome copies in oysters, there was around a 2 log decrease of PV genome copies (and a 1 log decrease in PFU). Love et al. (2010) and Provost et al. (2011) also showed that PV was removed at a faster rate than HAV in *C. virginica.*

Drouaz et al. (2015) investigated the comparative depuration of Tulane virus (TV), Mengovirus (MgV) and NoV (GI) from Pacific oysters when maintained in large scale commercial depuration tanks at 11°C for eight weeks. Oysters were fed phytoplankton throughout the trial and samples were

collected weekly. PCR methods were used for the detection of all three viruses. NoV (GI) was found to be more persistent in oysters than TV or MgV, with half-lives of 7.56 days, 4.65 days and 2.17 days respectively. The authors hypothesised that TV may behave more like a NoV GII strain (rather than GI trialled in the study), due to differences in the HBGAs that GII and GI strains bind to.

In summary, comparative elimination studies to date have shown that surrogate viruses are more rapidly depurated than NoV and HAV under a variety of depuration conditions, including temperatures of 8 - 25°C, times varying between 23 h and eight weeks, and using both recirculating and flow through systems which have UV and or filtration disinfection. As part of this review, the time required to reduce NoV and HAV by 1 log in each depuration study was estimated (Tables 1.4 and 1.5), this was also estimated for the surrogate viruses (Table 1.6). A comparison of the days to achieve 1 log reduction, shows that the number of days to reduce NoV/HAV (mean = 19 days for NoV, 12 days for HAV) is greater than that recorded for the surrogate viruses (mean = 7.5 days). Given the more rapid depuration of indicator viruses tested to date, they may not be suitable surrogates for assessing the virological safety of depurated oysters.

Table 1.6. Levels of surrogate viruses in oysters following depuration and relaying and the number of days of purification estimated to achieve a one log reduction.

Virus type	Oyster species	Depuration period	Seawater temperature	Type of depuration	Seawater treatment	Feeding	Virus concentration post accumulation	Virus concentration post depuration	Days to achieve 1 log reduction	Author
Phage (salmonella WG49 host)	*C. gigas*	7 days	9°C	NS	NS	No	a. 3.4 x 10^4 PFU/100g b. 2.8 x 10^3 PFU/100g	a. 1.4 x 10^4 PFU/100g b. 4.2 x 10^2 PFU/100g	a. 1.8 b. LR	Dore et al. (1998)
	C. gigas	7 days	18°C	NS	NS	No	a. 3.4 x 10^4 PFU/100g b. 2.8 x 10^3 PFU/100g	a. 6.6 x 10^2 PFU/100g b. <30 PFU/100g	a. 4.8 b. 1.0	
	C. gigas	4 weeks R + 48 h D	NS	R+D	NS	No	a. 1.9 x 10^4 PFU/100g b. 1.8 x 10^4 PFU/100g	a. <30 PFU/100g b. 6 x 10^2 PFU/100g	a. 5.0 b. 18.9	
	O. edulis	4 weeks R + 48 h D	NS	R+D	NS	No	a. 2 x 10^4 PFU/100g b. 2.9 x 10^3 PFU/100g	a. <20 PFU/100g b. <30 PFU/100g	a. 5.0 b. 14.1	
Phage (salmonella WG49 host)	*C. gigas*	14 days	8°C	RC	UV	No	9 x 10^3 PFU/g	1.2 x 10^3 PFU/g	14.6	Neish (2013)
	C. gigas	14 days	16°C	RC	UV	No	9 x 10^3 PFU/g	65 PFU/g	6.5	
Phage MS2 (*E. coli* host)	*C. gigas*	7 days	22°C	RC	F + UV	No	2 x 10^3 PFU/g	42 PFU/g	4.1	Loisy et al. (2005)
Phage MS2 (*E. coli* host)	*C. virginica*	5 days	25°C	FT	NA	No	NS	NS	2.1	Love et al. (2010)
Poliovirus	*C. virginica*	5 days	25°C	FT	NA	No	NS	NS	6.9	
Poliovirus	*C. gigas*	23 h	20°C	RC	None	No	1.2 x 10^5 PCR units/g 5.8 x 10^3 PFU/g	8.5 x 10^3 PCR units/g 1.7 x 10^3 PFU/g	0.6 1.7	McLeod et al. (2009a)
Rotavirus virus like particles	*C. gigas*	7 days	22°C	RC	F + UV	No	1.1 x 10^6 particles/g	1 x 10^5 particles/g	6.6	Loisy et al. (2005)
Tulane virus	*C. gigas*	8 days	8 – 10°C	NS	NS	No	3 x 10^3 copies/g	1.2 x 10^3 copies/g	LR	Drouaz et al. (2015)
Tulane virus	*C. gigas*	8 days	8 – 10°C	NS	NS	Yes	6.9 x 10^4 copies/g	1.1 x 10^4 copies/g	LR	
Tulane virus	*C. gigas*	8 weeks	11°C	RC	F	Yes	6.7 x 10^4 copies/g	<100 copies/g	11.5	
Mengovirus	*C. gigas*	8 weeks	11°C	RC	F	Yes	1.5 x 10^5 copies/g	<100 copies/g	11.4	
Feline calicivirus	*C. gigas*	10 days	10°C	FT	Filtration	No	a. 2.2 x 10^3 copies/g b. 5 x 10^3 copies/g	Not detected Not detected	<0.9	Ueki et al. (2007)

R = relaying; D = depuration; NS = not stated in publication; RC = recirculating system; FT = flow through system; F = Filtration; UV = ultra violet radiation; LR = limited reduction, observed loss did not allow a prediction of a log reduction time.

5.4 Possible reasons for variation in the persistence of different types of viruses

Comparative studies demonstrate significant differences in depuration rates of different viruses from oysters (Drouaz et al., 2015; Loisy et al., 2005; Love et al., 2010; McLeod et al., 2009a; Nappier et al., 2008; Neish, 2013; Provost et al., 2011; Ueki et al., 2007). There are several potential reasons that may account for these differences and the prolonged retention in oysters of some viruses such as NoV and HAV.

Viral localisation studies have demonstrated the presence of viruses, including NoV and HAV, in the lumen and epithelium of the digestive tract tissue (stomach, intestine and digestive diverticula), in connective tissue, and in phagocytic blood cells of oysters (haemocytes) (Fisher, 1986; Fries and Tripp, 1970; Le Guyader et al., 2006a; McLeod et al., 2009b; Provost et al., 2011; Romalde et al., 1994). Further research has demonstrated that NoV binds to certain histo blood group like antigens within oyster tissues: GI and GII NoV bind to group A like antigens in the digestive tract, which is considered to facilitate their accumulation and retention (Maalouf et al. 2010b, 2011). This specific binding may inhibit entry of NoV into the digestive system and thus protect it from degradation through the digestive process. Viruses that do not bind to these specific ligands may therefore potentially be more susceptible to elimination from oysters due to their easy entry into the digestive system, and subsequent acidic digestion within haemocytes and/or excretion through the lumen of the digestive tract.

Oyster haemocytes contain acidic vesicles which aid in the digestion of small food particles that are phagocytosed, therefore haemocytes have low pH. Provost et al. (2011) hypothesised that for viruses to persist within shellfish and haemocytes, they must be resistant to acidic digestion within haemocytes. Consistent with this hypothesis the authors demonstrated that more acid tolerant viruses persisted for longer periods in *C. virginica* (HAV>MNV>PV>FCV). Thus differences in acid tolerance between viruses may also, at least partially, account for variations in the persistence of different viruses within oysters.

5.5 Summary of factors that may influence depuration efficiency of norovirus

A variety of factors may impact the rate at which viruses are eliminated from oysters during depuration, such factors include:

- oyster species
- temperature
- salinity
- food availability (plankton)
- contamination level and length of the contamination period.

Modulation of temperature, salinity, and food availability are known to lead to changes in the filtration and clearance rates (the volume of seawater that is cleared of particles in a certain time) of bivalves. Changes in filtration rate and the consequential effects that this has on the oyster digestion rate and enzymatic activity, is likely to affect the capacity of shellfish to inactivate and eliminate viruses and likely explains changes in the depuration rate of viruses.

5.5.1 Oyster species

Few studies have directly compared the depuration rate of viruses in different oyster species, with studies generally focusing on just one species.

Nappier et al. (2008) compared uptake and retention of FRNA bacteriophage, HAV, MNV, PV and NoV in two species of oysters (*C. virginica* and *C. ariakensis*) that were depurated for a period of one

month at 20 - 23°C in separate tanks. The depuration system was a flow through system in which seawater was re-circulated back to the tanks following filtration. Oysters were fed weekly during the depuration period. *C. ariakensis* was found to be 8.4 and 11.4 times more likely to harbour NoV and HAV than *C. virginica* during depuration. Similar results were obtained when the two species of oysters were accumulated and depurated in the same tank system, with NoV remaining for 29 days in *C. ariakensis,* and 14 days in *C. virginica* (Nappier et al., 2010). These studies demonstrate that different oyster species do have varying capacity to accumulate and depurate viruses.

To our knowledge, no studies have been undertaken directly comparing the depuration rates of NoV and HAV in the two oyster species commonly found in the UK, *O. edulis* and *C. gigas*.

5.5.2 Temperature

Pacific oysters have been reported to increase clearance/filtration rates as temperatures rise from 5 to 20°C, but further increases from 20 to 32°C resulted in declining rates. With regards to native oysters (*O. edulis*), which are also commercially produced in the UK, clearance rates are reported to increase up to 30°C (Haure et al., 1998).

Consistent with this, several studies have demonstrated that higher seawater temperatures increase the rate at which viruses are depurated from oysters. It has been demonstrated that FRNA bacteriophage depurates from Pacific oysters more rapidly at higher temperatures; at 18°C levels of phage were reduced to 2% of the initial contamination level, but at 9°C, 40% of the phage remained. Similarly, Neish et al. (2013) found that a temperature of 16°C increased the depuration rate of phage when compared to a temperature of 8°C. NoV depuration was also enhanced at 16°C compared to 8°C, however the NoV reduction was still very small over 14 days (<0.5 log). Choi et al. (2016) demonstrated the persistence of NoV for six weeks in *C. virginica* depurated at 7 and 15°C, whereas NoV was only detected for four weeks in oysters held at 25°C. Love et al. (2010) also found that increased temperatures resulted in higher depuration rates for HAV, FRNA bacteriophage and poliovirus from *C. virginica.*

5.5.3 Salinity

Oysters are euryhaline and can thrive in a wide range of salinities. The salinity optima for *C. gigas* and *C. virginica* are in the range 23 - 25 ppt and 14 - 28 ppt respectively (reviewed in Gosling (2003)). The feeding rate of *O. edulis* was noted to decline at salinities above 28 ppt, and ceased below 16 ppt.

While the physiology of oysters is clearly impacted by salinity, the results of studies to date on the impact of variable salinity on viral depuration rates are not clear. Love et al. (2010) investigated the depuration of FRNA bacteriophage (MS2), PV and HAV from *C. virginica* under variable salinity (8, 18 and 28 ppt). The depuration rate of FRNA bacteriophage was found to be higher at 18 ppt than at 8 ppt, however the depuration rate of HAV and PV did not change with salinity. Nappier et al. (2008) found that salinity (8, 12 and 20ppt) did not impact virus accumulation and retention in *C. ariakensis*, but *C. virginica* was salinity dependent, with NoV and HAV not being efficiently bioaccumulated at 8 and 20 ppt, compared to 18 ppt. Love et al. (2010) and Nappier et al. (2008) both recorded mortality of *C. virginica* at 8 ppt, which corresponds with the salinity tolerance of that species.

5.5.4 Food availability

The filtration and clearance rates of oysters are known to increase with food availability, but then decrease with further rises in food concentration (Barille et al., 1997; Pascoe et al., 2009; Riisgard et al., 2003; Strohmeier et al., 2009).

Commercial depuration is normally conducted in the absence of added food, although some processes may include plankton that naturally occurs in untreated seawater. Several studies have investigated the impact of presenting oysters with a phytoplankton food source during depuration. The impact of feeding on the efficiency of NoV, TV and MgV depuration from Pacific oysters was investigated, no significant difference (P=0.25) in NoV concentrations between oysters that were fed phytoplankton (4.6×10^3 copies/g) or starved (7.6×10^3 copies/g) were observed after eight days depuration. Similarly, no difference was observed for MgV between oysters that were fed or starved, but a small difference (approximately 1 log) in TV concentration was observed (Drouaz et al., 2015).

The persistence of HAV in *C. virginica* was studied over a six-week depuration period in which the oysters were maintained at a relatively high temperature of 18°C in standard depuration conditions for the USA, with UV disinfection (Kingsley and Richards, 2003). Several groups of oysters were fed with microalgae daily phytoplankton daily (*Isochrysis* and *Tetraselmis*) to evaluate the impact of feeding on depuration. Infectious HAV (as determined by cell culture) was detected following three weeks of depuration with feeding. The presence or absence of food did not appear to affect viral persistence as determined by PCR, with HAV detected for six weeks regardless of feeding. Similarly, Love et al. (2010) found that feeding *C. virginica* with microalgae (*Isochrysis*) had no impact on the depuration of HAV, FRNA bacteriophage or PV.

6 New Approaches for Viral Depuration

Depuration was initially developed to reduce and control bacterial pathogens that were responsible for shellfish-associated outbreaks of cholera and typhoid fever. Thus, for decades the development and validation of the depuration process has focused on bacterial pathogens. It has become increasingly apparent that the traditional process does not adequately eliminate certain viruses like NoV and HAV from shellfish, and this poses a major food safety challenge. The on-going nature of viral illness outbreaks attributed to shellfish consumption, including those that have been depurated, highlights the need for improved water quality throughout the world, but also for new post-harvest treatment processes that would ensure the virological safety of shellfish. This section is focused on new potential approaches to viral depuration that may hold promise for the future.

6.1 Traditional depuration process

The traditional depuration strategy relies on the natural filter feeding process and excretion activities of bivalves to eliminate pathogenic bacteria from their digestive tracts through normal excretion when they are placed in clean seawater in conditions which allow the shellfish to function normally. As discussed, numerous modifications to the standard depuration process have been trialled, including various time, temperature and salinity regimes, feeding shellfish during the depuration process, and different disinfection approaches (e.g. UV, ozone, chlorine, iodophors). Whilst some of these modifications result in slight improvements in viral depuration rates, they have not resulted in significant reductions of the two viruses that are the predominant cause of illness outbreaks – NoV and HAV. The overwhelming finding is that the depuration process does not sufficiently eliminate NoV and HAV, with many studies showing no, or very low levels of viral reduction (Section 5.2). Given the success of depuration in reducing typhoid and cholera outbreaks, it is clear that the requirements for elimination of faecal indicator bacteria (i.e. faecal coliforms and/or *E. coli*) are significantly different to those required for viral elimination.

While the requirements for efficient bacterial and viral depuration are clearly different, there is some commonality in the initial stages of depuration. Viral depuration has been described by several authors as being 'two phase', with elimination in the first few days being more rapid than subsequent days (Love et al., 2010; Polo et al., 2014b; Polo et al., 2015; Provost et al., 2011). The first rapid phase of viral depuration is likely related to extracellular digestion and purging of the digestive tract; this process is governed by physiological traits related to the shellfish species involved which are common to both bacterial and viral depuration, including the filtration and clearance rate of the species, the digestion rate, and enzymatic activity. In this sense, the optimisation of the physiological state of the shellfish and the adjustment of the different parameters previously named (i.e. temperature, salinity and time) for each shellfish species are necessary and important to optimise reductions in viral contamination. However, the persistence of a proportion of the viral load in shellfish (the second slower phase of elimination), highlights that other properties are at play. As discussed (Section 4.3), this likely relates to the ability of some viruses and virus strains to bind to molecular receptors/ligands (HBGAs) that are present on shellfish gastrointestinal cells, and penetrate into non-conductive parts of the digestive tissue, connective tissue and other organs. This particular issue regarding viral binding represents a barrier to enhancing viral depuration and is a key point for consideration in the development of future depuration processes (Le Guyader et al., 2012; Maalouf et al., 2011; Nappier et al., 2008; Polo et al., 2014a; Polo et al., 2014b; Provost et al., 2011).

6.2 Potential new approaches

6.2.1 Enzymatic pre-treatment

Considering the specific binding of NoV to HBGAs present in oyster tissues, an approach that may have potential to enhance depuration rates is the application of certain enzymes which are known to

degrade the ligands involved. Such enzymes could be diluted in seawater and the oysters immersed for a short period of time prior to the depuration step, potentially leading to the degradation of the ligands, and enhanced release of NoV from the oyster tissues during the standard depuration process. This approach is discussed in detail in Part 2 of this report, which describes the results of pilot experiments to evaluate the efficacy of the enzymatic pre-treatment approach.

6.2.2 Bacteria

Another avenue that may be worthy of further research is the potential for particular bacteria to produce active agents/compounds that have antiviral activity, and/or induce a physiological or immune response by the shellfish that facilitates viral elimination. Various bacteriocins with antiviral activity have already been reported against several viruses, including herpes simplex type 1 (Todorov et al., 2010), influenza virus (Serkedjieva et al., 2000) and New Castle Disease Virus (Saeed et al., 2007). Lange-Starke et al. (2014) also reported a 1.25 log reduction of MNV when a lactic acid bacteria was applied, however the antiviral metabolite could not be identified.

Recently, it has been reported that a bacteria was isolated from the mussel *Mytilus galloprovincialis* and identified as *Enterococcus hirae* (designated as 3M21). The bacteria showed an antibacterial activity against *Listeria monocytogenes*, *Listeria innocua* and *Enterococcus faecalis* (Fajardo et al., 2014). In the same publication the authors reported that the bacteria showed antiviral activity against HAV and MNV-1. The active substance, which was proteinaceous in nature, has been successfully microencapsulated in alginate and is reported to be effectively ingested by oysters (Prado-Alvarez et al., 2015; Darmody et al., 2014). The efficacy of the active substance in reducing HAV and NoV titres during depuration or other post-harvest treatments has not been reported.

6.2.3 Microencapsulation

A key issue with regards to the efficacy of enzymes or bacteria in cleaving the ligand-virus specific bonds, relates to ensuring that an adequate concentration is targeted at the sites of interest within the shellfish body. Since viruses are principally concentrated in the digestive tissue, if the introduction of certain bacteria or enzymes are to have an effect it would be necessary to ensure that bacteria survive and reach the digestive gland, and that the enzymes are present in sufficient quantities at the site of interest. Additionally, chlorine, ozone and UV are all commonly used in depuration systems, and may act to reduce the viability of any bacteria.

Microencapsulation represents one possible avenue for ensuring that enzymes or bacteria applied during the depuration process reach the sites of interest in the shellfish digestive tissue. Microencapsulation techniques have been used widely in the aquaculture sector and were initially focused on overcoming marine larval feeding problems. However, their use has evolved to assist in the delivery of probiotic bacteria and bacterial substances, immunostimulants, vaccines and enzymes to target species (Polk et al. 1994; Skjermo and Vadstein 1999; Rosas-Ledesma et al. 2012; Darmody et al., 2014; Borgogna et al. 2011). Three main polymers have been broadly used for microencapsulation in the human and aquaculture sectors: chitosan, alginate and PLGA (Poly Lactic-co-Glycolic Acid) (Borgogna et al. 2011; Plant and LaPatra 2011; Luzardo-Alvarez et al., 2010; Behera et al., 2010).

While the use of probiotic bacteria, bacteriocins and inmunostimulants in encapsulated form is gaining in popularity in the aquaculture industry for targeting specific bacterial and protozoan pathogens (Martínez-Cruz et al., 2012; Darmody et al., 2014; Prado-Alvarez et al., 2015; Fajardo et al., 2014), its application in viral shellfish depuration is practically non-existent. Darmody et al. (2014) demonstrated the efficacy of delivering fluorescent particles encapsulated in alginate to target oyster

tissues. The study revealed the presence of fluorescent microbeads within the gills, digestive tubules, connective tissue and haemocytes. Similar results were also reported after the oral administration of alginate microcapsules containing inmunoestimulants in *Ostrea edulis* against the protozoan parasite *Bonamia ostreae* (Prado-Alvarez et al., 2015).

The successful ingestion of alginate microbeads by the oysters, their absorption across digestive epithelium and the release of their contents into surrounding tissues such as connective tissues, and into haemocytes (a potential virus repository), suggest that microencapsulation could represent a viable tool for the transport of antiviral substances directly to these areas.

7 Summary of Data Gaps and Limitations

Several information gaps and limitations have been identified through the literature review and are summarised below:

1. A multitude of studies have been undertaken to investigate the persistence of NoV in oysters following short term and long term depuration and relaying. These have been conducted using RT-PCR methods, as no practical culture method exists for NoV at this time. Some information on the infectivity of NoV following depuration can be inferred from illness outbreaks following depuration, a lack of illness following relaying, and from studies using culturable NoV surrogate viruses. However, the rates at which infectivity of NoV in oysters declines following depuration and relaying, under different depuration regimes (i.e. variable temperature, salinity, disinfection regimes etc), is not known.

2. Until recent times, the quantitative approach used in many depuration studies (particularly historical older research) may not have been appropriate. This is due to the lack of viral reference standards to enable accurate quantification and inappropriate sampling plans which didn't account for variation in uptake between oysters. Further studies using the standard ISO method for quantitation or new technologies, such as digital PCR, may improve our understanding of depuration rates and enable direct comparisons to be made between studies.

3. NoV persists for long periods of time in oysters due to specific binding to ligands within the oyster digestive tract, and possibly because of its high resistance to acidic conditions that are experienced within the oyster digestive system. This review demonstrates that HAV is also retained for long periods of time within oysters. The biological basis of why HAV is retained in oysters for a long time is less well understood than NoV, with no information currently available on the potential existence of ligands within oysters that HAV may bind to.

4. Numerous illness outbreaks of NoV and HAV have occurred from the consumption of depurated oysters (Table 1.3). Examination of the conditions used for depuration in these outbreaks could provide inferential information on the effectiveness of certain depuration processes. However, for most outbreaks information on the depuration conditions used is not recorded, thus it is difficult to evaluate the efficacy of the processes.

5. Many studies have investigated the impact of depuration process parameters such as temperature, time, salinity, and the feeding and disinfection approach, through modulation of one factor at a time. Only a few studies however, have sought to optimise the physiology of Pacific oysters and use a combination of conditions that promote optimal clearance rates in *C. gigas*. If further studies were conducted, care would need to be taken that conditions do not favour proliferation of potentially harmful bacteria such as *Vibrio* spp.

6. The relative rates of NoV and HAV depuration from *O. edulis* and *C. gigas* (the oyster species of relevance to the UK) have not been determined.

7. Potential differences in depuration rate for oysters that have bioaccumulated NoV in the natural environment, potentially over extended periods of time, vs. oysters that have been artificially contaminated in laboratory uptake work, is not well understood.

8 Conclusions and Recommendations

The introduction of depuration in the late 1800s was highly successful in reducing outbreaks of typhoid and cholera, however depuration has not been successful in reducing outbreaks of viral related gastroenteritis and hepatitis. There have been fifteen published illness outbreaks of NoV and HAV clearly linked to oysters that have been through standard depuration processes in a variety of countries. The oysters implicated in the outbreaks were subjected to differing depuration processes, with a range of temperatures (e.g. 8 - 12°C in France, 18°C in Australia), timeframes (ranging from 36 h to 15 days), salinities, and disinfection approaches used (e.g. UV, aeration). This information infers that NoV is resistant to depuration across a broad range of operational parameters. For six of the published outbreaks the concentration of NoV following depuration was reported, with concentrations between 1.7×10^2 and 8×10^3 NoV genome copies/g, far in excess of the infectious dose which is estimated to be as low as 10 viral particles.

While illnesses continue to occur following depuration, relaying appears to be a more successful approach, with several papers reporting significant reductions of NoV following the relay of oysters into clean open seawater for periods between 17 days and four weeks. Importantly, no illnesses were reported to be associated with relayed product, despite trace levels of NoV being detected following relaying (around the LoD of 100 genome copies/g).

A variety of factors (i.e. temperature, salinity, time) have been found to influence the rate of bacterial depuration, this largely relates to optimising depuration conditions to suit the physiology of oysters and maximise clearance rates and digestion. Optimising these environmental parameters also improves the depuration rates for most surrogate viruses (such as FRNA bacteriophage, MNV etc), but has only resulted in small (or no) improvements for NoV and HAV. This is likely related to some differences in the basic biology of the interactions between NoV and oysters, and surrogate viruses/bacteria and oysters.

Many studies have noted that depuration is a two phase process, with an initial rapid decline in contaminant level, followed by a protracted phase of slow decline. The initial rapid phase is considered to be related to extracellular digestion and purging of the digestive tract, it is thus intimately related to the physiological status of the oysters, including their clearance/filtration rate and digestion rate. Thus optimising depuration conditions such as temperature and salinity should improve viral depuration rates in the first phase of reduction – which may account for the small improvements in NoV elimination noted in studies to date. However, the retention of NoV and HAV in oysters over a long period of time during the second slow phase of elimination demonstrates that other factors are also at play for these particular contaminants (in contrast to bacteria and other surrogate viruses which are more readily excreted).

Recent publications by Le Guyader et al. (2012) and Provost et al. (2011) provide insights into the main factors that govern NoV persistence in oysters, which clearly involves a special relationship. This relationship includes the binding of GI and GII NoV to an HBGA A-like ligand present in the gut of oysters, this is considered to facilitate accumulation and retention of the virus. GII NoVs have been shown to be less well accumulated by oysters than GI NoVs, this may be related to the binding of GII NoV to sialic acid residues in the gills, which is hypothesised to lead to NoV elimination. Further, it has been demonstrated that haemocytes (key sites of intracellular digestion in oysters) are repositories of viruses in oyster tissues, thus it is considered that the high acid resistance of NoV and HAV (unlike most surrogate viruses and bacteria) may also partially account for their persistence. It is likely that these special factors that govern NoV (and HAV) retention in oysters are not influenced by basic changes in the physiology of oysters that arise due to optimising operational parameters such as temperature or feeding.

The difference between the way in which NoV (and HAV) interacts with oysters, compared to other surrogate viruses, is highlighted by the findings of this review which shows that NoV and HAV are retained for longer periods of time than a variety of surrogate viruses (including FRNA bacteriophage, FCV, MNV, PV, MgV and TV) in all comparative elimination studies to date. A comparison of the days required to achieve a 1 log reduction in virus concentration across studies to date (excluding those which show no reduction) shows that NoV takes an average of 19 days to reduce in concentration by 1 log, HAV takes 12 days, and surrogate viruses take 7.5 days.

There was no reduction of NoV in oysters noted in 50% of the studies published on NoV elimination, for those in which a reduction was demonstrated it took between 9 and 45.5 days to reduce levels by 1 log. For HAV, the number of days estimated to achieve a 1 log reduction was between 7 and 16.1 days, with two of the five published experiments showing no loss of HAV (Table 1.5). The timeframes required to achieve a 1 log reduction are clearly much longer than that routinely used for depuration, which in the UK is recommended to be 42 h. Further, illness outbreaks commonly involve NoV concentrations of around 10^3 viral copies/g. Thus a one log reduction is unlikely to be sufficient to protect public health, and further time would be required to reduce levels sufficiently. In contrast, relaying oysters to areas with clean seawater over a four-week period appears to be successful in reducing NoV levels to around the LoD (100 genome/copies), and no illnesses have been reported following the consumption of relayed oysters to date.

In light of the special relationship between NoV (and HAV) and oysters, and the limited success of studies which have focused on optimising operational parameters of the depuration process (i.e. temperature, salinity, feeding etc), it is suggested that the following topics be given priority when considering future research to support the production of virologically safe oysters:

- In a detailed review of depuration undertaken by Richards in 1988 he comments that "depuration was not intended for grossly polluted shellfish or for shellfish harvested from grossly polluted waters". This comment remains as valid today as it was in 1988. Bearing this in mind, the major focus should be placed on improvements in water quality to avoid NoV and HAV contamination of shellfish at source. To this end, further collaboration between water companies, local authorities and the shellfish industry should be prioritised to improve wastewater treatment, and processes governing discharges and communication of these to all affected parties.
- Improvement of our understanding of the special virus-oyster relationship and binding interactions, particularly for viruses of high concern such as HAV, for which less information is known.
- Investigations into post-harvest interventions that exploit the mechanisms by which NoV is retained (binding to HBGA a-like antigens) and potentially eliminated (binding to sialic acid ligands) from oysters.
- Limited information currently exists regarding the time over which NoV infectivity is retained in oysters during depuration and relaying. Some inferential information is available from HAV studies (which have involved culture methods), and from epidemiological observations following the consumption of depurated and relayed oysters. However, more direct information on the infectivity of NoV over the course of purification would be informative for risk management purposes. Recent advances in the US have resulted in a cell culture method for NoV being developed. This is not likely to be amenable to routine use for the analysis of shellfish, however, it may be useful to have access to this capacity through collaboration on limited studies that investigate viral infectivity during depuration/relaying.

Part Two: Pilot Laboratory Study: Can Enzymatic Pre-treatment Increase Depuration Efficiency?

Authors: David Polo, Catherine McLeod, Jean-Claude Le Saux and Françoise S. Le Guyader

1 Introduction

Norovirus (NoV) persists in shellfish tissues for longer periods of time than faecal bacteria. Recent studies have demonstrated selective accumulation and retention of different NoV strains in oysters via specific binding to carbohydrate ligands known as histo blood group antigens (HBGAs). These ligands are mainly localised in the digestive tissues (DT) of the oyster (but also in the gills and labial palps) and are similar to receptors involved in human NoV infections (Le Guyader et al., 2006a; Le Guyader et al., 2012; McLeod et al., 2009a, 2009b; Tian et al., 2006). GI and GII NoV strains are known to bind to Type-A HBGAs, while GII strains also bind to sialic acid residues. Thus, shellfish use HBGAs to actively accumulate particular virus types, and even strains. Consistent with this, molecular epidemiology of shellfish related outbreaks show a high prevalence of some NoV GI strains, compared to general outbreaks in which GII strains dominate (Yu et al., 2015). The specific binding of NoV to HBGAs probably explains viral persistence and the failure of different depuration strategies tested to date (Polo et al., 2014a; Richards et al., 2010).

Certain enzymes have the ability to degrade HBGAs, which are complex carbohydrates, through the cleavage of certain bonds in the carbohydrate structure. Thus, application of particular enzymes to NoV contaminated oysters could result in destruction of the HBGAs and destabilisation of the specific NoV-HBGA linkages. This component of the project describes a series of pilot experiments conducted to investigate the hypothesis that an enzymatic pre-treatment step prior to depuration will damage the HBGAs in oysters, leading to the release of NoV particles and enhanced depuration rates.

1.1 Identification and selection of compounds

The composition and structure of HBGAs is important to consider when selecting the most promising enzymes to trial. The HBGAs are carbohydrates that contain structurally related saccharide moieties. Type-A antigens (GalNAcα1-3(Fuc-α1-2)Gal) are generated by transfer of GalNAc and Gal residues to a core structure of (Galβ1-3GlcNAcβ) in type 1A antigens, or (Galβ1-4GlcNAcβ) in type 2A antigens (Shirato, 2011). Sialic acid (SA) is a generic term for the N- or O-substituted derivatives of neuraminic acid, a monosaccharide with a nine-carbon backbone. The most common member of this group is the N-acetylneuraminic acid (Neu5Ac) (Varki, 1992). A variety of compounds that have the ability to degrade Type-A and SA antigens within oyster tissues has been identified from the literature; these are presented in Appendix 3 (Davies et al., 2005; Sova et al., 2013; Bakunina et al., 2012; Feng et al., 2013).

The first group of compounds with the potential to act against the HBGAs of concern are the proteases. Proteases are extensively used for the modification of red blood cell membranes and are frequently used in blood group serology. They cleave proteins at defined sites along peptide sequences. The broad activity and low specificity of proteases (even in the presence of various salts) makes them promising candidates for targeting the protein structures involved in NoV binding. Different types of proteases are able to be commercially sourced, including those of animal (trypsine, lipase) and plant origin (proteinase K, papain) (Appendix 3).

The second group of promising compounds are the glycosidases (glycosyl hydrolases). Glycosidases catalyse the breakage of glycosidic bonds in complex sugars. Depending on the cleavage site,

glycosidases can be classified as endo- (non-terminal residues) or exo-glycosidases (terminal residues). Although these compounds are more specific than proteases, they have great potential to destroy the carbohydrate moieties that are integral components of the HBGAs. Of particular interest are α-amylase, which acts upon α(1,4)-D-glucosidic linkages, and β-galactosidase hydrolyses β-glycosidic bonds, which may favour the hydrolysis of type-A HBGAs (Appendix 3).

Compounds that degrade the sialyl groups that are present in some HBGAs may favour the release of NoV GII strains, which bind to SA residues. Sodium periodate ($NaIO_4$) may show promise in this regard, as it has an oxidising effect on glycans that contain sialyl groups and is non-enzymatic and therefore more stable in seawater than proteases and glycosidases.

Eight of these compounds were selected for use in the pilot experiments, including $NaIO_4$, two types of proteinase K (PK), α-amylase, β-galactosidase, lipase, trypsin and papain. The rationale for selecting these compounds is: (1) their potential to disrupt the NoV-HBGA linkage; (2) the compounds selected represent each of the major groups identified as being potentially effective (animal and plant proteases, glycosidases and $NaIO_4$) (blue shading, Appendix 3); and (3) the compounds can be sourced in sufficient quantities for the experiments.

1.2 Food safety considerations

A consideration with respect to the potential use of enzymes/bacteria during the depuration process is whether these substances are safe for human consumption if residual levels remain in market-ready oysters. To fully evaluate the safety aspects, it is necessary to undertake a risk assessment to evaluate the toxicology of the compounds involved, the quantities that may be present in oysters following treatment, and the potential for acute and/or chronic impacts in consumers. Such an assessment is not possible at this time, as antiviral substances that are effective would first need to be identified, and the parameters of their use in the depuration process defined. However, it is noted that several of the substances that may be effective are already used in the food industry as processing aids and additives, which indicates that their application during the depuration process may not necessarily pose an undue risk to consumers. The following provides an overview of some of the compounds that may potentially degrade NoV ligands and their current application in the food processing industry.

Serine proteases (Enzyme Commission number: EC 3.4.21), such as trypsin, α-chymotrypsine and PK, are the largest group of proteases (Hedstrom, 2002). They are extensively used in the food and animal feed industries due to their optimal activity at neutral or alkaline pH (pH 7 - 11). They are used to produce protein hydrolysates from whey, casein, soy, keratinous materials, as well as scraps from meat and fish processing. They are also used for the development of flavour during ripening of dairy products (Dalev, 1994; Wilkinson and Kilcawley, 2005).

Sulfhydryl proteases, also known as thiol or cysteine proteases (EC 3.4.22) (e.g. papain, chymopapain, bromelain, ficin), tend to be optimally active at neutral pH (pH 6 - 7.5) and are relatively heat stable, which accounts for their use as meat tenderisers. These proteases may be applied in various ways to achieve meat tenderisation e.g. blending, dipping, dusting, soaking, spraying, injection and vascular pumping (Etherington and Bardsley, 1991). Sulfhydryl proteases are also used in the brewing industry to control haziness and improve the clarity of beer, and in the baking industry to improve the elasticity and firmness of dough through the modification of gluten (Mathewson, 1998).

Metalloproteases (EC 3.4.24), like pronase E or O-sialoglycoprotease, are characterised by having metal ions in their active sites. Some of the industrial applications of metalloenzymes include the synthesis of peptides for use as low calorie sweeteners. Pronase E is also used, alone or in combination with other proteases (protease cocktails), for the production of food protein

hydrolysates, flavour-enhancing peptides, and for accelerating the ripening of dry sausages (Fernandez *et al*., 2000).

Glycosidases (EC 3.2.1) are carbohydrate processing enzymes (or carbohydrases) that catalyse the hydrolysis of glycosidic bonds to liberate monosaccharides and oligosaccharides. α-amylases are the most common carbohydrases used in food processing; they are used by the starch, alcoholic beverages and sugar industries. Lactases or β-galactosidases are also common and are used to catalyse the breakdown of the milk sugar, lactose, into its constituent monosaccharides, galactose and glucose. β-galactosidases are widely used in the dairy industry to produce fermented milks, ice-cream, milk drinks and lactose-reduced milk (Simpson *et al*., 2012). O-glycosidases are used for flavour enhancement in beverages such as fruit juices and particularly in wine processing (Sarry and Gunata, 2004).

Lipases, or triacylglycerol acylhydrolases (EC 3.1.1.3), are hydrolytic enzymes that catalyse the breakdown of ester bonds in biomolecules such as triglycerides, phospholipids, cholesterol esters and vitamin esters. Lipases are water-soluble proteins (Wang and Hartsuck, 1993). Lipases are used in the manufacture of dairy products e.g. milk, cheese and butter to facilitate the ripening process and develop flavouring. They are also used to improve the flavour or aroma of bakery foods and beverages and for quality improvement of mayonnaises, dressings and whippings (Schmidt and Verger, 1998; Sharma *et al*., 2001).

1.3 Regulatory considerations

Substances that are added to foods are classified differently in the EU, depending on their purpose and the way they are used in the manufacturing process.

Food ingredients are generally considered to be substances that are used in the manufacture or preparation of a food and that are still present in the finished product, even in an altered form.

Food additives are substances that are not normally consumed as food or used as a characteristic ingredient of food, but are added to the food intentionally for a technological purpose. Regulation (EC) No. 1333/2008 defines a food additive and lists the approved food additives and sets condition of use.

Processing aids are substances that are not consumed as foods, but are used in the processing of raw materials, foods or their ingredients, for a technological purpose, and residues of the substance (or derivatives) may be found in the final product. Any residues should be safe and not have a technological effect in the final product. Processing aids are defined in the food additive legislation, but this legislation does not control their use.

Food enzymes are defined as substances capable of catalyzing a specific biochemical reaction and are added to food for a technological purpose. The rules for the use of enzymes in the EU is covered under Regulation (EC) No. 1332/2008 and this covers both enzymes used as food ingredients and as processing aids. The European Commission is working towards the establishment of a positive list of food enzymes.

An assessment would need to be made as to the classification of a substance applied during depuration under EU law. For food enzymes it would be necessary to ensure that data required for risk assessment and risk management is gathered (consistent with the requirements of Regulation (EC) No. 234/2011). This would enable the European Food Safety Authority (EFSA) to assess the safety of the enzyme and for the European Commission (and Member States) to form an opinion as to

whether the enzyme in question should be included on the eventual EU approved list for food enzymes.

1.4 Objectives and approach

The main objective of the pilot experiments is to evaluate if the treatment of NoV-contaminated oysters with specific compounds prior to depuration improves NoV reduction rates. To investigate the efficacy of various compounds, two different experimental approaches were pursued.

Firstly, ten bioaccumulation and depuration trials were performed using oysters contaminated with either NoV GII, or a mixture of NoV GI and GII. Contaminated oysters were immersed for one or two hours in each of the eight different bioactive compounds as a pre-treatment step prior to depuration. Two concentrations of each compound were evaluated, using three different seawater temperatures.

Secondly, due to the time intensive nature of the bioaccumulation and depuration experiments, an *in vitro* approach was developed whereby the DT of NoV contaminated oysters were treated with the bioactive compounds in 24-well cell culture plates. If compounds that are effective in destabilising the HBGAs are applied, there is a reduction in the NoV levels in the DT (or an increase in Ct value[2]). This *in vitro* assay enabled additional screening of the candidate compounds.

[2]The relationship between cycle threshold values (Ct) obtained using real time RT-PCR and the concentration of viral particles is an inverse relationship – that is - the higher the Ct value, the lower the concentration of viral genomes present.

2 Materials and Methods

2.1 Bioaccumulation

Pacific oysters (*Crassostrea gigas*) were sourced from a single supplier to minimise natural variation in uptake rates between oysters. The oysters were first acclimatised to being maintained in tanks with natural seawater and continuous aeration for a period of four hours until all the oysters showed correct filtering activity (open shells) and were then subjected to a bioaccumulation step for 24 hours to contaminate them with NoV.

A total of ten bioaccumulation trials were performed, nine using NoV GII.3 and one using NoV GI.1 and GII.3 as a mixture. These strains were selected as they have been previously used for ligand characterisation and tissue distribution studies; it is known that they accumulate efficiently and can therefore, be considered as a 'worst' case (compared to Nov GII.4, for example). A stool suspension was prepared at the beginning of the study and aliquots of 10 ml were frozen allowing for good reproducibility of the bioaccumulation process. Each bioaccumulation trial was performed with approximately 50 oysters. The oysters were uniformly dispersed in a monolayer in tanks along with 10 litres of natural seawater. NoV (5×10^8 RNA copies) was then added to the tanks containing the oysters for a period of 24 h. Bioaccumulation of the oysters was undertaken in seawater maintained at ambient room temperature (around 15 ± 3°C). The NoV concentration after bioaccumulation (and prior to treatment and depuration) was determined for each trial to verify viral uptake by the oysters (see Section 3.2 and Tables 2.1 and 2.2 for results). Contaminated oysters were relocated to tanks with clean seawater and washed to remove intra-valvular fluid and associated viruses that had not been internalised.

Oysters from each trial were then divided into groups of eight to ten individuals, resulting in five to seven groups per trial (with the exception of trial 4, which is discussed further below). Each group of eight to ten individuals was treated as a single pooled sample, using this 'pooled sample' approach assisted in overcoming potential issues relating to variation in NoV uptake by individual oysters. For each trial, one sample (i.e. one group of 8 -10 oysters) acted as a control sample and was subjected to depuration without exposure to the bioactive compounds prior to purification. The viral content of control samples is shown in Tables 2.1 and 2.2. The remaining four – six samples (per trial) were subjected to treatment with the bioactive compounds, as discussed in further detail for each trial below.

2.2 Depuration trials

As noted, the efficacy of eight different compounds was evaluated (Section 1.1). Two different concentrations of each of the compounds were trialled, at three temperatures (14°C, 20°C and 27°C). The higher temperatures were used in the initial experiments to mimic, as far as possible, the optimal activity conditions of the enzymes. Latter experiments were conducted at 14°C to reflect temperatures commonly used for depuration in commercial settings in the UK, as identified in Part 1 of this report (Part 1, Section 3.2.2). All bioaccumulation and depuration trials were conducted in seawater at pH 7.5 and salinity of 35 ppt.

Treatment with each of the eight compounds was performed for either one (trials 1-6) or two hours (trials 7 - 10), followed by depuration in clean seawater for either 24 hours, 48 hours or one week at the temperatures noted above (control samples were subjected to the same depuration conditions). Depuration was performed in small tanks (around 50 litres of seawater), with aeration using filtered natural seawater (with no feeding or UV treatment). Table 2.1 gives details of the compounds and conditions tested in each of the ten depuration trials, in summary:

- Trial 1: Two concentrations of PK from *Tritirachum album* (10 and 100 mg/L; 300 and 3000 units (U), respectively) and $NaIO_4$ (1 and 10 millimolar (mM)) were tested using seawater at room temperature (around 20°C). PK at the highest concentration (100mg/L) was also tested at 27°C, in an attempt to be closer to the optimal activity of PK (37°C) within the physiological capacity of the oysters. NoV GI.1 and GII.3 was used for the bioaccumulation step. The treatment and depuration periods were 1 and 24 h respectively.
- Trial 2: Two different types of PK from *Tritirachum album* (PK in powder and PK glycerol solution) were tested. Four treatments were performed: (1) PK in powder for 1 h (100 mg/L; 3000U); (2) PK in glycerol solution for 1 h (100 mg/L; 3000U); (3) a mix of both types of PK for 1 h (200 mg/L; 6000U); and (4) a sequential treatment (200 mg/L; 6000U) involving treating the oysters for 1h with PK in powder, relocating the oysters to clean seawater for 1 h, then adding PK in glycerol solution and treating the oysters for an additional 1h. The temperature of the seawater was 27°C. The depuration period was 24 h.
- Trial 3: Two concentrations of α-amylase from porcine pancreas (300 and 3000 mg/L; 3000 and 30000U, respectively) were tested. This trial was performed at two seawater temperatures (20 and 27°C). The treatment and depuration periods were 1 and 24 h respectively.
- Trial 4: β-galactosidase (50 mU) from *Bacteroides fragilis* (expressed in *E. coli*) was trialled. Due to the special characteristics of this compound it is commercially available in very small quantities, thus this trial was carried out using very small samples numbers (five oysters). Three oysters were analysed individually to determine the initial concentration of NoV after the bioaccumulation step and one oyster was used as a control (and the NoV concentration post depuration with no treatment was determined). The remaining oyster was treated with β-galactosidase. The treatment and depuration periods were 1 and 24 h respectively.
- Trial 5: Lipase from *Candida rugosa* was tested at two concentrations (100 and 1000 mg/L) and two seawater temperatures 20 and 27°C. The treatment and depuration periods were 1 and 24 h respectively.
- Trial 6: Papain from *Carica papaya* latex (100 mg/L) was tested at two different temperatures of seawater (20 and 27°C) and trypsin from bovine pancreas (100 and 1000 mg/L) was tested at 27°C. The treatment and depuration periods were 1 and 24 h respectively.
- Trial 7: Four concentrations of $NaIO_4$ (0.05, 0.1, 0.5 and 1 mM) were tested using seawater at 14°C. The treatment time was 2 h. The depuration time was 24 h. Gill and DT were analysed.
- Trial 8: Three concentrations of PK from *Engyodontium album* (500, 1500 and 5000U) were tested using seawater at 14 °C. Two additional batches were included using PK that had been previously activated in distilled water at 37°C. The treatment time was 2 h. One of the additional batches was subjected to depuration for 48 h and the other batch was depurated for one week (in this case seawater was changed every two days). Gill and DT were analysed.
- Trial 9: Three concentrations (300, 3000 and 6000 mg/L) of α-amylase from porcine pancreas were tested. The treatment time was 2 h and the depuration period was 2 4 h. Seawater temperature was 14°C. DT were analysed.
- Trial 10: A further trial was undertaken using α-amylase from porcine pancreas with 2 h treatments. Enzyme treatment was followed by 24 h of depuration (6000 mg/L), and 48 h depuration (3000 mg/L and 6000 mg/L) at 14°C.

Table 2.1. Summary of the compounds tested and conditions used in depuration experiments

Trial	Compound	Source of compound	Concentration	Temperature of seawater (°C)	Treatment period	Depuration period	Tissues analysed
1	Proteinase K	*Tritirachum album*	300 Units 3000 Units	20 20 and 27	1 hour	24 hours	DT
	$NaIO_4$	Not applicable	1mM 10mM	20	1 hour	24 hours	DT
2	Proteinase K (powder form) Proteinase K (glycerol form) Proteinase K (powder + glycerol forms) Proteinase K (powder + glycerol forms)	*Tritirachum album*	3000 Units 3000 Units 6000 Units 6000 Units	27	1 hour 1 hour 1 hour[a] 1 hour[b]	24 hours	DT
3	α-amylase	Porcine pancreas	300 mg/L 3000 mg/L	20 and 27	1 hour	24 hours	DT
4	β-galactosidase	*Bacteroides fragilis*	50 mU	27	1 hour	24 hours	DT
5	Lipase	*Candida rugosa*	100 mg/L 1000 mg/L	20 and 27	1 hour	24 hours	DT
6	Papain	*Carica papaya* latex	100 mg/L	20 and 27	1 hour	24 hours	DT
	Trypsin	Bovine pancreas	100 mg/L 1000 mg/L	27	1 hour	24 hours	DT
7	$NaIO_4$	Not applicable	0.05, 0.1, 0.5, 1 mM	14	2 hours	24 hours	DT and gill
8	Proteinase K	*Engyodontium album*	500 Units 1500 Units 5000 Units	14[c]	2 hours	24 hours, 48 hours or 1 week	DT and gill
9	α-amylase	Porcine pancreas	300 mg/L 3000 mg/L 6000 mg/L	14	2 hours	24 hours	DT
10	α-amylase	Porcine pancreas	3000 mg/L 6000 mg/L	14	2 hours	48 hours 24 and 48 hours	DT

[a]Mix of both types of PK (PK in powder and in glycerol) for 1 h

[b]Sequential treatment (200 mg/L; 6000U) involving treating the oysters for 1h with PK in powder, relocating the oysters to clean seawater for 1h, then adding PK in glycerol solution and treating the oysters for an additional 1 h

[c]Two additional batches were included using PK that had been previous activated in distilled water at 37°C.

2.3 Evaluation of compound efficacy using an *in vitro* method

Oysters bioaccumulated NoV GII.3 as detailed in Section 2.1. The DT of the oysters was dissected and carefully chopped into small pieces and gently washed with phosphate buffered saline (PBS) to remove virus that was not firmly associated with the DT. DT pieces were then distributed into each well in a 24-well cell culture plate, to obtain a weight of 200 mg of DT/well, and each well was treated with the selected compounds for 1 h. Control wells were included and were treated only with PBS. In some *in vitro* assays, gills were also analysed in the same manner as the DT.

After the treatment period the supernatant component (ST) and DT (or gill) component were recovered and then analysed separately to determine what proportion of virus remains attached to the DT, and what proportion has been 'cleaved' and eluted into the ST. Increases in Ct values (i.e. a decrease in viral concentration) in the DT samples following treatment indicates that the compounds have cleaved the virus from the ligand. Viral recovery from the DT and ST, RNA extraction and quantification by real-time RT-PCR was carried out according to the ISO/TS 15216-1 method with minor modifications (Section 2.4). A schematic outline of the final *in vitro* process used is shown in Figure 2.1.

An initial experiment was undertaken to investigate the reproducibility and practicality of the *in vitro* method using Eppendorf tubes, or 24-well cell culture plates. This involved investigating the impact of three concentrations of $NaIO_4$ on NoV levels in triplicate samples of DT and ST using Eppendorf's and 24-well plates. Both assay formats gave reproducible results with relatively low standard deviations (SD) (Appendix 4), however the 24-well plate format was easier to perform and less time consuming and was therefore used for all experiments.

2.4 Method of analysis

Viral recovery from shellfish, RNA extraction and quantification by real-time RT-PCR (RT-qPCR) was carried out according to the ISO/TS 15216-1 method with minor modifications (Le Guyader et al., 2009). For the extraction step, mengovirus (MgV) was used as an extraction control to measure the recovery (%) of viruses from the oysters. NoV quantification was performed using real-time RT-PCR as previously described (ISO/TS15216-1), using standard curves in each amplification series. Additionally, RNA extracted from the oysters was analysed 'neat' (undiluted) and diluted (1/10) to assist in overcoming any issues relating to reaction inhibition. The extraction efficiency was checked for each extraction to avoid potential technical problems due to the use of enzymes. If required, extractions were repeated.

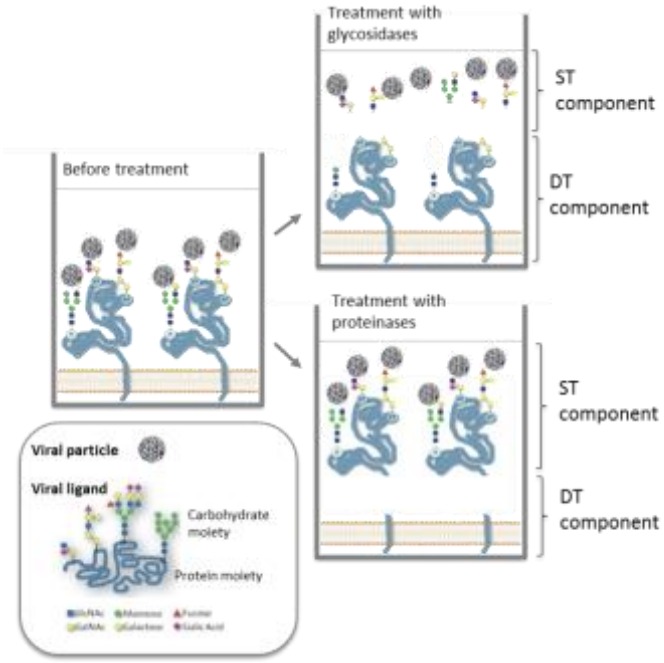

Figure 2.1: Schematic outline of *in vitro* process used. The figure shows a virtual cell-culture well containing the oyster digestive tissue (DT) and supernatant (ST) portions and associated glycoproteins (composed of a carbohydrate moiety and protein moiety) where viral particles bind. If the compound used is effective, the viral particles will be cleaved from the carbohydrate moiety (glycosidases) or from the protein moiety (proteinases).

3 Results

3.1 Quality control for RNA extraction and real-time RT-PCR

Extraction efficiency values, determined by the addition of a known amount of MV in each RNA extraction series, showed that samples were suitable for quantification and the data were consistent. Following the ISO/TS 15216-1 method recommendations, only extraction efficiency values greater than 1% were considered to be acceptable and included in the study. Notably, in a recent field study involving the analysis of 168 oyster samples, we found no relationship between samples showing acceptable but poor (1-5%) extraction efficiencies and the quantity of NoV detected, compared to those with better extraction efficiencies (>5%) (Le Mennec et al., 2016). This indicates that quantification of NoV in samples with extraction efficiencies above 1% is valid. Extraction efficiency values for DT were between 1.2% and 61.8% for NoV GII.3 and between 9.8% and 25.2% for NoV GI.1.

3.2 Virus concentration following bioaccumulation

The concentration of NoV GII.3 in oysters following bioaccumulation (but prior to enzyme treatment and depuration) in trials 1 – 6 was between 3.5 $\times 10^4$ and 5.6 $\times 10^5$ RNA c/g DT, with a geometric mean titre (GMT) of 9.8 $\times 10^4$ RNA c/g DT. For trial 1 there was 2.0 $\times$ 10^3 RNA c/g DT of NoV GI.1 present. For the experiments conducted at 14°C (trials 7 to 10), the initial NoV GII.3 levels were between 1.2 $\times 10^3$ and 1.3 $\times 10^6$ RNA c/g DT, with a GMT of 1.9 $\times 10^4$ RNA c/g DT. In these trials, gill tissue was also analysed and the initial NoV GII.3 concentrations prior to enzyme treatment and depuration were between the limit of quantification (100 RNA c/g DT) and 1.9 $\times 10^2$ RNA c/g gill.

3.3 Depuration trials

The depuration trials were conducted using three different temperatures: trials 1 to 6 were carried out from March 2015 to June 2015 at 20°C and 27°C. Trials 7 to 10 were carried out from November 2015 to March 2016 at 14 ± 1°C, this temperature was selected to more closely mimic industry practices. Compounds and conditions used in each trial are shown in Table 1, with respective results presented in Tables 2.2 (Trials 1 – 6) and 2.3 (Trials 7 – 10), and Figures 2.2 and 2.3.

For experiments conducted at 20 and 27°C, the GMT of NoV GII.3 in oysters following bioaccumulation was 9.8 x 10^4 RNA c /g of DT, and for NoV GI.1, the GMT was 2 x 10^3 RNA c/g of DT. Generally, enzymatic pre-treatment followed by depuration did not result in large decreases in NoV concentrations in the DT (Tables 2.2 and 2.3), however small reductions were observed for some compounds, particularly papain, $NaIO_4$, PK and trypsin. Figure 2.2 illustrates data presented in Table 2.2 and shows that NoV GII.3 was reduced by a further 68% in oysters that were treated with 100 mg/L papain when the seawater temperature was 20°C when compared to the control sample (only subjected to depuration). NoV was reduced by a further 56% in oysters treated with 1 mM $NaIO_4$, compared to control samples which were depurated at 20°C. Oysters treated with 100 mg/L trypsin or 100 mg/L PK showed higher reduction rates than control samples when depurated at 27°C (68% and 65% further reduction in NoV respectively).

For two trials (PK in trial 1 and amylase in trial 3) an apparent increase in NoV GII.3 levels was observed following some treatments (Table 2.2). However, the increase was small and likely due to variation in the viral content of the samples after bioaccumulation. The variability in the initial viral loads among trials might be explained by natural variation in individual physiological activity, shellfish age and the condition of each oyster. These factors can affect the filtering and uptake processes, even when experimental lab conditions are identical. These phenomena have been previously observed in different studies using a variety of viruses and shellfish species (Canzonier, 1971; Hernroth and Allard, 2007; Love et al. 2010; Ueki et al. 2007; Polo et al. 2014a, b). This variation should be considered

when interpreting the results of the reduction studies, because small decreases in NoV may be a function of variability rather than the effect of the compounds. Considering this, PK, trypsin and papain showed the largest reductions and were the most promising compounds trialled at higher temperatures; while the initial trials with $NaIO_4$ at 20°C showed reductions, further trials at 14°C exhibited no enhancement of depuration – as discussed further below.

For NoV GI.1 (Table 2.3), only two compounds (PK and $NaIO_4$) were tested (trial 1), PK did not enhance the depuration of NoV, and $NaIO_4$ at 10 mM (20°C) showed a very marginal enhancement in viral reduction (14.5 %).

Table 2.2. NoV GII.3 levels in oysters following immersion for one hour in various compounds and depuration for 24 hours at either 20 and 27°C. Control samples shown were depurated, but not subjected to treatment with the compounds.

Trial	Compound	Temp (°C)	mg/L[a]	Eff. Ext.[b] (%)	Mean Ct[d] value	RNAc/g DT[d]
1	*Initial load*[c]	20	-	*10*	*24.6*	*3.6 x 10^5*
	Control		*0*	*17*	*24.6*	*3.7 x 10^5*
	PK		10	13	23.9	5.9 x 10^5
	PK		100	14	23.7	6.4 x 10^5
	$NaIO_4$ (1mM)		214	24	25.8	1.7 x 10^5
	$NaIO_4$ (10mM)		2140	21	25.1	2.7 x 10^5
	Control	27	*0*	*10*	*27.9*	*4.4 x 10^4*
	PK		100	25	27.6	5.3 x 10^4
2	*Initial load*[c]	20	-	*9.5*	*27.3*	*6.7 x 10^4*
	Control		*0*	*21*	*26.2*	*1.3 x 10^5*
	PK		100	12	27.8	4.8 x 10^4
	PK glycerol		100	6	26.9	8.3 x 10^4
	PK mix		200	27	27.0	7.7 x 10^4
	PK sequential	27	200	7	26.4	1.1 x 10^5
3	*Initial load*[c]	20	-	*19.9*	*27.6*	*5.4 x 10^4*
	Control		*0*	*9*	*28.3*	*3.5 x 10^4*
	α-Amylase		300	24	28.8	2.6 x 10^4
	α-Amylase		3000	10	27.6	5.2 x 10^4
	Control	27	*0*	*23*	*28.4*	*3.3 x 10^4*
	α-Amylase		300	23	28.1	3.8 x 10^4
	α-Amylase		3000	22	27.7	4.9 x 10^4
4	*Initial load*[c]	27	-	*47.8*	*23.9*	*5.6 x 10^5*
	Control		*0*	*43*	*24.6*	*3.6 x 10^5*
	β-galactosidase		50 mU	50	23.9	5.7 x 10^5
5	*Initial load*[c]	20	-	*39.7*	*28.3*	*3.5 x 10^4*
	Control		*0*	*19*	*27.2*	*7.0 x 10^4*
	Lipase		100	28	26.7	9.4 x 10^4
	Lipase		1000	27	28.2	3.7 x 10^4
	Lipase		100	15	27.2	7.0 x 10^4
	Lipase	27	1000	22	27.2	7.0 x 10^4
6	*Initial load*[c]	20	-	*14.4*	*28.3*	*3.5 x 10^4*
	Control		*0*	*32*	*25.9*	*1.6 x 10^5*
	Papain		100	6	27.7	5.0 x 10^4
	Papain		100	9	27.1	7.5 x 10^4
	Trypsin		100	15	27.7	5.0 x 10^4
	Trypsin	27	1000	10	26.9	8.3 x 10^4

[a]Compounds tested and their final concentrations; [b]the extraction efficiency; [c]NoV concentration following bioaccumulation (no treatment or depuration); [d]Results (expressed as Ct values, and RNA copies/g DT) for each trial are shown. PK = proteinase K. $NaIO_4$ = sodium periodate.

Table 2.3. NoV GI.1 levels in oysters following immersion for one hour in various compounds and depuration for 24 hours at either 20 and 27°C. Control samples shown were depurated, but not subjected to treatment with the compounds.

Trial	Compound	Temp (°C)	mg/L[a]	Eff. Ext. (%)[b]	Mean Ct value[d]	RNAc/g DT[d]
1	*Initial load*[c]	20	-	*10*	*32.8*	*2.0 x 10^3*
	Control		-	*17*	*33.7*	*1.1 x 10^3*
	PK		10	13	31.7	4.1 x 10^3
	PK		100	14	32.2	2.8 x 10^3
	$NaIO_4$ (1mM)		214	24	33.7	2.0 x 10^3
	$NaIO_4$ (10mM)		2140	21	32.7	9.4 x 10^2
	Control	27	-	*10*	*33.9*	*9.8 x 10^2*
	PK		100	25	33.7	1.1 x 10^3

[a]Compounds tested and their final concentrations; [b]the extraction efficiency; [c]NoV concentration following bioaccumulation (no treatment or depuration); [d]Results (expressed as Ct values, and RNA copies/g DT) for each trial are shown. PK = proteinase K. $NaIO_4$ = sodium periodate.

To investigate the effect of the compounds when depuration is conducted at lower temperatures, reflecting those more routinely used in the UK, some experiments were repeated (trials 7 – 10) at 14°C (Table 2.4, Figure 2.3). For these experiments the GMT for NoV in oysters following bioaccumulation was 1.9 x 10^4 RNA c /g of DT and 90 RNA c/g of gills.

At 14°C, two compounds showed an enhanced reduction in viral levels in comparison to non-treated control samples: PK (500U) with 60% reduction; and α-amylase at 3000 and 6000 mg/L, with 61 and 80% reduction respectively (Figure 2.3). α-amylase at a concentration of 6000 mg/L was the compound that showed the highest level of reduction (trial 9), however this was not able to be repeated and confirmed in a subsequent experiment (trial 10), corroborating results obtained with trial 3 which showed no reduction at 20 - 27°C. $NaIO_4$ did not enhance virus removal confirming that the small decrease in NoV observed at 20 - 27°C may relate to the higher seawater temperature and an increase in the oysters physiological activity.

For trial 8 at 14°C, a sample of oysters treated with PK was maintained in clean seawater under aeration for a week to investigate if long term depuration would lead to further NoV reductions, however, no further decrease was observed (final concentration of 3.92 x 10^4 RNA c/g of DT).

As gills are one of the first organs to come into contact with contaminated seawater, the effect of two compounds (PK and $NaIO_4$) on the reduction of NoV in the gills following depuration was examined (trials 7 and 8). Only low levels of NoV were detected in the gills following bioaccumulation (Table 2.4), confounding interpretation, however it appears that neither $NaIO_4$ nor PK is effective in enhancing depuration of NoV from this tissue.

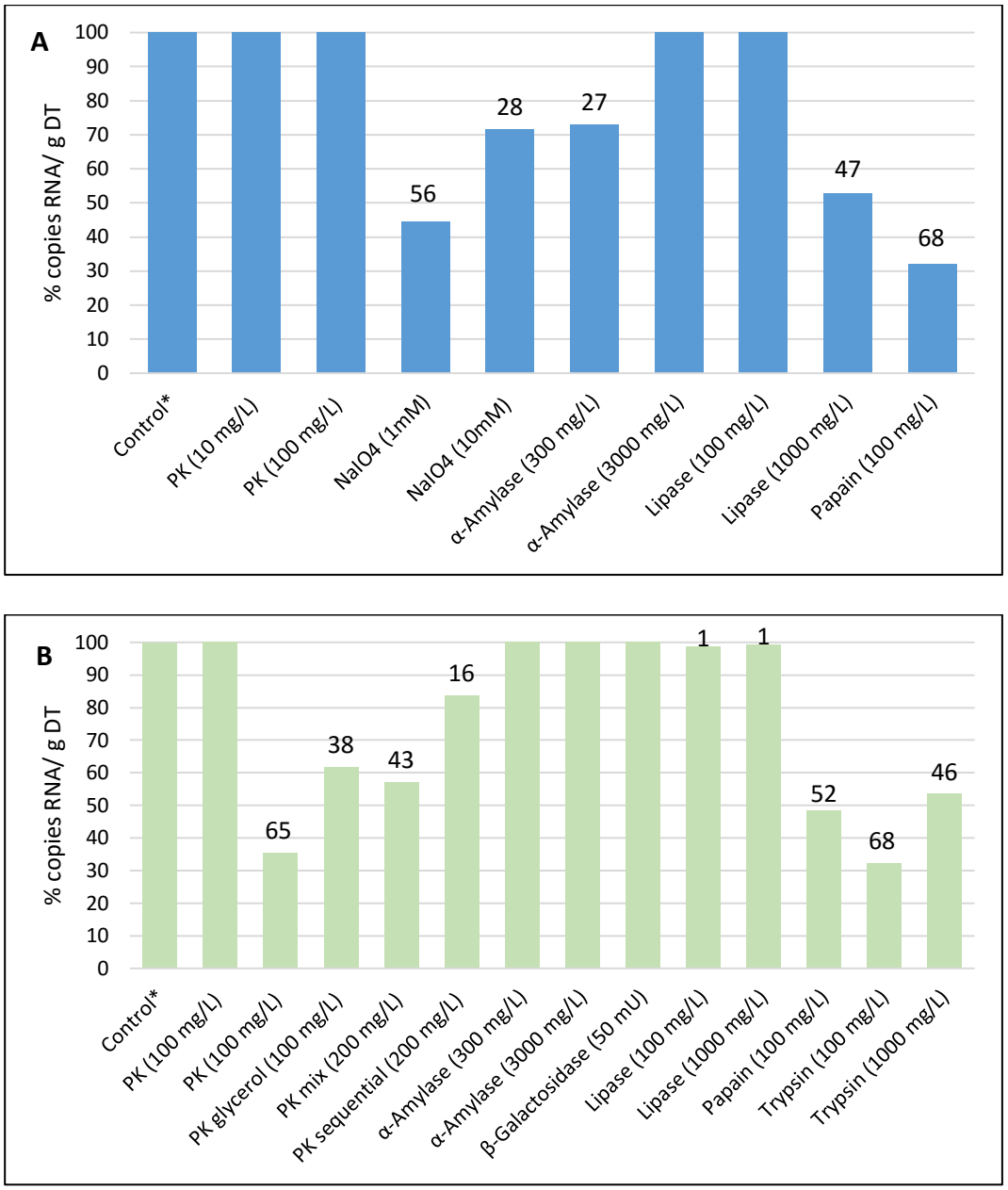

Figure 2.2: Schematic representation of NoV GII.3 removal in oyster DT following a one hour enzyme treatment step and 24 hours of depuration at 20°C (A) and 27°C (B). The bars show the amount (as a %) of NoV remaining in oysters following treatment when compared to control samples (which were not subjected to enzymatic pre-treatment) i.e. smaller bars indicate a larger reduction in NoV. Values above bars show the decrease in NoV (as a %), compared to control samples.

Table 2.4. Concentration of NoV GII.3 in oysters following treatment for two hours with various compounds and depuration at 14°C.

Digestive Tissue					
Trial	**Compound**	**mg/L[a]**	**Eff. Ext. (%)[b]**	**Mean Ct value**	**RNAc/ g DT**
	Initial load[c]		*15.0*	*20.9*	*1.33 x 10^6*
7	*Control*	-	*17*	*23.62*	*2.30 x 10^5*
	NaIO4 (0.05 mM)	10	16	21.98	6.46 x 10^5
	NaIO4 (0.1 mM)	20	12	21.51	8.83 x 10^5
	NaIO4 (0.5 mM)	100	9	22.71	4.70 x 10^5
	NaIO4 (1 mM)	200	13	22.18	5.66 x 10^5
	Initial load[c]		*7.4*	*30.5*	*7.9 x 10^3*
8	*Control*	-	*9*	*27.57*	*4.90 x 10^4*
	PK (500U)	16.6	5	29.07	1.94 x 10^4
	PK (1500U)	50	7	27.65	4.80 x 10^4
	PK (5000U)	166.6	7	27.05	7.02 x 10^4
	PK (5000U) (48h)	166.6	34	28.52	1.81 x 10^4
	Initial load[c]		*18.7*	*29.5*	*1.0 x 10^4*
9	*Control*	-	*14*	*28.22*	*2.20 x 10^4*
	α-Amylase	300	62	27.10	4.56 x 10^4
	α-Amylase	3000	20	29.93	8.49 x 10^3
	α-Amylase	6000	43	31.49	4.45 x 10^3
	Initial load[c]		*5.8*	*30.7*	*1.2 x 10^3*
10	*Control*	-	*7*	*31.48*	*7.24 x 10^2*
	α-Amylase	6000	13	31.55	6.94 x 10^2
	α-Amylase (48h)	3000	18	31.51	7.10 x 10^2
	α-Amylase (48h)	6000	6	30.62	1.25 x 10^3
Gills					
Trial	**Compound**	**mg/L[a]**	**Eff. Ext.[b](%)**	**Mean Ct value**	**RNAc/ gDT**
	Initial load[c]		*2.2*	*33.5*	*1.9 x 10^2*
7	*Control*	-	*2*	*34.10*	*1.71 x 10^1*
	NaIO4 (0.05 mM)	10	2	36.05	4.97 *x 10^1*
	NaIO4 (0.1 mM)	20	1	34.74	1.08 *x 10^2*
	NaIO4 (0.5 mM)	100	1	32.36	5.60 *x 10^2*
	NaIO4 (1 mM)	200	2	32.22	5.34 *x 10^2*
	Initial load[c]		*3.3*	*38.6*	*<LQ*
8	*Control*	-	*7*	*36.50*	*1.83 x 10^2*
	PK (500U)	16.6	7	37.52	9.11 *x 10^1*
	PK (1500U)	50	5	36.79	1.38 *x 10^2*
	PK (5000U)	166.6	5	35.31	3.92 *x 10^2*

[a]Compounds tested and their final concentrations; [b]The extraction efficiency; [c]NoV concentration following bioaccumulation (no treatment or depuration); [d]Results (expressed as Ct values, and RNA copies/g DT) for each trial are shown. PK = proteinase K. $NaIO_4$ = sodium periodate.

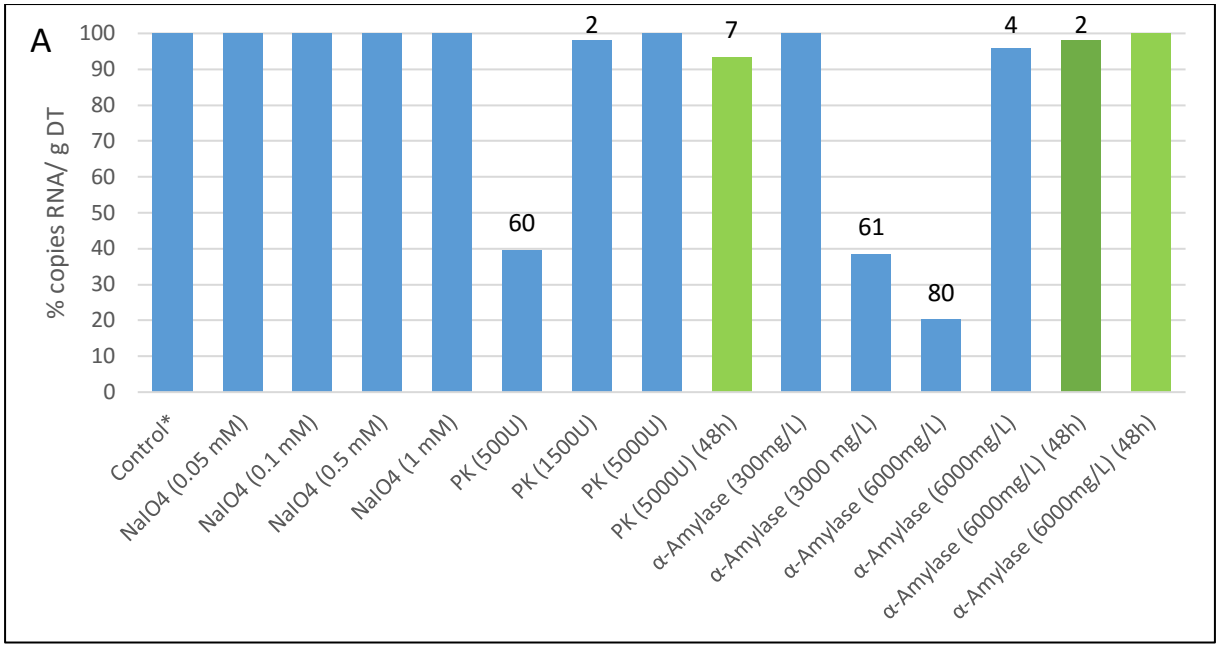

Figure 2.3: NoV GII.3 reductions in oyster DT following a 2-hour enzyme treatment step and depuration at 14°C. The bars show the amount (as a %) of NoV remaining in oysters following treatment when compared to control samples (which were not subjected to enzymatic pre-treatment) i.e. smaller bars indicate a larger reduction in NoV. Values above bars show the decrease in NoV (as a %), compared to control samples. Blue bars = depuration for 24 hours; green bars = depuration for 48 hours.

3.4 Evaluation of compound efficacy using an *in vitro* method

The efficacy of four compounds was evaluated using an *in vitro* method (Section 2.3), namely $NaIO_4$, PK, α-amylase and papain, at different concentrations (Table 2.5, Figure 2.4). The results show reductions in NoV GII.3 in the DT component in wells treated with $NaIO_4$, PK and to a lesser extent with papain. The highest concentration (100 mM) of $NaIO_4$ resulted in a decrease of NoV (RNA copies) in the DT component of about 10 fold (on average). However, the concentrations in the ST did not increase proportionately, suggesting a disruption of the capsid when high concentrations are used. Lower concentrations of $NaIO_4$ had a reduced effect.

Only the highest concentration of PK (1000 mg/L) showed a reduction in RNA copies in the DT component. Similarly, for papain only the highest concentration (100 g/L) resulted in a decrease in NoV. The *in vitro* experiments with α-amylase showed no differences in NoV concentration in the DT component following treatment (Table 2.5, Figure 2.4), confirming the results of the depuration trials.

The average concentration of virus in the corresponding ST for each treatment generally increased proportionately with the enzyme concentration, which indicates the release of NoV from the DT and its migration to the ST. This was generally the case for $NaIO_4$ (except the highest concentration), α-amylase and papain. Interestingly for PK, when the concentration of virus in the DT component decreased, levels in the ST also decreased and this was proportionate to the enzyme concentration (Figure 2.4). The observed reduction in NoV in the ST may indicate that the virus is being destroyed by the PK.

With regard to *in vitro* assays carried out with gill tissue, only $NaIO_4$ and PK were tested. While $NaIO_4$ was ineffective in reducing NoV levels, all concentrations of PK resulted in small decreases of NoV in the gill component (Table 2.5).

Table 2.5. Efficacy of four compounds in reducing NoV levels in digestive tissue (DT) (top) and gills (bottom), as determined using an *in vitro* method. Results are expressed as NoV GII.3 RNA copies/200 mg of DT or gill component, or 500 µL of supernatant (ST) along with the corresponding standard deviation (*SD*). A decrease in RNA copies in the DT or gill component when compared to control concentrations indicates that the treatment is effectively cleaving the virus/ligand from the DT.

Digestive Tissue						
Trial	Compound	mg/L[a]	DT component	*SD* of DT component	ST component	*SD* of ST component
1	*Control*	-	*4.64 x 10³*	± 2.67E+03	*6.58 x 10²*	± 5.72E+02
	$NaIO_4$ (1 mM)	200	2.27 x 10³	± 1.34E+03	1.13 x 10³	± 1.02E+03
	$NaIO_4$ (10 mM)	2000	4.11 x 10²	± 9.59E+02	2.92 x 10²	± 4.18E+02
	$NaIO_4$ (100 mM)	20000	1.99 x 10²	± 1.65E+02	1.03 x 10³	± 7.66E+02
2	*Control*	-	*1.50 x 10⁴*	± 1,50E+04	*3.90 x 10³*	± 9,24E+02
	$NaIO_4$ (10 mM)	200	3.24 x 10⁴	± 8,48E+03	7.02 x 10³	± 1,93E+03
	$NaIO_4$ (100 mM)	20000	9.76 x 10²	± 9,70E+02	7.08 x 10³	± 1,51E+03
3	*Control*	-	*6.91 x 10⁵*	± 4,98E+05	*7.45 x 10⁴*	± 1,73E+04
	$NaIO_4$ (0,05 mM)	10	3.19 x 10⁵	± 1,36E+05	4.00 x 10⁴	± 2,35E+04
	$NaIO_4$ (0,1 mM)	20	8.51 x 10⁵	± 5,12E+05	9.94 x 10⁴	± 3,46E+03
	$NaIO_4$ (0,5 mM)	100	3.82 x 10⁵	± 1,15E+05	4.11 x 10⁴	± 1,30E+04
	$NaIO_4$ (1 mM)	200	5.04 x 10⁵	± 2,08E+05	1.56 x 10⁵	± 9,18E+04
	$NaIO_4$ (10 mM)	2000	2.84 x 10⁵	± 1,75E+05	3.42 x 10⁴	± 6,63E+03
4	*Control*		*6.69 x 10³*	± 3.52E+03	*1.23 x 10³*	± 6.10E+02
	PK (0,1U)	10	5.80 x 10³	± 6.52E+03	9.79 x 10²	± 9.66E+02
5	*Control*		*2.69 x 10⁵*	± 1,68E+05	*2.07 x 10⁴*	± 8,66E+03
	PK (1,5 U)	100	5.42 x 10⁴	± 1,35E+05	1.23 x 10⁴	± 2,02E+04
	PK (7,5 U)	500	1.28 x 10⁵	± 1,93E+05	1.01 x 10⁴	± 1,24E+04
	PK (15 U)	1000	3.60 x 10⁴	± 7,00E+04	5.49 x 10³	± 1,91E+04
6	*Control*		*7.35 x 10³*	± 4,99E+03	*2.18 x 10³*	± 6,48E+02
	α-Amylase	600	1.13 x 10⁴	± 4,89E+03	3.99 x 10³	± 2,84E+03
	α-Amylase	6000	6.36 x 10³	± 2,21E+03	3.26 x 10³	± 2,90E+03
	α-Amylase	12000	1.21 x 10⁴	± 5,44E+03	3.52 x 10³	± 1,06E+03
	Control		*3.58 x 10⁴*	± 1.56E+04	*1.09 x 10³*	± 6.10E+02
	α-Amylase	20000	3.26 x 10⁴	± 2.47E+03	2.30 x 10³	± 9.19E+02
7	*Control*		*1.67 x 10⁴*	± 1.42E+04	*1.34 x 10³*	± 9.28E+02
	Papain	10000	1.01 x 10⁴	± 2.23E+03	1.47 x 10³	± 1.15E+03
	Papain	100000	4.92 x 10³	± 6.12E+03	2.58 x 10³	± 5.00E+03
Gills						
Trial	Compound	mg/L[a]	Gill component	*SD* of gill component	ST component	*SD* of ST component
1	*Control*	-	*3.69 x 10²*	± 2,91E+02	*1.64 x 10²*	± 3,50E+02
	$NaIO_4$ (0.05 mM)	10	5.59 x 10²	± 6,10E+02	3.08 x 10¹	± 2,18E+01
	$NaIO_4$ (0.1 mM)	20	5.60 x 10²	± 3,69E+02	1.52 x 10²	± 1,66E+02
	$NaIO_4$ (0.5 mM)	100	7.74 x 10²	± 9,20E+02	4.37 x 10¹	± 1,30E+02
	$NaIO_4$ (1 mM)	200	3.97 x 10²	± 2,53E+02	1.30 x 10²	± 1,18E+02
	$NaIO_4$ (10 mM)	2000	3.50 x 10²	± 2,06E+02	9.18 x 10¹	± 7,53E+01
2	*Control*	-	*2.28 x 10²*	± 2,73E+02	*<LQ*	-
	PK (1.5 U)	10	<LQ	-	<LQ	-
	PK (7.5 U)	50	1.41 x 10²	± 4,22E+01	<LQ	-
	PK (15 U)	1000	1.10 x 10²	± 1,09E+02	<LQ	-

[a]Compounds tested and their final concentrations. LQ = limit of quantification (50 RNAc/g of DT). PK = proteinase K. $NaIO_4$ = sodium periodate.

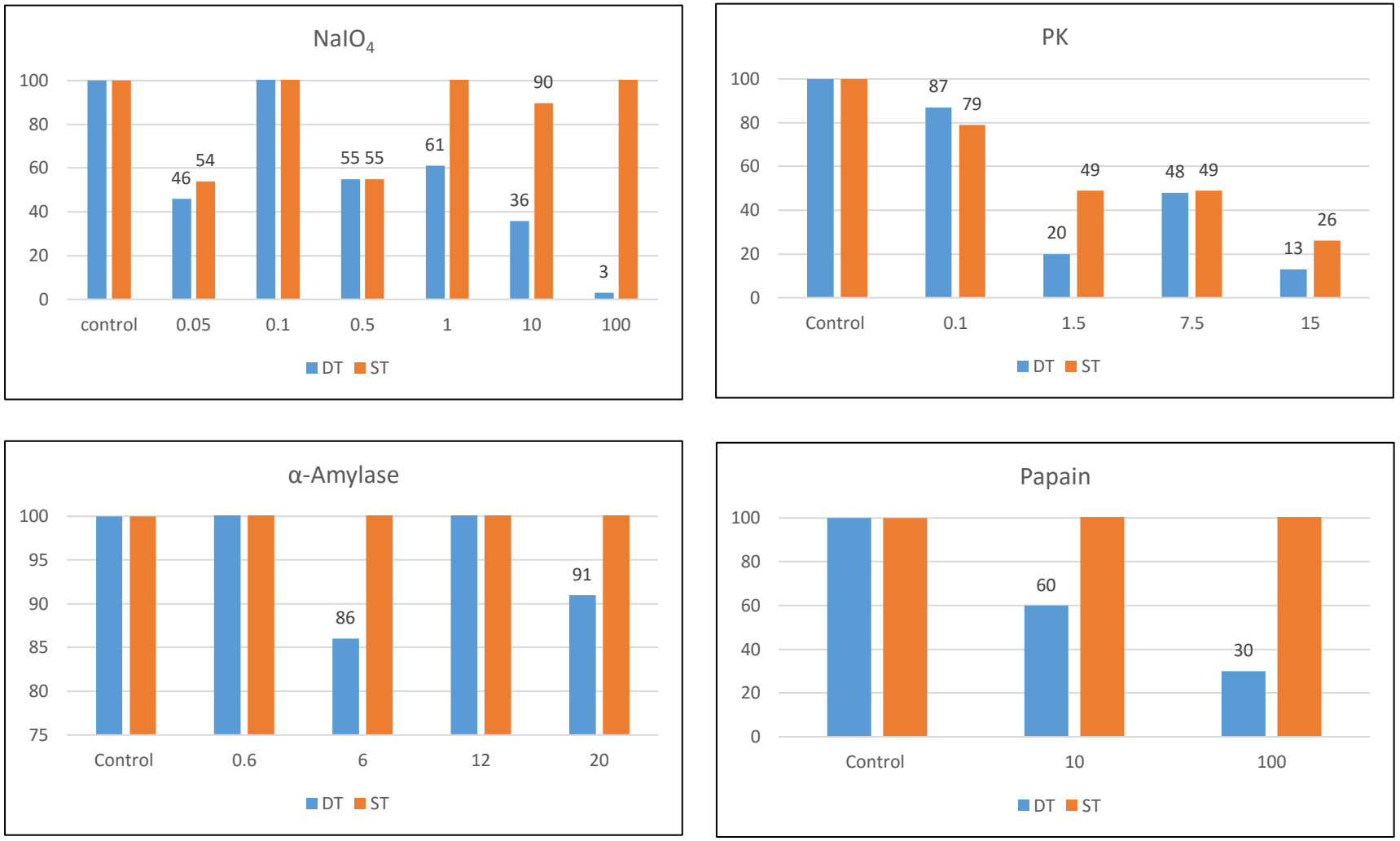

Figure 2.4: Comparison of NoV GII.3 levels (detected by an *in vitro* assay) in the digestive tissue (DT) (blue bars) and supernatant (ST) components (orange bars) of oysters treated with various compounds. Levels are expressed as a % of that detected in control wells in which no compounds were applied. Compound concentrations are expressed in mM for $NaIO_4$, in units for PK (proteinase K), and mg/L for α-amylase and papain.

4 Discussion

Two different types of experiments were undertaken to identify the best candidate compounds: (1) live oysters contaminated with NoV were held in tanks, a selection of compounds were added at different concentrations using a range of conditions, and depuration was then undertaken; and (2) *in vitro* assays were conducted to evaluate the potential for the compounds to reduce NoV levels in the digestive and gill tissues.

The study began with the selection of compounds that can potentially disrupt NoV ligands in oyster tissues, that are safe for consumers and environmentally friendly. Norwalk virus (GI.1) binds to the DT of *C. gigas* via an A-type HBGA (Maalouf et al., 2010b). As in humans, NoV GII recognises a slightly different ligand and displays a different tissue distribution than GI strains. Higher concentrations of GII are detected in gill and mantle tissues, where binding involves SA, whereas in the DT the interaction involves both SA and an A-type HBGA (Maalouf et al., 2010b; Zakhour et al., 2010). Based on this knowledge, $NaIO_4$ was selected for further investigation because it cleaves sialylated carbohydrate motifs. PK was selected because of its efficiency in disrupting viral particles as part of the extraction step in different analytical methods. Enzymes such as papain and α-amylase were selected because of their activity against HBGAs, and also the ability to readily source these compounds for trials.

Secondly, consideration was given to the strains of NoV to use for the experiments. NoV GII.3 and GI.1 were chosen because they are efficiently bioaccumulated by oysters (Maalouf et al., 2011; Yu et al., 2015). All experiments were thus performed with two stools that have been previously characterised and are known to exclusively contain GII.3 and GI.1 NoV (Drouaz et al., 2015; Maalouf et al., 2011; Maalouf et al., 2010b). This approach eliminated variability that could be due to the presence of different NoV strains and varying bioaccumulation efficiencies in oysters.

Quantification of the viruses in oysters was conducted using real-time RT-PCR, following the ISO/TS standard method. Standard precautions were adopted to ensure accuracy, including multiple extractions in different series, incorporation of quantified nucleic acid standards and the use of inhibition controls. However, as with many microbiological quantification techniques, comparing levels within a log can be challenging due to the variability of the method. Thus, small changes in viral levels could be 'masked' by method variability, as suggested by some results observed in this study (particularly for gill samples which had low levels of NoV). During a recent field study, for which triplicate extractions and triplicate amplifications of each extract were performed, high variability was observed for oysters with lower levels of contamination (Le Mennec et al., 2016). To avoid such variability and be within the working range of the method, oysters in this study were contaminated at quite high levels, which may not be representative of usual contamination levels.

One of the main challenges in using enzymes for treating NoV contaminated oysters is the difficulty associated with optimising conditions to maximise enzyme activity. Thus, some depuration trials were conducted at higher temperatures (20°C and 27°C) than those routinely used in the UK; this raises the possibility that observed NoV reductions may be partially due to the high temperature and potential increases in oyster filtration rates. However, the control oysters, to which no enzyme treatment was applied, showed no noticeable NoV depuration, even at 27°C. This confirms that the effect (i.e. enhanced depuration) observed for some compounds, particularly PK, trypsin and papain, was not related to increases in physiological activity of the oysters alone. PK also showed an effect at the lower temperature of 14°C (trypsin and papain not tested at lower temperatures). $NaIO_4$ improved the NoV depuration rates at 20°C or 27°C, but there was no noticeable effect at 14°C. For $NaIO_4$, the increased physiological activity of the oysters at higher temperatures may have contributed to the higher depuration rates observed (Bougrier et al., 1995), because there is no evidence to suggest that the activity of $NaIO_4$ increases at higher temperatures. It should also be noted, that temperature is

not the only parameter influencing the activity of the compounds used in this study, other factors such as the pH of the seawater may also have an impact and could be investigated in future trials.

The conditions (e.g. temperature) used during treatment and depuration are important to consider, as good filtration activity needs to be maintained to ensure that the compounds reach the target tissue and exert their effect on the HBGA-virus bond. Most compounds tested showed no effect on oyster filtration activity, however we did note that the lipase and amylase treatments formed foam upon aeration resulting in slightly foggy seawater. In some trials, $NaIO_4$ impacted the oysters filtration behaviour, when oysters came in contact with $NaIO_4$ they closed their valves and ceased filtering. This behaviour was observed for all concentrations used with $NaIO_4$, but it was more striking for the highest level. This may explain why NoV reduction was highest in DT using a low $NaIO_4$ concentration, rather than higher concentrations. We did not observe a significant reduction in NoV in the gills of oysters treated with $NaIO_4$, the reduction in oyster filtering may have impaired any potential reductions making it difficult to evaluate the effect of the $NaIO_4$. This finding highlights the need to find a balance between the optimal conditions for the compound activity, such as concentration, solubility in seawater, temperature and the physiological conditions of the oysters to maximise their filtration activity. These factors, in addition to the physiological condition of the oysters at the outset, may also contribute to variability in NoV levels.

To further evaluate the efficacy of the selected compounds an *in vitro* method was developed that demonstrated that PK and papain can reduce NoV GII.3 levels in DT. This corroborated results from the depuration trials, which showed that PK, papain and trypsin were effective in enhancing depuration (note, trypsin was not trialled using the *in vitro* method). The *in vitro* test also demonstrated that $NaIO_4$ can reduce GII.3 levels in DT (similar to depuration trials at elevated temperatures), however very high concentrations of $NaIO_4$ were needed for significant elution of GII.3, exceeding $NaIO_4$ levels needed to destroy SA ligands (Maalouf et al., 2010b). This observation may suggest that after 24 hours bioaccumulation, the NoV particles are not just binding to SA, but may also be entrapped in the tissue. Depuration trials for $NaIO_4$ gave mixed results, with no enhanced reductions at 14°C and negative impacts on oyster filtration. Thus, despite the advantages of $NaIO_4$ being a chemical compound of a non-enzymatic nature, which is more stable in seawater and able to disrupt/destroy SA, this compound is not a good candidate. PK and papain (plant proteases) showed the most promise in both the depuration trials and *in vitro* assays. This raises the question of whether plant proteases are more resistant than animal proteases to seawater conditions and thus potentially more adequate for use in depuration processes.

The impact of longer contact times with some compounds (including PK) was evaluated in this study, with the 'dipping' period increased from one to two hours. The depuration period was also lengthened, from 24 hours used in initial experiments, to a 48-hour period (more similar to the 42 h period routinely practiced in the UK). However, the increments in dipping and depuration times did not further enhance the reduction of NoV.

5 Conclusions and Recommendations

This study aimed to identify compounds that enhance NoV depuration and provides promising initial results to support future in-depth investigations. Firstly, from a technical perspective, the development of an *in vitro* method will be useful for further studies. Secondly, the *in vitro* method and depuration trials identified two plant proteases, PK and papain, that show promise in reducing NoV in oysters. Depuration trials demonstrated that trypsin may also be effective. While the initial studies are encouraging, further research is required to confirm and optimise the effect of these compounds, thus the following specific recommendations are offered:

1. The preliminary experiments were conducted on oysters contaminated with high levels of NoV – this enabled the reduction in NoV to be accurately quantified in most trials. Further experiments are required on oysters that contain lower levels of NoV, such as those that are naturally contaminated, to confirm the effect of these compounds in all oyster tissues. Quantification of low levels of NoV in DT and other tissues using the existing real time RT-PCR method is subject to considerable variation, thus it is likely that future studies would need to use a more precise method. Recent advances have been made on the development of a digital PCR (dPCR) method for the detection of NoV in oysters (Polo et al., 2016). This method precludes the need for standard curves and is less prone to inhibition, and thus may be less variable that the standard ISO/TS real time PCR based method. Additionally, the dPCR method has been used for naturally contaminated oysters, and it may be suitable for future studies on the impact of various compounds (Polo et al., 2016).

2. Further studies that seek to optimise the physiological activity of oysters and the enzymatic activity of PK, papain and trypsin should be conducted, including optimisation of conditions such as seawater temperature, pH, enzyme concentration, and dipping and depuration periods.

3. Consideration could be given to conducting experiments which involve the immersion of contaminated oyster batches for short periods in warm seawater, at temperatures at which the enzymes will be more efficient (generally >30°C). Higher temperatures than those trialled to date (27°C) could be tested, but for very short times to avoid spawning or increases in the growth of pathogenic bacteria such as Vibrio.

4. A key issue to consider when applying treatments such as enzymes to oysters, is the need to ensure that adequate amounts of enzyme reach the sites of interest inside the oyster. Microencapsulation (Part 1, Section 6.2.3) has been used for the delivery of vaccines and probiotic bacteria in the aquaculture sector and holds potential for ensuring active enzymes reach the HBGA-NoV complexes in oysters. Future studies could be conducted to explore the feasibility of microencapsulation of the enzymes, and to evaluate if this facilitates further NoV reductions in oysters.

Lastly, while enzymatic pre-treatment holds potential for enhancing NoV reduction through the depuration process, it is clear that NoV can persist for long periods in oysters due to the specific interactions between HBGAs and NoV. Therefore, it is considered that the major focus should be placed on improvements in water quality to avoid NoV and HAV contamination of shellfish at source.

References

de Abreu Corrêa, A., Albarnaz, J.D., Moresco, V., Poli, C.R., Teixeira, A.L., Simões, C.M.O., Barardi, C.R.M., 2007. Depuration dynamics of oysters (*Crassostrea gigas*) artificially contaminated by Salmonella enterica serovar Typhimurium. Marine environmental research 63(5), 479-489.

Ang, L.H., 1998. An outbreak of viral gastroenteritis associated with eating raw oysters. Commun Dis Public Health 1(1), 38-40.

Anon, 2009. Guidance for inspection of shellfish purification systems for Local Food Authorities, 2 ed. Food Standards Agency: Scotland, p. 45.

Anon, 2012. Norovirus (NoV) in oysters: methods, limits and control options. EFSA Journal 10(1), 39.

Anon, 2015a. WHO estimates of the global burden of foodborne diseases: foodborne disease burden epidemiology reference group 2007-2015, Geneva. p. 268. http://www.who.int/foodsafety/publications/foodborne_disease/fergreport/en/

Apweiler R, Hermjakob H, Sharon N. (1999). On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. Biochim. Biophys. Acta 1473: 4-8.

Atmar, R.L., Neill, F.H., Romalde, J.L., Le Guyader, F., Woodley, C.M., Metcalf, T.G., Estes, M.K., 1995. Detection of Norwalk virus and hepatitis A virus in shellfish tissues with the PCR. Applied and Environmental Microbiology 61(8), 3014-3018.

Atmar, R.L., Opekun, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H., Graham, D.Y., 2008. Norwalk virus shedding after experimental human infection. Emerging Infectious Diseases 14(10), 1553-1557.

Atmar, R.L., Opekun, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H., Ramani, S., Hill, H., Ferreira, J., Graham, D.Y., 2014. Determination of the 50% human infectious dose for Norwalk virus. J Infect Dis 209(7), 1016-1022.

Bakunina, I.Y., Nedashkovskaya, O., Kim, S., Zvyagintseva, T., Mikhailov, V., 2012. Diversity of glycosidase activities in the bacteria of the phylum Bacteroidetes isolated from marine algae. Microbiology 81(6), 688-695.Bansal V, Sharma PK, Sharma N et al. (2011). Applications of chitosan and chitosan derivatives in drug delivery. Adv Biol Res 5:28–37

Barille, L., Prou, J., Heral, M., Razet, D., 1997. Effects of high natural seston concentrations on the feeding, selection, and absorption of the oyster *Crassostrea gigas* (Thunberg). Journal of Experimental Biology and Ecology 212, 149-172.

Bedford, A.J., Williams, G., Bellamy, A.R., 1978. Virus accumulation by the rock oyster *Crassostrea glomerata*. Appl Environ Microbiol 35(6), 1012-1018.

Behera, T, P.K. Nanda, C. Mohanty, D. Mohapatra, P. Swain, B.K. Das, P. Routray, B.K. Mishra, S.K. Sahoo. (2010). Parenteral immunization of fish, Labeo rohita with Poly D, L-lactide-co-glycolic acid (PLGA) encapsulated antigen microparticles promotes innate and adaptive immune responses. Fish and shellfish inmunology 28: 320-325.

Bell, A., 2005. The retention of picoplankton by the Pacific oyster, *Crassostrea gigas*, and implications for oyster culture, School of Biological Sciences. University of Auckland, Auckland, p. 200.

Bellou, M., Kokkinos, P., Vantarakis, A., 2013. Shellfish-borne viral outbreaks: a systematic review. Food Environ Virol 5(1), 13-23.

Berg, D.E., Kohn, M.A., Farley, T.A., McFarland, L.M., 2000. Multi-state outbreaks of acute gastroenteritis traced to fecal-contaminated oysters harvested in Louisiana. The Journal of Infectious Diseases 181(supplement 2), S381-S386.

Bidhan C, De Meena DK, Behera BK, Das P, Das Mohapatra PK, Das Sharma AP. Probiotics in fish and shellfish culture: immunomodulatory and ecophysio- logical responses. Fish Physiol Biochem 2014;40:921e71.

Boom, R., Sol, D.J.A., Salimans, M.M.M., Jansen, D.L., Wertheim van Dillen, P.M.E., van der Noordaa, J., 1990. Rapid and simple method for purification of nucleic acids. Journal of Clinical Microbiology 28, 495-503.

Borgogna M, Bellich B, Cesáro A. (2011). Marine polysaccharides in microencapsulation and application to aquaculture: ''From Sea to Sea''. Mar Drugs 9:2572–2604.

Bougrier, S., Geairon, P., Deslous-Paoli, J., Bacher, C., Jonquières, G., 1995. Allometric relationships and effects of temperature on clearance and oxygen consumption rates of *Crassostrea gigas* (Thunberg). Aquaculture 134(1), 143-154.

Canzonier, W.J., 1971. Accumulation and elimination of coliphage S-13 by the hard clam *Mercenaria mercenaria*. Applied Microbiology 21(6), 1024-1031.Choi, C., Kingsley, D.H., 2016. Temperature-Dependent Persistence of Human Norovirus Within Oysters (Crassostrea virginica). Food and Environmental Virology, 1-7.

Conaty, S., Bird, P., Bell, G., Kraa, E., Grohmann, G., McAnulty, J.M., 2000. Hepatitis A in New South Wales, Australia from consumption of oysters: the first reported outbreak. Epidemiol Infect 124(1), 121-130.

Dalev PG. (1994). Utilization of waste feathers from poultry slaughter for production of a protein concentrate. Bioresour. Technol. 48: 265-267.

Darmody G, Maloy AP, Lynch SA, Prado-Alvarez M, Cotterill J, Wontner-Smith T, et al. Tissue targeting of the European flat oyster, Ostrea edulis, using microencapsulated microbeads as a biological proxy. Aquac Int. http://dx.doi.org/10.1007/s10499-014-9842-y.

Davies, G.J., Gloster, T.M., Henrissat, B., 2005. Recent structural insights into the expanding world of carbohydrate-active enzymes. Current opinion in structural biology 15(6), 637-645.Di Girolamo, R., Liston, J., Matches, J., 1977. Ionic bonding, the mechanism of viral uptake by shellfish mucus. Appl Environ Microbiol 33(1), 19-25.

Dodgson, R., 1936. Shellfish and the public health. British medical journal 2(3942), 169.

Dore, B., Keaveney, S., Flannery, J., Rajko-Nenow, P., 2010. Management of health risks associated with oysters harvested from a norovirus contaminated area, Ireland, February-March 2010. Eurosurveillance 15(19), 1-4.

Dore, W.J., Henshilwood, K., Lees, D.N., 1998. The development of management strategies for control of virological quality in oysters. Water Science and Technology 38(12), 29-35.

Doyle, A., Barataud, D., Gallay, A., Thiolet, J., Le Guyader, F., Kohli, E., Vaillant, V., 2004. Norovirus foodborne outbreaks associated with the consumption of oysters from the Etang de Thau, France, December 2002. Euro Surveillance 8(3), 1-7.

Drouaz, N., Schaeffer, J., Farkas, T., Le Pendu, J., Le Guyader, F.S., 2015. Tulane Virus as a Potential Surrogate To Mimic Norovirus Behavior in Oysters. Applied and environmental microbiology 81(15), 5249-5256.

EFSA, 2011. Scientific Opinion on An update on the present knowledge on the occurrence and control of foodborne viruses. EFSA Journal 9(7), 2190.

Espinosa, E.P., Hassan, D., Ward, J.E., Shumway, S.E., Allam, B., 2010a. Role of epicellular molecules in the selection of particles by the blue mussel, *Mytilus edulis*. The Biological Bulletin 219(1), 50-60.

Espinosa, E.P., Perrigault, M., Ward, J.E., Shumway, S.E., Allam, B., 2009. Lectins associated with the feeding organs of the oyster *Crassostrea virginica* can mediate particle selection. The Biological Bulletin 217(2), 130-141.

Espinosa, E.P., Perrigault, M., Ward, J.E., Shumway, S.E., Allam, B., 2010b. Microalgal cell surface carbohydrates as recognition sites for particle sorting in suspension-feeding bivalves. The Biological Bulletin 218(1), 75-86.

Etherington DJ, Bardsley RG. (1991). Enzymes in the meat industry. In: Enzymes in Food Processing. Tucker GA, Woods LFJ (Eds.). Blackie Academic & Professional, London, pp. 144-191.

Fajardo P., Atanassova M., Garrido-Maestu A., Wortner-Smith T., Cotterill J., Cabado A. G. (2014). Bacteria isolated from shellfish digestive gland with antipathogenic activity as candidates to increase the efficiency of shellfish depuration process. Food Control 46: 272-281.

Feng, C., Ghosh, A., Amin, M.N., Giomarelli, B., Shridhar, S., Banerjee, A., Fernández-Robledo, J.A., Bianchet, M.A., Wang, L.-X., Wilson, I.B., 2013. The galectin CvGal1 from the eastern oyster (Crassostrea virginica) binds to blood group A oligosaccharides on the hemocyte surface. Journal of Biological Chemistry 288(34), 24394-24409.

Fernandez M, Ordonez JA, Bruna JM, Herranz B, de la Hoz L. (2000). Accelerated ripening of dry fermented sausages. Trends Food Sci. Technol. 11: 201-209.

Fisher, W., 1986. Structure and functions of oyster hemocytes, In: Brehelin, M. (Ed.), Immunity in Invertebrates. Springer, Berline, pp. 25-35.

Fitzgerald, T.L., Zammit, A., Merritt, T.D., McLeod, C., Landinez, L., White, P.A., Munnoch, S.A., Durrheim, D.N., 2014. An outbreak of norovirus genogroup II associated with New South Wales oysters. Communicable disease intelligence 38(1), 9-15.

Fries, C.R., Tripp, M.R., 1970. Uptake of viral particles by oyster leukocytes in vitro. J Invertebr Pathol 15(1), 136-137.

Gallimore, C.I., Cheesbrough, J.S., Lamden, K., Bingham, C., Gray, J.J., 2005. Multiple norovirus genotypes characterised from an oyster-associated outbreak of gastroenteritis. Int J Food Microbiol 103(3), 323-330.

Gentry, J., Vinje, J., Guadagnoli, D., Lipp, E.K., 2009. Norovirus distribution within an estuarine environment. Applied and Environmental Microbiology 75(17), 5474-5480.

Gill, O., Cubitt, W., McSwiggan, D., Watney, B., Bartlett, C., 1983. Epidemic of gastroenteritis caused by oysters contaminated with small round structured viruses. British Medical Journal 287, 1532-1534.

Glass, R.I., Parashar, U.D., Estes, M.K., 2009. Norovirus gastroenteritis. N Engl J Med 361(18), 1776-1785.

Gosling, E., 2003. Bivalve Molluscs. Blackwell Publishing, Oxford, United Kingdom.

Greening, G., Hewitt, J., Hay, B., Grant, C., 2003. Persistence of Norwalk-like viruses over time in Pacific oysters grown in the natural environment, In: Villalba, A., Reguera, B., Romalde, J., Beiras, R. (Eds.), International Conference on Molluscan Shellfish Safety. Consellería de Pesca e Asuntos Marítimos da Xunta de Galicia & Intergovernmental Oceanographic Commission of UNESCO, Santiago de Compostela, Spain, pp. 367-377.

Greening, G.E., Hewitt, J., 2008. Norovirus detection in shellfish using a rapid, sensitive virus recovery and real-time RT-PCR detection protocol. Food Analytical Methods 1(2), 109-118.

Grodzki, M., Ollivier, J., Le Saux, J.C., Piquet, J.C., Noyer, M., Le Guyader, F.S., 2012. Impact of Xynthia tempest on viral contamination of shellfish. Applied and Environmental Microbiology 78(9), 3508-3511.

Grohmann, G.S., Murphy, A.M., Christopher, P.J., Auty, E., Greenberg, H.B., 1981. Norwalk virus gastroenteritis in volunteers consuming depurated oysters. Australian Journal of Experimental Biology and Medical Science 59(2), 219-228.

Guillois-Becel, Y., Couturier, E., Le Saux, J.C., Roque-Afonso, A., Le Guyader, F., Le Goas, A., Pernes, J., Bechec, S., Briand, A., Robert, C., Dussaix, E., Pommepuy, M., Vaillant, V., 2009. An oyster-associated hepatitis A outbreak in France in 2007. Euro Surveillance 14(10), 1 - 6.

Haure, J., Penisson, C., Bougrier, S., Baud, J., 1998. Influence of temperature on clearance and oxygen consumption rates of the flat oyster *Ostrea edulis*: determination of allometric coefficients. Aquaculture 169(3), 211-224.

Hedstrom L. (2002). Serine protease mechanism and specificity. Chem. Rev. 102: 4501-4524.

Heller, D., Gill, O.N., Raynham, E., Kirkland, T., Zadick, P., Stanwell-Smith, R., 1986. An outbreak of gastrointestinal illness associated with consumption of raw depurated oysters. British medical journal 292, 1726 - 1727.

Henshilwood, K., Green, J., Gallimore, C., Brown, D., Lees, D., 1998. The development of polymerase chain reaction assays for detection of small round structured and other human enteric viruses in molluscan shellfish. Journal of Shellfish Research 17(5), 1675-1678.

Herdman, W. A., & Scott, A. (1896). Report on the investigations carried out in 1895 in connection with the Lancashire Sea-Fisheries Laboratory at the University College, Liverpool. *Proceedings Trans. Liverpool Biological Society, 10,* 103-174.

Hernroth, B., Allard, A., 2007. The persistence of infectious adenovirus (type 35) in mussels (*Mytilus edulis*) and oysters (*Ostrea edulis*). Int J Food Microbiol 113(3), 296-302.

Hirenkumar K. Makadia1 and Steven J. Siegel (2011). Poly Lactic-*co*-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier. Polymers (Basel). 3: 1377-1397. doi:10.3390/polym3031377.

Ho, B.S., Tam, T.-Y., 2000. Natural depuration of shellfish for human consumption: a note of caution. Water Research 34(4), 1401-1406.

Huppatz, C., Munnoch, S.A., Worgan, T., Merritt, T.D., Dalton, C., Kelly, P.M., Durrheim, D.N., 2008. A norovirus outbreak associated with consumption of NSW oysters: implications for quality assurance systems. Communicable diseases intelligence 32(1), 88-91.

Jothikumar, N., Lowther, J.A., Henshilwood, K., Lees, D.N., Hill, V.R., Vinjé, J., 2005. Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. Applied and Environmental Microbiology 71(4), 1870-1875.

Kingsley, D.H., Richards, G.P., 2003. Persistence of hepatitis A virus in oysters. Journal of Food Protection 66(2), 331-334.

Kohn, M.A., Farley, T.A., Ando, T., Curtis, M., Wilson, S.A., Jin, Q., Monroe, S.S., Baron, R.C., McFarland, L.M., Glass, R.I., 1995. An outbreak of Norwalk virus gastroenteritis associated with eating raw oysters: Implications for maintaining safe oyster beds. JAMA 273(6), 466-471.

Koopmans, M., 2008. Progress in understanding norovirus epidemiology. Current opinion in infectious diseases 21(5), 544-552.

Lange-Starke, A, A. Petereit, U. Truyen, P. G. Braun, K. Fehlhaber, T. Albert (2014). Antiviral Potential of Selected Starter Cultures, Bacteriocins and D,L-Lactic Acid. Food Environ Virol (2014) 6:42–47.

Le Guyader, F., Loisy, F., Atmar, R.L., Hutson, A.M., Estes, M.K., Ruvoen, N., Pommepuy, M., Le Pendu, J., 2006a. Norwalk virus-specific binding to oyster digestive tissues. Emerging Infectious Diseases 12(6), 931-936.

Le Guyader, F.S., Atmar, R.L., Le Pendu, J., 2012. Transmission of viruses through shellfish: When specific ligands come into play. Current Opinion in Virology 2(1), 103-110.

Le Guyader, F.S., Bon, F., DeMedici, D., Parnaudeau, S., Bertone, A., Crudeli, S., Doyle, A., Zidane, M., Suffredini, E., Kohli, E., Maddalo, F., Monini, M., Gallay, A., Pommepuy, M., Pothier, P., Ruggeri, F.M., 2006b. Detection of multiple noroviruses associated with an international gastroenteritis outbreak linked to oyster consumption. J Clin Microbiol 44(11), 3878-3882.

Le Guyader, F.S., Krol, J., Ambert-Balay, K., Ruvoen-Clouet, N., Desaubliaux, B., Parnaudeau, S., Le Saux, J.C., Ponge, A., Pothier, P., Atmar, R.L., Le Pendu, J., 2010. Comprehensive analysis of a norovirus-associated gastroenteritis outbreak, from the environment to the consumer. J Clin Microbiol 48(3), 915-920.

Le Guyader, F.S., Le Saux, J.C., Ambert-Balay, K., Krol, J., Serais, O., Parnaudeau, S., Giraudon, H., Delmas, G., Pommepuy, M., Pothier, P., Atmar, R.L., 2008. Aichi virus, norovirus, astrovirus, enterovirus, and rotavirus involved in clinical cases from a French oyster-related gastroenteritis outbreak. J Clin Microbiol 46(12), 4011-4017.

Le Mennec, C., Parnaudeau, S., Rumebe, M., Le Saux, J.-C., Piquet, J.-C., Le Guyader, S.F., 2016. Follow-Up of Norovirus Contamination in an Oyster Production Area Linked to Repeated Outbreaks. Food and Environmental Virology, 1-8.

Le Pendu, J., Nystrom, K., Ruvoen-Clouet, N., 2014. Host-pathogen co-evolution and glycan interactions. Curr Opin Virol 7, 88-94.

Lee KY, Heo TR (2000) Survival of Bifidobacterium longum immobilized in calcium alginate beads in simulated gastric juices and bile salt solution. Appl Environ Microbiol 66:869–873

Lee, R., Lovatelli, A., Ababouch, L., 2008. Bivalve depuration: fundamental and practical aspects. Food and Agriculture Organization of the United Nations. P. 139.

Lee, R., 2010. Cefas protocol for inspection and approval of purification (depuration) systems - England and Wales., 8 ed. CEFAS, p. 41.

Lees, D., Younger, A., Dore, B., 2010a. Depuration and relaying. Safe management of shellfish and harvest waters, 145-181.

Lees, D., 2010b. International standardisation of a method for detection of human pathogenic viruses in molluscan shellfish. Food and Environmental Virology 2(3), 146-155.

Lewis, G.D., Metcalf, T.G., 1988. Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus, from oyster, water, and sediment samples. Appl. Environ. Microbiol. 54(8), 1983-1988.

Loisy, F., Atmar, R.L., Le Saux, J.C., Cohen, J., Caprais, M.P., Pommepuy, M., Le Guyader, F.S., 2005. Use of rotavirus virus-like particles as surrogates to evaluate virus persistence in shellfish. Appl Environ Microbiol 71(10), 6049-6053.

Love, D.C., Lovelace, G.L., Sobsey, M.D., 2010. Removal of Escherichia coli, Enterococcus fecalis, coliphage MS2, poliovirus, and hepatitis A virus from oysters (Crassostrea virginica) and hard shell clams (Mercinaria mercinaria) by depuration. International journal of food microbiology 143(3), 211-217.

Lowther, J.A., Avant, J.M., Gizynski, K., Rangdale, R.E., Lees, D.N., 2010. Comparison between quantitative real-time reverse transcription PCR results for norovirus in oysters and self-reported gastroenteric illness in restaurant customers. Journal of Food Protection 73(2), 305-311.

Lowther, J.A., Gustar, N.E., Powell, A.L., Hartnell, R.E., Lees, D.N., 2012. Two-year systematic study to assess norovirus contamination in oysters from commercial harvesting areas in the United Kingdom. Appl Environ Microbiol 78(16), 5812-5817.

Luzardo-Alvarez A, Otero-Espinar FJ, Blanco-Mendez J. Microencapsulation of diets and vaccines for cultured fishes, crustaceans and bivalve mollusks. J Drug Del Sci Tech 2010;20:277e88.

Maalouf, H., Schaeffer, J., Parnaudeau, S., Le Pendu, J., Atmar, R.L., Crawford, S.E., Le Guyader, F.S., 2011. Strain-dependent norovirus bioaccumulation in oysters. Applied and Environmental Microbiology 77(10), 3189-3196.

Maalouf, H., Pommepuy, M., Le Guyader, F.S., 2010a. Environmental conditions leading to shellfish contamination and related outbreaks. Food and Environmental Virology 2(3), 136-145.

Maalouf, H., Zakhour, M., Pendu, J.L., Le Saux, J.C., Atmar, R.L., Le Guyader, F.S., 2010b. Distribution in tissue and seasonal variation of norovirus genogroup I and II ligands in oysters. Applied and Environmental Microbiology 76(16), 5621-5630.

Maalouf, H., Schaeffer, J., Parnaudeau, S., Le Pendu, J., Atmar, R.L., Crawford, S.E., Le Guyader, F.S., 2011. Strain-dependent norovirus bioaccumulation in oysters. Applied and Environmental Microbiology 77(10), 3189-3196.

Martínez-Cruz P, Ibáñez AL, Monroy Hermosillo OA et al (2012) Use of probiotics in aquaculture. ISRN Microbiology 2012(2012):1–13. Article ID 916845, doi:10.5402/2012/916845

Mathewson PR (Ed.), Eagan Press Handbook Series, American Chemical Society, St. Paul.

Mathewson PR. (1998). Enzymes-practical guides for the food industry. In: Enzymes.

McDonnell, S., Kirkland, K.B., Hlady, G., Aristeguieta, C., Hopkins, R., Monroe, S., Glass, R.I., 1997. Failure of cooking to prevent shellfish-associated viral gastroenteritis. Arch Intern Medicine 157, 111-116.

McIntyre, L., Galanis, E., Mattison, K., Mykytczuk, O., Buenaventura, E., Wong, J., Prystajecky, N., Ritson, M., Stone, J., Moreau, D., Youssef, A., Outbreak Investigation, T., 2012. Multiple clusters of norovirus among shellfish consumers linked to symptomatic oyster harvesters. J Food Prot 75(9), 1715-1720.

McLeod, C., 2014. A critical review of the current evidence for the use of indicator shellfish species for the purposes of biotoxin and chemical contaminants monitoring in the Scottish shellfish production areas. Seafood Safety Assessment Ltd., Scotland, pp. 1 - 124.

McLeod, C., Hay, B., Grant, C., Greening, G., Day, D., 2009a. Inactivation and elimination of human enteric viruses by Pacific oysters. Journal of Applied Microbiology 107(6), 1809-1818.

McLeod, C., Hay, B., Grant, C., Greening, G., Day, D., 2009b. Localization of norovirus and poliovirus in Pacific oysters. Journal of Applied Microbiology 106(4), 1220-1230.

Metcalf, T., Mullin, B., Eckerson, D., Moulton, E., Larkin, E., 1979. Bioacummulation and depuration of enteroviruses by the soft-shelled clam, *Mya arenaria*. Applied and Environmental Microbiology 38(2), 275-282.

Morrison, C.M., Dial, S.M., Day, W.A., Joens, L.A., 2012. Investigations of *Salmonella enterica* serovar newport infections of oysters by using immunohistochemistry and knockout mutagenesis. Applied and environmental microbiology 78(8), 2867-2873.

Murphy, A.M., Grohmann, G.S., 1980. Epidemiological studies on oyster-associated gastroenteritis. Food Technology in Australia 32(2), 86-88.

Nappier, S.P., Graczyk, T.K., Schwab, K.J., 2008. Bioaccumulation, retention, and depuration of enteric viruses by Crassostrea virginica and Crassostrea ariakensis oysters. Appl Environ Microbiol 74(22), 6825-6831.

Nappier, S.P., Graczyk, T.K., Tamang, L., Schwab, K.J., 2010. Co-localized Crassostrea virginica and Crassostrea ariakensis oysters differ in bioaccumulation, retention and depuration of microbial indicators and human enteropathogens. J Appl Microbiol 108(2), 736-744.

Neish, A., 2013. Investigative trials on the purification of oysters to identify ways of reducing norovirus. CEFAS, Weymouth, UK, p. 25.

Pascoe, P.L., Parry, H.E., Hawkins, A.J.S., 2009. Observations on the measurement and interpretation of clearance rate variations in suspension-feeding bivalve shellfish. Aquatic Biology 6, 181-190.

Pales-Espinosa E, Barille_ L, Allam B. Use of encapsulated live microalgae to investigate pre-ingestive selection in the oyster Crassostrea gigas. J Exp Mar Biol Ecol 2007;343:118e26.

Plant KP, LaPatra SE (2011) Advances in fish vaccine delivery. Dev Comp Immunol 35:1256–1262.

Polk AE, Amsden B, Scarratt DJ, Gonzal A, Okhamafe AO, Goosen MFA (1994) Oral delivery in aquaculture: controlled release of proteins from chitosan-alginate microcapsules. Aquac Eng 13:311–323.

Polo, D., Álvarez, C., Díez, J., Darriba, S., Longa, Á., Romalde, J.L., 2014a. Viral elimination during commercial depuration of shellfish. Food Control 43, 206-212.

Polo, D., Avarez, C., Vilarino, M.L., Longa, A., Romalde, J.L., 2014b. Depuration kinetics of hepatitis A virus in clams. Food Microbiol 39, 103-107.

Polo, D., Feal, X., Romalde, J.L., 2015. Mathematical model for viral depuration kinetics in shellfish: an useful tool to estimate the risk for the consumers. Food Microbiol 49, 220-225.

Polo, D., Schaeffer, J., Fournet N., Le Saux J-C., Parnaudeau S., McLeod, C., Le Guyader, F.S. 2016. The use of diigital PCR to quantify norovirus in oyster samples implicated in French outbreaks. Emerging and Infectious Disease. Accepted, in press.

Prado-Alvarez M, Lynch S.A., Kane A., Darmody G., Pardo B.G., Martínez P., Cotterill J., Wontner-Smith T., Culloty S.C. (2015). Oral immunostimulation of the oyster Ostrea edulis: Impacts on the parasite Bonamia ostreae. Food Control 1-9.

Provost, K., Dancho, B.A., Ozbay, G., Anderson, R.S., Richards, G.P., Kingsley, D.H., 2011. Hemocytes are sites of enteric virus persistence within oysters. Appl Environ Microbiol 77(23), 8360-8369.

Rajko-Nenow, P., Keaveney, S., Flannery, J., Mc, I.A., Dore, W., 2014. Norovirus genotypes implicated in two oyster-related illness outbreaks in Ireland. Epidemiol Infect 142(10), 2096-2104.

Richards, G., McLeod, C., Le Guyader, F., 2010. Processing Strategies to Inactivate Enteric Viruses in Shellfish. Food and Environmental Virology 2(3), 183-193.

Richards, G.P., 1985. Outbreaks of shellfish-associated enteric virus illness in the United States: requisite for development of viral guidelines. journal of Food Protection 48(9), 815-823.

Richards, G.P., 1988. Microbial purification of shellfish: A review of depuration and relaying. Journal of Food Protection 51(3), 218-251.

Richards, G.P., 1991. Shellfish depuration, In: Ward, D.R., Hackney, C. (Eds.), Microbiology of marine food products. Avi, New York, pp. 395-428.

Riisgard, H., Kittner, C., Seerup, D., 2003. Regulation of opening state and filtration rate in filter-feeding bivalves (*Cardium edule, Mytilus edulis*, *Mya arenaria*) in response to low algal concentration. Journal of Experimental Biology and Ecology 284, 105-127.

Romalde, J.L., Estes, M.K., Szucs, G., Atmar, R.L., Woodley, C.M., Metcalf, T.G., 1994. In Situ detection of hepatitis A virus in cell cultures and shellfish tissues. Applied and environmental microbiology 60(6), 1921-1926.

Rosas-Ledesma P, Leo´n-Rubio JM, Alarco´n FJ et al (2012) Calcium alginate capsules for oral administration of fish probiotic bacteria; assessment of optimal conditions for encapsulation. Aquac Res 43:106–116

Saeed, S., Rasool, S. A., Ahmad, S., Zaidi, S. Z., & Rehmani, S. (2007). Antiviral activity of staphylococcin 188: a purified bacteriocin like inhibitory substance isolated from Staphylococcus aureus AB188. Research Journal of Microbiology, 2(11), 796-806.

Sarry J-E, Gunata Z. (2004). Plant and microbial glycoside hydrolases: Volatile release from glycosidic aroma precursors. Food Chem. 87: 509-521.

Schmid RD, Verger R. (1998). Lipases: interfacial enzymes with attractive applications. Angew. Chem. Int. Ed. 37: 1608-1633.

Schwab, K.J., Neill, F.H., Estes, M.K., Metcalf, T.G., Atmar, R.L., 1998. Distribution of Norwalk virus within shellfish following bioaccumulation and subsequent depuration by detection using RT-PCR. Jounal of Food Protection 61(12), 1674-1680.

Seamer, C., 2007. The biology of virus uptake and elimination by Pacific Oysters (*Crassostrea gigas*), School of Biological Sciences. Victoria University of Wellington, Wellington, p. 239.

Serkedjieva, J., Danova, S., & Ivanova, I. (2000). Antiinfluenza virus activity of a bacteriocin produced by Lactobacillus delbrueckii. Applied Biochemistry and Biotechnology, Part A Enzyme Engineering and Biotechnology, 88(1-3), 285-298.

Sharma R, Chisti Y, Banerjee UC. (2001). Production, purification, characterization and applications of lipases. Biotechnol. Adv. 19: 627-662.

Shieh, Y., Monroe, S.S., Fankhauser, R.L., Langlois, G.W., Burkhardt, W., 3rd, Baric, R.S., 2000. Detection of norwalk-like virus in shellfish implicated in illness. J Infect Dis 181 Suppl 2, S360-366.

Shieh, Y.C., Calci, K., Baric, R.S., 1999a. A method to detect low levels of enteric viruses in contaminated oysters. Appl. Environ. Microbiol. 65, 4709-4714.

Shieh, Y.S., Calci, K.R., Baric, R.S., 1999b. A method to detect low levels of enteric viruses in contaminated oysters. Applied and Environmental Microbiology 65(11), 4709-4714.

Shirato H, Norovirus and Histo-Blood Group Antigens. 2011. Jpn. J. Infect. Dis., 64, 95-103.

Shumway, S., Cucci, T., Newell, R., CM, Y., 1985. Particle selection, ingestion and absorption in filter-feeding bivalves. Journal of Experimental Biology and Ecology 91, 77-92.

Siebenga, J.J., Lemey, P., Kosakovsky Pond, S.L., Rambaut, A., Vennema, H., Koopmans, M., 2010. Phylodynamic reconstruction reveals norovirus GII.4 epidemic expansions and their molecular determinants. PLoS Pathog 6(5), e1000884.

Simmons, G., Greening, G., Gao, W., Campbell, D., 2001. Raw oyster consumption and outbreaks of viral gastroenteritis in New Zealand: evidence for risk to the public's health. Aust N Z J Public Health 25(3), 234-240.

Simpson BK, Rui X, Klomklao S. (2012). Enzymes in food processing. In: Food biochemistry and food processing, 2nd Ed. Simpson B et al. (Eds). John Wiley and Sons, Inc.

Skjermo J, Vadstein O (1999) Techniques for microbial control in the intensive rearing of marine larvae. Aquaculture 177:333–343

Smith, A.J., McCarthy, N., Saldana, L., Ihekweazu, C., McPhedran, K., Adak, G.K., Iturriza-Gomara, M., Bickler, G., O'Moore, E., 2012. A large foodborne outbreak of norovirus in diners at a restaurant in England between January and February 2009. Epidemiol Infect 140(9), 1695-1701.

Sobsey, M.D., Oglesbee, S.E., Wait, D.A., 1985. Evaluation of methods for concentrating hepatitis A virus from drinking water. Applied and Environmental Microbiology 50(6), 1457-1463.

Sova, V., Pesentseva, M., Zakharenko, A., Kovalchuk, S., Zvyagintseva, T., 2013. Glycosidases of marine organisms. Biochemistry (Moscow) 78(7), 746-759.Stafford, R., Strain, D., Heymer, M., Smith, C., Trent, M., Beard, J., 1997. An outbreak of Norwalk virus gastroenteritis following consumption of oysters. Communicable disease intelligence 21(21), 317 - 320.

Strohmeier, T., Strand, Ø., Cranford, P., 2009. Clearance rates of the great scallop (*Pecten maximus*) and blue mussel (*Mytilus edulis*) at low natural seston concentrations. Marine biology 156(9), 1781-1795.

Teunis, P.F.M., Moe, C.L., Liu, P., Miller, S.E., Lindesmith, L., Baric, R.S., Le Pendu, J., Calderon, R.L., 2008. Norwalk virus: How infectious is it? Journal of Medical Virology 80(8), 1468-1476.

Thebault, A., Teunis, P.F., Le Pendu, J., Le Guyader, F.S., Denis, J.-B., 2013. Infectivity of GI and GII noroviruses established from oyster related outbreaks. Epidemics 5(2), 98-110.

Tian, P., Bates, A.H., Jensen, H.M., Mandrell, R.E., 2006. Norovirus binds to blood group A-like antigens in oyster gastrointestinal cells. Lett Appl Microbiol 43(6), 645-651.

Todorov, S. D., Wachsman, M., Tome, E., Dousset, X., Destro, M. T., Dicks, L. M. T., et al. (2010). Characterisation of an antiviral pediocin-like bacteriocin produced by Enterococcus faecium. Food Microbiology, 27(7), 869-879.

Traore, O., Arnal, C., Mignotte, B., Maul, A., Laveran, H., Billaudel, S., Schwartzbrod, L., 1998. Reverse transcriptase PCR detection of astrovirus, hepatitis A virus, and poliovirus in experimentally contaminated mussels: comparison of several extraction and concentration methods. Appl. Environ. Microbiol. 64, 3118-3122.

Ueki, Y., Shoji, M., Suto, A., Tanabe, T., Okimura, Y., Kikuchi, Y., Saito, N., Sano, D., Omura, T., 2007. Persistence of caliciviruses in artificially contaminated oysters during depuration. Appl Environ Microbiol 73(17), 5698-5701.

Varki A. (1992). Diversity in the sialic acids. Glycobiology, 2: 25-40.

Wang, D., Wu, Q., Kou, X., Yao, L., Zhang, J., 2008. Distribution of norovirus in oyster tissues. J Appl Microbiol 105(6), 1966-1972.

Wang CS, Hartsuck JA. (1993). Bile salt-activated lipase. A multiple function lipolytic enzyme. Biochim. Biophys. Acta 1166: 1-19.

Ward, J., Levinton, J., Shumway, S., Cucci, T., 1997. Site of particle selection in a bivalve mollusc. Nature 390, 131-132.

Ward, J., Levinton, J., Shumway, S., Cucci, T., 1998. Particle sorting in bivalves: in vivo determination of the pallial organs of selection. Marine Biology 131, 283-292.

Wilkinson MG, Kilcawley KN. (2005). Mechanism of enzyme in corporation and release of enzymes in cheese during ripening. Intl. Dairy J. 15: 817-830.

Yu, Y., Cai, H., Hu, L., Lei, R., Pan, Y., Yan, S., Wang, Y., 2015. Molecular Epidemiology of Oyster-Related Human Noroviruses and Their Global Genetic Diversity and Temporal-Geographical Distribution from 1983 to 2014. Appl Environ Microbiol 81(21), 7615-7624.

Zakhour, M., Maalouf, H., Di Bartolo, I., Haugarreau, L., Le Guyader, F.S., Ruvoen-Clouet, N., Le Saux, J.-C., Ruggeri, F.M., Pommepuy, M., Le Pendu, J., 2010. Bovine Norovirus: Carbohydrate Ligand, Environmental Contamination, and Potential Cross-Species Transmission via Oysters. Appl. Environ. Microbiol. 76(19), 6404-6411.

Zheng, D.P., Ando, T., Fankhauser, R.L., Beard, R.S., Glass, R.I., Monroe, S.S., 2006. Norovirus classification and proposed strain nomenclature. Virology 346(2), 312-323.

Appendix One: Evaluation of key publications considered in the literature review

An evaluation of the most relevant papers considered in the literature review was undertaken (Tables A1 and A2). The scientific findings of each paper are discussed in the literature review section of this report (Part 1). The methodology (Section 2) provides details on the selection process for publications included in the evaluation and the approach used to evaluate the papers. Briefly, the selected papers were critiqued against a series of pre-determined questions. The questions used to evaluate the papers were:

Question 1: Were appropriate analytical test methodologies used for NoV (i.e. the ISO standard method or equivalent)?
Question 2: Were depuration parameters noted in the study (e.g. temperature, salinity, time, disinfection approach, flow rate, loading density)?
Question 3: Did the study design, data and statistical treatment support the conclusions?

For NoV uptake and depuration studies:
Question 4: Were oysters maintained in appropriate conditions to ensure they were alive and functioning optimally?
Question 5: Was the number of sampling occasions and/or oyster samples analysed sufficient to support conclusions regarding relative elimination efficiency for NoV?

For reports on illness outbreaks related to NoV in oysters:
Question 4: Does the epidemiology evidence presented strongly implicate oysters as the vector (i.e. were the epidemiology investigations analytical and have statistics presented)?
Question 5: Were oysters tested for the presence of NoV?

For each of the papers considered, the questions above were assessed and a score of 0 (no), 1 (acceptable/generally) or 2 (yes) was allocated for each question. A total score was calculated for each paper, thus high scoring papers are suggestive of robust results and conclusions (a maximum score of 10 is possible). Tables A1 and A2 provide the results for the evaluation.

Table A1 Summary of evaluation undertaken for key norovirus depuration publications considered in the literature review

	Q1: Appropriate methods used?	**Q2:** Depuration parameters noted?	**Q3:** Study design appropriate to support conclusions?	**Q4:** Oysters maintained appropriately?	**Q5:** Sampling numbers sufficient?	TOTAL SCORE
McLeod et al. (2009a)	2 (real time PCR)	2	2	2	1 (sampling could have been extended beyond 24 hours)	**9**
Schwab et al. (1998)	1 (PCR, semi-quan)	2	2	2	2	**9**
Drouaz et al. (2015)	2 (real time PCR)	1 (type of system not noted)	2	2	2	**9**
Ueki et al. (2007)	2 (real time PCR)	2	2	2	2	**10**
Neish et al. (2013)	2 (real time PCR)	2	1 (A statistically significant reduction in NoV noted, but the small decrease may have been biologically insignificant)	2	2	**9**
Choi and Kingsley (2016)	2 (real time PCR)	2	2	2	2	**10**
Drouaz et al. (2015)	2 (real time PCR)	2	2	2	2	**10**

Table A2 Summary of evaluation undertaken for key NoV outbreak publications considered in the literature review

	Q1: Appropriate methods used?	**Q2:** Depuration parameters noted?	**Q3:** Study design appropriate to support conclusions?	**Q4**: Is epidemiological evidence strong?	**Q5:** Were oysters tested for NoV?	**TOTAL SCORE**
Rajko-Nenow et al. (2014)	2	0	2	1 (not specified)	2	**7**
Fitzgerald et al. (2014)	2	0	2	1 (descriptive)	2	**7**
Dore et al. (2010)	2	0	2	1 (not specified)	2	**7**
Smith et al. (2012)	2	0	2	2 (analytical)	2	**8**
Le Guyader et al. (2010)	2	0	2	2 (analytical)	2	**8**
Lowther et al. (2010)	2	0	2	1 (not specified)	2	**7**
Huppatz et al. (2008)	1 (not specified)	1 (time and disinfection noted)	2	2 (analytical)	1 (different batch tested)	**7**
Le Guyader et al. (2008)	2	0	2	2	2	**8**
Gallimore et al. (2005)	NA	1 (time, temp noted)	2	1 (descriptive)	0	**4**
Le Guyader et al. (2006b)	2	0	2	2	2	**8**
Ang (1998)	NA	0	2	2	0	**4**
Stafford et al. (1997)	1 (not specified)	0	2	2	1 (tested, not detected)	**6**
Heller et al. (1986)	NA	1 (time, UV noted)	2	2	0	**5**
Gill et al. (1983)	NA	2	2	2	0	**6**
Grohmann et al. (1981)	NA	1 (time noted)	2	2	0	**5**

Appendix Two: International and UK depuration surveys

Part 1: UK Depuration Systems Questionnaire

Seafood Safety Assessment Ltd
'Hillcrest', Kilmore, Isle of Skye, IV44 8RG
E-mail: seafoodsafetyassessment@gmail.com
Tel: 01471 844 725

Seafood Safety Assessment Ltd (SSA Ltd) has been commissioned by the UK Food Standards Agency (FSA) to review the effectiveness of depuration in removing norovirus from oysters and to investigate alternative approaches to virus removal.

One element of this study is to identify current depuration practice in the UK, including whether the system is operator designed/constructed or purchased 'off the shelf' from one of the established manufacturers and actual operational parameters of temperature, UV power, oxygen levels, use of ozone, length of depuration cycle, etc. The Questionnaire (attached) also aims to gather industry observations about 'real world' problems and issues concerning the practical operation of these systems.

The output from this research into current depuration systems aims to be a comprehensive summary of the typical values and range of current operational criteria, plus a synopsis of industry concerns associated with the process.

Completion of this Questionnaire by you, a licensed depuration operator, will contribute significantly to the accuracy and all-inclusive nature of the output. In addition, the responses will enable researchers to 'replicate' commercial reality when testing potential alternative approaches to virus depuration.

SSA Ltd is grateful for your participation in this research, which will assist in efforts to improve the public health status of oysters harvested in the UK. The survey of industry practice is supported and endorsed by the main representative trade organisations, the Shellfish Association of Great Britain (SAGB) and the Association of Scottish Shellfish Growers (ASSG).

Completed Questionnaires should be returned to the address above by the end of March 2015 at the latest. Please note that individual company responses will remain confidential and will not be published – only a summary of UK-wide results will be incorporated into the project.

Questionnaire on Oyster Depuration Practices in the UK

Name:	
Position/role:	
Company Name:	
Address:	
E-mail:	
Phone:	
Mobile:	

Please place a cross in the boxes and/or complete answers in the space provided.

1. In which year was your depuration system commissioned?_____

2. Is your depuration system self-designed/constructed?

Yes ☐ *If Yes, Go to Q.4*
No ☐

3. Is your depuration system a standard Seafish 'off the shelf' design, from a third party manufacturer?

Yes ☐ *If Yes, please identify the manufacturer:* _____
No ☐

4. Which species of oysters are depurated at your location?

Crassostrea gigas ☐
Ostrea edulis ☐
Other ☐ *Please specify:* _____

5. How are the shellfish loaded into the depuration system?

Loose on trays ☐
Bags/sacks ☐
Small mesh boxes ☐
Other ☐ *Please specify:* _____

6. What is the stocking density/container? _____ Kg per Tray/Bag/Box

7. What is the maximum amount of oysters (in Kilograms) that can be purified in the system in a single cycle? _____Kg

8. What volume of water is contained in each tank (_____m3) and the total system (_____m3) when fully loaded with oysters?

9. Typically, how long (hours) is each depuration cycle?

- In summer:_____Hrs.
- In winter: _____ Hrs.

10. What is the flow rate through the system during the cycle period?

- In summer:_____Litres/sec
- In winter:_____Litres/sec

11. At what water temperature(s) do you operate the system?

- Max:_____
- Min:_____

12. Do these vary between summer and winter operation?

Yes ☐
No ☐

If Yes, what are the seasonal temperatures:

- Summer Max:_____ Min:_____
- Winter Max:_____ Min:_____

13. At what dissolved oxygen level(s) in the tank water do you aim to operate at (start/finish of cycle)?

- Start of cycle: _____mg/L
- Finish of cycle:_____ mg/L.

14. Is the water supply for the depuration process locally sourced seawater, tanker supplied or artificial seawater?

Local supply ☐
Tanker supplied ☐
Artificial ☐

15. What is the typical salinity of the seawater used? _____ ‰

16. Do you test the water supply (before UV treatment) for:

- Microbiological contamination?

Yes ☐
No ☐

- Turbidity?

Yes ☐
No ☐

If Yes, what threshold level(s) would cause the depuration process to be halted?

- Microbiological:_____
- Turbidity:_____

17. Do you use in-line filters on inlet water pipework?

Yes ☐
No ☐

If Yes, are the filters:

Cartridge ☐
Sand ☐

18. Do you re-use seawater after completion of a cycle?

Yes ☐
No ☐

If Yes, how many times is the water typically reused? _____

19. What power of UV lamps is typically used? _____

20. What dose does this result in:

- New UV lamp? _____mJ/cm2
- At 80% efficiency? _____mJ/cm^2

21. Do you use ozone treatment?

Regularly ☐ *If Yes, at what concentration?* _____
Occasionally ☐ *If Yes, at what concentration?* _____
Never ☐

22. What problems/difficulties/issues have you experienced with the practical operation of depuration facilities? _____

23. Do you believe that depuration is potentially an effective process to remove viruses from oysters and if so, what criteria (temperature, cycle time, oxygen levels, etc.) would be required to effect significant reduction? _____

24. Have you participated in any viral reduction studies/projects using your depuration system?

Yes ☐
No ☐

If Yes, can you supply a reference/publication for the research: _____

25. Would you be willing to discuss the issues surrounding viral reduction through depuration and/or alternative approaches with a representative of SSA Ltd?

Yes ☐
No ☐

If Yes, please indicate the best day and time for an initial telephone contact: _____

SSA Ltd appreciates the time and effort taken to complete the Questionnaire, which we hope will prove to be a significant contribution to improving the quality of oysters placed on the market.

****

Part 2: International Depuration Questionnaire

Seafood Safety Assessment Ltd
'Hillcrest', Kilmore, Isle of Skye, IV44 8RG
E-mail: seafoodsafetyassessment@gmail.com
Tel: 01471 844 725

Seafood Safety Assessment Ltd (SSA Ltd) has been commissioned by the UK Food Standards Agency (FSA) to review the effectiveness of depuration in removing norovirus from oysters and to investigate alternative approaches to virus removal.

One element of this study is to identify current depuration practices in the UK; in addition the project aims to include an international comparison of operational parameters. To this end SSA Ltd would be grateful if you could take the time to complete (as far as relevant) this questionnaire on depuration operations as practiced by the oyster industry (producers and processors) in your country. If you are interested in further background information on the project, please visit:
http://www.food.gov.uk/science/research/foodborneillness/p01programme/fs101068

Questionnaire on Depuration Systems

Name:……
Position/Role:……..
Organisation:……
Address:……..
Contact Details: E-mail ……..; Tel: ……

1. What proportion of oysters placed on the market in your country would you estimate are depurated (defined as: 'Purification carried out under controlled conditions')?
   All
   75%
   50%
   25%
   None

   Comments:

2. Are the depuration systems used by industry generally 'self-constructed' or "off the shelf" purchases from third party manufacturers using recognised technical standards? If the latter, which manufacturers, whose standards?
   Off the shelf
   Self constructed

   Comments:

3. What is the scale of depuration operations, i.e. what amount of oysters (Kilograms) can be purified in a typical system in a single cycle?

   - Average:……..Kg/cycle
   - Range:……..Kg/cycle

4. Which species of oyster are generally depurated, e.g. Crassostrea gigas, Ostrea edulis, etc.? ……
   ……

5. For each oyster species depurated, could you specify typical parameters as listed below:

   - How long is the depuration cycle for each species? …….
   ……
   - At what temperatures are the systems operated? ……..
   ……
   - At what salinity are the systems operated? ……..
   ……
   - Is ozone used (Frequently? Occasionally?) and at what concentration(s)?
   ……..
   ……

Species	Depuration period (hours)	Seawater temperature	Salinity	Ozone used	Ozone concentration
				Yes, no, sometimes	

6. Is there anything unique or different about the design or operation of depuration units in your country when compared with other international operators?

7. Have there been any problems/difficulties/issues experienced by operators with the practical operation of depuration facilities?
   ……
   ……
   ……

8. Are you aware of any norovirus outbreaks associated with depurated oysters from your country? Yes/No

   If 'Yes', please provide details of any associated publications/reports.

9. Are you aware of any research/experimental work/pilot projects targeting removal of Norovirus from oysters through depuration or other methods(published or unpublished)? Yes/No

   If Yes, please provide details if possible ……..
   ……

10. What are the references for any published studies/test results for work investigating removal of Norovirus? ……
    ……

SSA Ltd appreciates the time and effort taken to complete the questionnaire.

****

Appendix Three: Summary of compounds that may act as disruptors of NoV ligands in oyster tissues

Origin	Compound (synonymous)	Source	Compound family	Cleavage site	pH optimum	Observations
Animal proteases	Lipase	Porcine/Bovine pancreas, *Candida rugosa*	Esterase	Esters in aqueous solutions, hydrolysis of triacylglycerols	------	Ca2+ necessary for activity Also from plant, bacterial and fungi origin
	Trypsine	Porcine/Bovine pancreas	Serine Endoprotease	Peptides on the C-terminal side of Lys and Arg amino acid residues	7.0-9.0	Ca2+ retard the autolysis ability and maintain the stability in solution
	α-chymotrypsine	Bovine pancreas	Serine Endoprotease	Peptide bonds on the C-terminal side of Tyr, Trp, Phe, Leu	------	Ca2+ activates and stabilizes the enzyme
	Pronase XIV (Pronase E)	*Streptomyces grisseus*	Endo/exo-proteases	Non specific protease	7.0-8.0	Ca2+ is recommended for protection from autolysis. Much more effective in digestion of casein than trypsine, chymotrypsine and other proteases.
	O-sialo glycoprotease	*Pasteurella haemolytica*	Endo glycosidase	O-glycosylated proteins on Ser and thr residues, removing sialyl groups	7.4	
Plant proteases	Proteinase K	*Tritirachum album*	Serine Endoprotease	Peptide bonds adjacents to the carboxyl group of aliphatic and aromatic amino acids	7.5-12	Ca2+ necessary for activation (1-5mM Ca2+) Maximum activity at 37ºC; 80% loss of activity at 20-60ºC
	Papain	Papaya latex *Carica papaya*	Cysteine Endoprotease	Peptide bonds of basic amino acids, esters and amides, especially at bonds involving Arg, Lys, Glu, His, Gly and Tyr.	6.0-7.0	Much more effective than pancreatic proteases. Upon prolonged incubation further bonds are split. The addition of L-cystein (0.5% w/v; 5mM) is essential for enzyme activity.
	Ficin	Fig tree latex	Thiol protease	Carboxyl side of Gly, Ser, Thr, Met, Lys, Arg, Tyr, Ala, Asn, Val	------	
	Bromelain	Pineapple stem	Cysteine Endoprotease	Broad protein specificity	------	
Glycosidases	α-Amylase	Porcine/Human pancreas	Endo glycosidase	α(1,4)-D-glucosidic linkages in polysaccharides of ≥3 of α(1,4) linked D-glucose units	7.0	Ca^{2+} necessary for stability; Chlorine ions necessary for stability; Also from human saliva and Bacterial origin
	B-Galactosidase (Lactase)	*Bacteroides fragilis,* expressed in *E. coli*	Endo glycosidase	β-glycosidic bond formed between a galactose and its organic moiety	6.0-8.0	Mg^{2+} and Na^{2+} are activators. The optimal concentration of Mg^{2+} can range from 0,1 to 10 mM, depending upon the sodium concentration
	O-Glycosidase	*Streptococcus pneumoniae*	Endo glycosidase	O-Glycans. N-acetylgalactosamine glycosidic linkage	6.0-8.0	

Origin	Compound (synonymous)	Source	Compound family	Cleavage site	pH optimum	Observations
	Neuraminidase (Sialidase)	*Vibrio cholerae*	Exo glycosidase	Preferentially α(2→3), but also α(2→6) and α(2→8) linkages between neuraminic acid and galactose	5.0	Ca^{2+} necessary for activity
	α-L Fucosidase	Bovine kidney	Exo glycosidase	α(1→ (2,3,4,6)) linked fucose from N- and O linked glycans.	5.5-5.8	It cleaves α-1→6 linked fucose on the trimannosyl core of N-linked glycans more efficiently than other α-fucose linkages.
	$NaIO_4$		Sodium salt of periodic acid	Sialic acids and carbon-carbon bonds of a wide range of carbohydrates	------	more effective in dark conditions

Appendix Four: Reproducibility of the *in vitro* method

Table A4 NoV levels in digestive tract (DT) and supernatant (ST) components of samples treated with three concentrations of sodium periodate ($NaIO_4$) in 24-well cell culture plates and Eppendorf tubes. Results are expressed as NoV GII.3 RNA copies/200 mg of DT component, or 500 µL of ST along with the corresponding standard deviation (*SD*).

Assay format	Compound	mg/L[a]	DT component	*SD* of DT component	ST component	*SD* of ST component
24-well plate	*Control*	-	4.36×10^3	$\pm 1.61 \times 10^3$	4.42×10^2	$\pm 2.34 \times 10^2$
	$NaIO_4$ (1 mM)	200	1.32×10^3	$\pm 2.27 \times 10^2$	4.09×10^2	$\pm 2.82 \times 10^2$
	$NaIO_4$ (10 mM)	2000	1.47×10^2	$\pm 5.26 \times 10^2$	7.11×10^2	$\pm 1.54 \times 10^2$
	$NaIO_4$ (100 mM)	20000	6.55×10^1	$\pm 4.67 \times 10^1$	1.10×10^3	$\pm 1.36 \times 10^2$
Eppendorf	*Control*	-	2.49×10^3	± 6.75	4.66×10^2	$\pm 1.55 \times 10^2$
	$NaIO_4$ (1 mM)	200	2.15×10^3	$\pm 7.17 \times 10^2$	3.99×10^2	$\pm 1.22 \times 10^2$
	$NaIO_4$ (10 mM)	2000	2.02×10^3	$\pm 1.91 \times 10^2$	1.18×10^3	$\pm 1.01 \times 10^2$
	$NaIO_4$ (100 mM)	20000	9.51×10^1	$\pm 5.07 \times 10^1$	4.90×10^2	$\pm 1.32 \times 10^2$