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Depuration and Relaying: A Review on Potential Removal of Norovirus from Oysters

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Abstract: Pollution of coastal waters can result in contamination of bivalve shellfish with human enteric viruses, including norovirus (NoV), and oysters are commonly implicated in outbreaks. Depuration is a postharvest treatment involving placement of shellfish in tanks of clean seawater to reduce contaminant levels; this review focuses on the efficacy of depuration in reducing NoV in oysters. There have been many NoV outbreaks from depurated oysters containing around 10^3 genome copies/g oyster tissue, far exceeding the median infectious dose (ID50). Half of the published NoV reduction experiments showed no decrease in NoV during depuration, and in the remaining studies it took between 9 and 45.5 d for a 1-log reduction—significantly longer than commercial depuration time frames. Surrogate viruses are more rapidly depurated than NoV; the mean number of days to reduce NoV by 1 log is 19, and 7.5 d for surrogates. Thus, surrogates do not appear to be suitable for assessing virological safety of depurated oysters; data on reduction of NoV infectivity during depuration would assist evaluations on surrogate viruses and the impact of methods used. The longer persistence of NoV highlights its special relationship with oysters, which involves the binding of NoV to histo-blood group-like ligands in various tissues. Given the persistence of NoV and on-going outbreaks, depuration as currently performed appears ineffective in guaranteeing virologically safe oysters. Conversely, relaying oysters for 4 wk is more successful, with low NoV concentrations and no illnesses associated with products. The ineffectiveness of depuration emphasizes the need for coastal water quality to be improved to ensure oysters are safe to eat.

Keywords: depuration, norovirus, oysters, purification, relaying

Introduction

There are 2 major routes by which contamination of raw food with microbes occurs, through infected food handlers and during the primary production process. Pollution of coastal waters with human effluent can result in contamination of bivalve molluscan shellfish with a variety of microbial pathogens, including human enteric viruses. More than 100 types of viruses can be shed in human stools, and several biological factors influence the efficiency of their transmission from person to person (Geoghegan and others 2016). The high levels of norovirus (NoV) shed in feces and its prevalence in the population (predominant cause of human gastroenteritis worldwide), means that this virus is a particular risk for environmental transmission. Indeed, recent estimates from the WHO on the global burden of foodborne diseases suggest that the most frequent causes of foodborne illness were diarrheal disease agents, particularly NoV and *Campylobacter* bacteria, and 7.6% of the total DALYs (Disability Adjusted Life Years) were attributed to NoV gastroenteritis (WHO 2015). NoV is the most common viral pathogen associated with shellfish illnesses globally (Bellou and others 2013; Yu and others 2015).

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While all bivalves can become contaminated through the process of filter feeding, oysters are more frequently implicated in illness outbreaks than other shellfish species (Bellou and others 2013). There are several possible reasons why oysters play a more dominant role in the transmission of NoV compared to other shellfish, including: (1) the mode of consumption (primarily raw); (2) the close proximity of intertidal 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 and others 2006a, 2012); and (4) relatively slow elimination of NoV from oyster tissues (McLeod and others 2009a, b; Richards and others 2010; Le Mennec and others 2017).

Oysters are an economically important food that is consumed worldwide. The Pacific oyster (*Crassostrea gigas*) is the most commonly produced oyster globally (including the EU), however, other species are also cultivated, including *Crassostrea virginica* (the Eastern oyster) in the United States and *Saccostrea glomerata* in Australia. Flat oysters (also known as native or dredge oysters), such as *Ostrea edulis,* are also produced in many countries (such as Ireland, the United Kingdom, and Croatia). In accordance with EU law, bivalve shellfish production areas are classified as A, B, or C based on the presence and levels of the fecal indicator bacterium *Escherichia coli*. Shellfish harvested from areas that are classified as B must be subjected to depuration, cooking, or relaying. In the U.S., shellfish production areas are classified based on fecal coliform levels in seawater, as approved, conditionally approved/restricted,

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restricted, or prohibited. Shellfish from conditionally restricted and restricted areas must be subjected to a postharvest treatment such as depuration or relaying.

While depuration is a highly effective postharvest treatment for removing *E. coli*, the removal of NoV by depuration is not considered efficient; several studies suggest low NoV reduction rates (Schwab and others 1998; Ueki and others 2007; McLeod and others 2009a), and some cases of NoV illness have occurred from the consumption of depurated oysters (Grohmann and others 1981; Stafford and others 1997; Gallimore and others 2005; Le Guyader and others 2010). These outbreaks, and continuing reliance on depuration as an end product control globally, have led to calls for an evaluation of NoV depuration in oysters (EFSA 2012) . Therefore, the primary aim of this review is to evaluate the effectiveness of depuration in removing NoV from oysters and, secondarily, to discuss possible explanations for the failure of depuration and to identify new potential approaches. For the purposes of this review, microbial elimination in a natural setting is referred to as relaying, and elimination in tanks is denoted as depuration (also frequently described as "purification").

Search Approach for a Review of the Current Literature

To review the efficacy of depuration in removing NoV from oysters, a series of literature searches were undertaken to identify studies and data of high relevance. The review focused on identifying papers that describe NoV illness outbreaks caused by depurated oysters, and the persistence of NoV in oysters following depuration and relaying. Given the historical lack of a culture method for NoV, studies on the depuration of surrogate viruses were also evaluated. Depuration studies of nonoyster shellfish species (such as mussels and clams) and bacterial microorganisms (such as *E. coli* and *salmonella)* were not considered in this review.

Literature searches began with a structured electronic search using the Google Scholar and PubMed search engines. Three electronic literature searches were undertaken and they 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); and
(3) (norovirus OR Norwalk virus) AND oyster AND (localization OR binding).

For the 1st literature search above, the titles and abstracts of the 1st 300 citations (sorted using the search engine on the basis of relevance) identified 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 only if it was clear that oysters implicated in outbreaks had been subjected to depuration; consequently, only 15 articles met this criterion. The outbreaks identified spanned the period 1979 to 2012 (while outbreaks through to 2016 were searched, illnesses from depurated oysters were only identified through 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 in which the oysters had been produced.
- Information regarding an epidemiological link between outbreak and oysters.
- Analytical confirmation of NoV in human feces and oysters.
- Concentrations of NoV present in oysters after depuration.

For the 2nd literature search noted above, the titles and abstracts of the 1st 300 citations (sorted using the search engine on the basis of relevance) identified by Google Scholar ($n = 1270$) were reviewed for relevance. All abstracts of the citations identified using PubMed ($n = 29$) were reviewed. Quantitative data on the reduction of NoV (9 publications) and viral surrogates in oysters during depuration and relaying were identified and collated (7 publications).

For the 3rd literature search on localization of NoV, the titles and abstracts of the 1st 300 citations (sorted using the search engine on the basis of relevance) identified 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 lists of the reviewed publications.

Analysis of Virus Reduction Rates

The time (days) required to achieve a 1-log reduction of NoV (and surrogate viruses) was estimated for different depuration and relaying studies; not all studies report this value, thus analysis of depuration data from published papers was required.

First, the virus concentration of oysters during depuration and relaying was identified in each previously published study from either: (1) raw data as reported in the publications; or (2) for publications in which raw data were not presented, the concentration values were estimated from graphs depicting viral loss through time using WebPlotDigitizer (http://arohatgi.info/WebPlotDigitizer/), which is an online programme that can determine raw data values from graphs. For each study, the actual and estimated virus concentration data were then tabulated against time (hours/days of depuration) in a spreadsheet (Excel 2016, Microsoft®).

Second, viral loss is exponential (Loisy and others 2005; Love and others 2010; Choi and Kingsley 2016), therefore a linear regression model was fitted to the estimated log 10 transformed viral concentrations (Excel 2016, Microsoft®). For depuration studies in which viral concentration dropped below the limit of detection (LoD), subsequent values below the LoD were ignored in the analysis. The slope of the fitted model provides the log reduction per day. Thus, the time (days) it would take to achieve a single log reduction of viruses within oyster tissues for each published study was calculated from the slope as follows:

$$\text{Days for single log reduction} = \frac{-1}{\text{Slope}}$$

Part 1: NoV Oyster Interactions

When considering the efficiency of NoV depuration, it is necessary to understand how NoV is ingested, retained, and excreted by oysters. Thus, this section presents biological information on NoV and its uptake by oysters, and the state-of-the-art regarding ligands that selectively bind NoV in the oyster digestive tract and other tissues.

NoVs are a group of highly diverse viruses that belong to the *Caliciviridae* family. They are nonenveloped icosahedral viruses with a single-stranded RNA genome of around 7.5 kb length

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(Le Guyader and others 2012; de Graaf and others 2016). NoV causes gastroenteritis characterized by vomiting, abdominal cramps, fever, watery diarrhea, headaches, chills, and myalgia, with an illness normally lasting 2 to 3 d (Glass and others 2009). For an infection in humans, NoV must 1st bind to histo-blood group antigens (HBGAs). These highly conserved glycans (carbohydrates) are present on a variety of gastrointestinal and epithelial cell types and are used as receptors by many viruses and bacteria (Le Pendu and others 2014).

NoVs are currently classified into 7 genogroups, of which 3 infect humans (GI, GII, and GIV) (Zheng and others 2006; de Graaf and others 2016). NoV is the main cause of gastroenteritis worldwide, GII.4 strains are responsible for most outbreaks (de Graaf and others 2016); however, GII.17 strains are becoming more prevalent (Zhang and others 2015). Information on NoV genotypes in stools and shellfish implicated in illness outbreaks was collated. This demonstrated that GI strains are more frequently detected in shellfish outbreaks compared to other NoV outbreaks (Le Guyader and others 2012). Similarly, Yu and others (2015) found that there are more GI sequences reported from oyster outbreaks (34%) than from nonoyster outbreaks for which 90% are GII strains.

NoV is one of the most infectious pathogens, as demonstrated through human volunteer studies. For example, a human trial involving a GI.1 strain determined that the median infectious dose (ID_{50}) was between 18 and 1015 genome copies (Teunis and others 2008). Using the same strain, an ID_{50} of approximately 1320 genome copies was determined for secretor positive persons who were blood type O or A (Atmar and others 2014). Thebault and others (2013) confirmed the high infectivity by statistically analyzing data from 5 outbreaks of NoV resulting from oyster consumption in France; median ID_{50} estimates ranged between 1.6 and 7.51 genome copies per oyster consumed, showing the need for very sensitive methods to identify contaminated oysters. Additionally, NoV (GI and GII strains) have been shown to be excreted at very high levels in the feces (up to 10^{11} copies/g feces) of both symptomatic and asymptomatic people for long periods (50% of people were shedding around 10^5 copies/g feces after 27 d) (Atmar and others 2008; Kirby and others 2014), contributing to its high prevalence in the community.

Contamination of oyster production areas and oyster feeding

Sewage can be introduced into oyster production areas in a variety of ways, including through the release of sewage from wastewater treatment plants, broken sewerage pipes, and pump stations (Doyle and others 2004), via leachate from septic tanks (Stafford and others 1997), following high rain fall (Murphy and Grohmann 1980; Doyle and others 2004), from people (such as harvesters) defecating directly into production areas (Kohn and others 1995; McDonnell and others 1997; Berg and others 2000; McIntyre and others 2012), and through release of sewage by vessels (Simmons and others 2001). When growing waters are contaminated with sewage, oysters can bioaccumulate pathogens that may be present.

The gills of oysters are involved in capture, selection, and transport of food particles, as well as respiration. Cilia on the gills create water currents, which draws seawater across the gills. Mucus then binds the particles in the seawater and carries them forward to the mouth. Factors such as nutritional value and size and charge of food particles are thought to influence the selection of food by bivalves, and unwanted particles are rejected in the pseudofeces prior to ingestion (Bedford and others 1978; Shumway and others 1985; Ward and others 1997, 1998). Interactions also occur between carbohydrates on the surface of algae and lectins within the mucus covering the feeding organs of mussels (*Mytilus edulis*) and oysters (*C. virginica*), which may represent a common mechanism for particle selection across bivalve taxa (Espinosa and others 2009, 2010a, b). Pacific oysters can efficiently capture food particles in the 4 to 10 μm size range (Bell 2005). However, oysters can also retain smaller particles, such as NoVs, which are rarely monodispersed in the environment. Indeed, like many enteric viruses, NoVs bind to particles depending on their isoelectric point, and thus are mainly present in the environment in clumps or bound to other particles (da Silva and others 2011). The adhesion of viruses to solids, including plankton, may enhance bioaccumulation by shellfish (Metcalf and others 1979; Gentry and others 2009).

Interaction of NoV with oyster ligands

The recognition that NoV persists for longer periods than bacteria when oysters are depurated (Schwab and others 1998; Ueki and others 2007) provided an early clue that different mechanisms may be governing NoV accumulation. The hypothesis that NoV binds specifically to oysters, thus increasing viral persistence, prompted investigations to identify NoV ligands in oysters, as already demonstrated in humans (Hutson and others 2002). First, a GI.1 strain was shown to bind to the midgut and digestive diverticula of Pacific oysters, but not to the other tissues (Le Guyader and others 2006a). In contrast, GII NoV binds to various tissue types, including the digestive diverticula, midgut (intestine), gills, mantle, and labial palps (Seamer 2007; Wang and others 2008; McLeod and others 2009b). Collectively, these results suggested strain-specific variations in binding patterns.

Immunochemistry experiments performed on thin-layer sections of Pacific oyster tissues demonstrated that GI.1 NoV binding can be inhibited by saturating binding sites with mucins, such as those contained in the saliva of individuals that are type A and O secretors, or by using *Helix pomatia* lectin (Maalouf and others 2010). Confirmatory tests using monoclonal antibodies and NoV particles with mutated capsids confirmed that GI.1 NoV was binding to oyster tissues *via* an A-like carbohydrate, similar to the HBGAs used for NoV attachment to human epithelial cells (Le Guyader and others 2006a). Similarly, binding of GI.1 NoV to *C. virginica* tissues was inhibited by an HBGA type A antibody, confirming that NoV binding occurs through an A-like carbohydrate (Tian and others 2006).

The binding of GII NoV strains to oyster digestive tissues (DTs) also occurs through an A-like carbohydrate, but binding to the gills and mantle is facilitated by a sialic acid (SA) residue (Maalouf and others 2010, 2011). Bioaccumulation studies demonstrated that both GII.3 and GII.4 strains are transiently captured in the gills and mantle before being almost exclusively localized in DTs, whereas the GI.1 strain is directly bioaccumulated in DTs (Maalouf 2011). The binding of GII strains to a SA ligand in the gills and mantle may facilitate partial destruction of the virus, or at least account for less efficient bioaccumulation of GII strains compared to the GI.1 strain (Maalouf and others 2011). Follow-up studies on ligand expression showed a seasonal pattern in expression of the GI.1 ligand, which correlated with a higher bioaccumulation efficiency of NoV (Maalouf and others 2011). In contrast, no seasonal variation was observed for the GII ligand, and GII strains were uniformly bioaccumulated all year long (Maalouf and others 2010).

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Thus, oysters are not just passive filters, they use specific ligands to selectively accumulate NoV strains. This specific binding may help to explain their prolonged retention in oysters, as observed in depuration and relaying studies to date, and may account for illness outbreaks attributed to depurated oysters.

Current detection method for NoV in oysters

Many detection methods have been developed for NoV in shellfish (Sobsey and others 1985; Boom and others 1990; Atmar and others 1995; Henshilwood and others 1998; Shieh and others 1999, 2000; Greening and Hewitt 2008; Lees 2010). In 2013, standard methods (ISO/TS 15216-1 and ISO/TS 15216-2) for the qualitative and quantitative detection of NoV in a variety of foods, including shellfish, were published (ISO 2013a, 2013b). After protease digestion for virus recovery, the RNA is purified using guanidine thiocyanate and silica adsorption. The method uses real-time polymerase chain reaction (PCR) to amplify a fragment of the viral genome sequence targeting the conserved region at the 5' end of open reading frame 2 (ORF2). This method, one of the 1st official molecular methods, includes a number of controls including extraction efficiency and inhibition controls. While based on RNA detection, the method enables comparison of results between laboratories/countries and food types, as it is an horizontal method covering several food matrices. Additional developments, such as the use of propidium monoazide PCR or mucin binding, may provide more information on capsid integrity and viral infectivity in the future (Tian and others 2008; Sánchez and others 2012), and digital PCR will provide more accurate quantification (Polo and others 2016). The recent advance in culturing NoV using human enteroid cells may answer some questions regarding the infectivity of NoV in the future (Ettayebi and others 2016).

Part 2: Efficacy of Depuration in Reducing NoV in Oysters

The depuration process

The depuration process involves placing shellfish into tanks (custom made, or off-the-shelf designs) with clean seawater, which either continuously flows through the tank or is recirculated and replenished periodically (Lee and others 2008). Seawater is generally disinfected to reduce bacterial build-up; a variety of methods or chemicals may be used, including chlorine, iodophores, or ozone (Richards 1988, 1991), but ultraviolet (UV) light is the most commonly used approach globally. During the process, shellfish should be able to open and shut their valves without encumbrance and filter water normally, and by doing so the shellfish will purge the contents of their digestive tract, including contaminants that may be associated with digested food and fecal matter (Lee and others 2008; Lees and others 2010). The shellfish feces settle to the bottom of the tank and are removed by cleaning following the depuration process. Depuration is generally conducted for relatively short periods, varying between 24 and 96 h depending on the location, time of year, and level of bacterial contamination.

Water quality factors that need to be controlled during depuration include dissolved oxygen levels, tank loading, water flow rate, salinity, temperature, turbidity, and pH. For the EU, Regulation (EC) 853/2004 (available at: http://eur-lex.europa.eu/homepage.html) contains depuration requirements that must be adhered to, including structural requirements such as tank location and type, the need for shellfish to be free of mud during depuration, tank loading guidance, and so on. For the United States, the Natl. Shellfish Sanitation Program Model Ordinance (available at: https://www.fda.gov/food/guidanceregulation/federalstatefoodprograms/ucm2006754.htm) contains requirements on how depuration must be conducted.

Depuration was 1st 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 U.K. and the U.S. (as reviewed by Richards (1988, 1991)). Some early systems were shown to be highly effective in reducing bacteria (Dodgson 1936; Richards 1988), and, since then, depuration has been used in many parts of the world 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 time frames such as 12 h for Salmonella, and 15 to 20 h for *E. coli* (Ho and Tam 2000; de Abreu Corrêa and others 2007). However, depuration may be not so efficient for vibrios or the *Salmonella enterica* serovar Newport (Richards 1991; Morrison and others 2012).

NoV illnesses related to depurated oysters

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. However, a limitation of these studies is that quantification performed by real-time PCR is not sufficiently precise and does not give information on the potential pathogenicity of NoV. Therefore, to provide some information on the potential infectivity of NoV in oysters after the depuration process, a literature search was undertaken to identify illness outbreaks that were attributed to oysters that had been depurated.

A summary of the NoV outbreaks caused by oysters that were depurated is shown in Table 1. Epidemiological data were presented for 12 of the 15 outbreaks. Laboratory investigations were conducted on clinical specimens (feces) for 14 of the 15 outbreaks, and on oysters for 10 of the 15 outbreaks. In most outbreaks (57 %), NoV was detected in both human fecal samples and oysters.

The reported outbreaks occurred in 4 countries, the United Kingdom, Ireland, Australia and France. The small geographical distribution likely relates to the literature search being narrowed to only outbreaks relating to oysters that had been depurated, and it perhaps reflects the relatively higher implementation of depuration in the United Kingdom, France, Ireland, and Australia compared to other countries, particularly those in East Asia. Technical details regarding the depuration conditions used were usually not reported, seawater temperature was identified for 5 of the outbreaks through contacting the corresponding authors. Temperatures used were "ambient," 12 to 13 °C, 18 °C for the 2012 outbreak in Australia (Zammit, personal communication), and between 4 to 6 °C and 8 to 10 °C in the 2006 and 2008 French outbreaks, respectively (Le Saux, personal communication). This demonstrates that depuration has been ineffective across a wide range of temperatures, up to 18 °C. UV disinfection is ubiquitously used in the U.K. and Australia (67% of the outbreaks came from these countries). For the 2006 French outbreak, UV was also used (Le Saux, personal communication). Thus, the use of UV disinfection in the depuration process appears ineffective in inactivating NoV, probably because NoV particles inside the oyster are not exposed to the UV; however, precise information on the UV treatment conditions is not known (such as exposure time, frequency at which lamps are changed and seawater quality). The depuration periods varied between 1 and 15 d (Table 1), suggesting that prolonged purification periods do not consistently reduce NoV to "safe" levels.

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Table 1–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 feces by PCR	Virus detected in oysters by PCR	Virus copies/g in oysters (postdepuration)[d]	Depuration time	Reference
18	2012	Pacific oysters	Ireland	NS	Y (GI and GII)	Y (GI and GII)	2.38×10^3	NS	Rajko-Nenow and others (2014)
8	2012	NS	Australia	Y	Y (GII)	Y (GII)	NS	39 h	Fitzgerald and others (2014)
70	2010	Pacific oysters	Ireland	NS	Y (GII)	Y (GI and GII)	2.35×10^3	NS	Dore and others (2010); Rajko-Nenow and others (2014)
≥ 240	2009	NS	United Kingdom[a]	Y	Y (GI and GII)	Y (GI and GII)	NS	NS	Smith and others (2012)
23	2008	Pacific oysters	France	Y	Y (GII)	Y (GII)	2.4×10^3	1 to 23 d[e]	Le Guyader and others (2010)
>93	2007	Pacific oysters	United Kingdom	NS	Y (GII)	Y (GII)	8×10^3	NS	Lowther and others (2010)
19	2007	NS	Australia	Y	Y	N	NA	36 h	Huppatz and others (2008)
205	2006	Pacific oysters	France	Y	Y (GI and GII)[b]	Y (GI and GII)[b]	2.4×10^3	1 to 12 d[e]	Le Guyader and others (2008)
15	2004	NS	United Kingdom	Y	Y (GI and GII)	NT	NA	42 h	Gallimore and others (2005)
329	2002	Pacific oysters	France	Y	Y (GI and GII)	Y (GI and GII)	1.7×10^2	48 h	Le Guyader and others (2006b)
9	1997	Pacific oysters	United Kingdom	Y	Y[c]	NT	NA	NS	Ang (1998)
97	1996	NS	Australia	Y	Y (GII)	N	NA	NS	Stafford and others (1997)
16	1985	Pacific oysters	United Kingdom	Y	NT	NT	NA	15 d	Heller and others (1986)
181	1983	Pacific oysters	United Kingdom[a]	Y	Y[c]	NT	NA	72 h	Gill and others (1983)
52	1979	*Saccostrea glomerata*	Australia	Y	Y	NT	NA	48 h	Grohmann and others (1981)

[a]Outbreak in the United Kingdom, location of supplier not noted.
[b]Other enteric viruses were also detected in the oysters and feces, including aichi virus, astrovirus, enterovirus, and rotavirus.
[c]Small round structured viruses (SRSV) were visualized in fecal samples.
[d]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).
[e]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.
NS, not stated; Y, yes; N, no; NT, not tested; NA, not applicable.

For 6 of the outbreaks, the NoV concentration following depuration was reported to be between 1.7×10^2 and 8×10^3 NoV genome copies/g of DT (Table 1). Given the low ID50, it is not surprising that these oysters were implicated in gastroenteritis outbreaks.

NoV persistence following relaying

Three studies have reported the efficiency of NoV elimination from Pacific oysters in the natural environment (open seawater) over an extended period. Table 2 presents a summary of NoV levels in oysters during relaying and depuration.

After a contamination event resulting in 205 cases of gastroenteritis in France, oysters were found to be contaminated with both NoV GI and GII. The follow-up over a 4-wk period showed a decrease in NoV from 3 log genome copies/g DT to around the limit of quantitation (LoQ) (70 genome copies/g DT), when seawater temperatures were between 8 and 10 °C (Le Guyader and others 2008). Similarly, GII NoV levels in oysters that were implicated in an illness outbreak declined from 2.9×10^3 to 492 copies/g following a 17-d relaying period (seawater temperature 15 to 17 °C), and were below the LoQ after a further 6 d of depuration (Dore and others 2010).

While NoV levels decreased in these relaying studies, NoV was still detectable in some oysters after 3 to 4 wk of purification, they were allowed to be sold for consumption following relaying, and no further human illnesses were reported (Le Guyader and others 2008; Dore and others 2010). This suggests that a relay period of around 4 wk may be sufficient to reduce GI and GII NoV to background levels in Pacific oysters, and that viral infectivity is reduced after this length of time. Other explanations such as the immunity of the local population, or the relationship between severity of symptoms and dose, could also contribute to the lack of reported illnesses (Atmar and others 2014; de Graaf and others 2016).

NoV persistence following depuration

Studies on in-tank depuration of NoV (GI and GII) in oysters have demonstrated that periods of between 23 h and 14 d result in negligible reductions (Table 2). Temperatures in the published studies ranged from 8 to 24 °C, and depuration at the higher temperatures (≥16 °C) did not result in significant NoV reductions (Schwab and others 1998; McLeod and others 2009a; Neish 2013); statistical analysis by analysis of variance (ANOVA; $P = 0.241$), and linear and quadratic regression ($P = 0.342$), determined that there was no significant reduction in NoV for the studies conducted by McLeod and others (2009a) and Neish (2013), respectively. However, an investigation into the impact of temperature on the reduction of GI NoV in Eastern oysters demonstrated a 2-log reduction of NoV at 7 and 15 °C over 6 wk, and at 25 °C NoV was not detected beyond week 4; the difference in depuration rates between oysters held at 7, 15, and 25 °C was found to be significant, as determined by ANOVA ($P < 0.01$) (Choi and Kingsley 2016). This suggests that elevated temperatures can increase depuration efficiency over longer time frames. UV and ozone disinfection appear to have limited impact on the depuration of NoV, with no decrease reported for oysters depurated at 8 °C, and only a 0.5 log reduction of GII NoV at 16 °C, in a UV system (Neish 2013); however, it is noted that NoV studies use PCR and do not provide information on potential reduction of viral infectivity.

Of the 16 published NoV reduction experiments, the observed loss of NoV genomes in 8 experiments was too limited to allow a prediction of the time required to reduce levels by 1 log. The

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Table 2–NoV levels reported for oysters following purification, and the number of days of purification estimated to achieve a 1-log reduction.

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

[a]The depuration system was a closed system with recirculating UV sterilized seawater, which was completely exchanged daily.
GII, genogroup II 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.

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estimated days to achieve a 1-log reduction of NoV (genomes) in the 8 remaining experiments were between 9 and 45.5 d (Table 2). A range of factors may influence the rate at which NoV is purged by oysters, including the oyster species involved (Nappier and others 2008, 2010), and seawater temperature and salinity (Nappier and others 2008; Love and others 2010; Choi and Kingsley 2016). The availability of food for oysters (phytoplankton) has also been hypothesized to improve NoV depuration; however, studies to date have not supported this, with similar viral depuration rates for oysters that were fed compared to starved animals (Kingsley and Richards 2003; Drouaz and others 2015).

Persistence of surrogate viruses

As previously noted, NoV is not yet able to be detected/quantified from shellfish tissues using cell culture methods. Thus, while it is clear that NoV genomes can persist for prolonged periods, it is not known how long NoV infectivity is retained within oyster tissues, except as inferred through the outbreak investigations noted above. To overcome this problem, studies have been conducted using surrogate viruses (which are generally able to be cultured) to assess the virological safety of depurated oysters. Table 3 presents a summary of depuration studies conducted using surrogate viruses.

Using Pacific oysters, no reduction of NoV was noted at 8 °C over 14 d, while F-specific ribonucleic acid (FRNA) bacteriophage was reduced by nearly 1 log (Neish 2013). A similar reduction was achieved at a higher temperature of 16 °C after 6.5 d for phage, while 45.5 d were needed for a 1-log reduction of NoV. The study suggests that there is a major difference in the behavior of phage and NoV during depuration, but the different detection methods used (culture and PCR) may have also contributed to the discrepancy in depuration rates.

Depuration of NoV and feline calicivirus (FCV) in oysters (*C. gigas*) were observed for 10 d at 10 °C in a flow-through system, while NoV levels did not decrease, FCV was rapidly depurated within 3 d (Ueki and others 2007). This is consistent with the results of Provost and others (2011), who used reverse-transcription PCR RT-PCR to demonstrate that FCV was undetectable 1 d after contamination of oysters (*C. virginica*), whereas hepatitis A virus was detected for >21 d. Murine norovirus (MNV) showed an intermediate persistence, and it was detected for up to 12 d (Provost and others 2011).

Comparing the elimination rates of NoV and poliovirus (PV) in Pacific oysters over 23 h of depuration in a recirculating system, no decline in NoV genome copies was observed, while PV genome copies decreased by 2 logs (McLeod and others 2009a). Tulane virus (TV), a recovirus belonging to the *Caliciviridae* family, binds to HBGAs in monkeys, similar to HBGA recognition by NoV in humans (Farkas and others 2010). As shown by genome detection, NoV GI was more persistent in Pacific oysters than TV or mengovirus (MgV), with half-lives of 7.56, 4.65, and 2.17 d, respectively, following depuration at 11 °C for 8 wk. The results suggested that TV may behave more like a NoV GII strain (rather than GI used in the study), due to differences in the HBGAs that GII and GI strains bind to (Drouaz and others 2015).

In summary, comparative elimination studies to date have shown that surrogate viruses (including phage, FCV, PV, MgV, and TV) are more rapidly depurated than NoV under a variety of depuration conditions, including temperatures of 8 to 25 °C, times varying between 23 h and 8 wk, and using both recirculating and flow-through systems that have UV and/or filtration disinfection (Table 3). A comparison of time needed to achieve a 1-log reduction shows that the number of days to reduce NoV (mean = 19 d; Table 2) is greater than that recorded for the surrogate viruses (mean = 7.5 d; Table 3). The rapid reductions noted for surrogate viruses may be partly attributable to the quantitation of infectious virions in some studies, whereas NoV quantitation is based on genome detection; however, several studies (Ueki and others 2007; McLeod and others 2009a; Drouaz and others 2015) have used genome detection for the analysis of both NoV and surrogate viruses (including FCV, PV, TV, and MgV) and large differences in reduction rates were still observed.

For a surrogate virus to provide useful information on NoV infectivity, 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. Given the more rapid depuration of surrogate viruses tested to date, they may not be suitable for assessing the virological safety of depurated oysters.

Possible reasons for variations in the persistence of different types of viruses

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

Viral localization studies have demonstrated the presence of viruses, including NoV 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 (hemocytes) (Fries and Tripp 1970; Fisher 1986; Romalde and others 1994; Le Guyader and others 2006a; McLeod and others 2009b; Provost and others 2011). Further research has demonstrated that NoV binds to HBGA-like ligands 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 and others 2010, 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 hemocytes and/or excretion through the lumen of the digestive tract.

Oyster hemocytes contain acidic vesicles that aid in the digestion of small food particles that are phagocytosed, therefore hemocytes have low pH. Provost and others (2011) hypothesized that for viruses to persist within shellfish, they must be resistant to acidic digestion within hemocytes. 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. The association of NoV with bacteria may also play a role in variable persistence; however, because bacteria are efficiently depurated it is unlikely that the adhesion of NoV to bacteria represents a significant excretion pathway (Miura and others 2013).

Autophagy is a normal physiological pathway that results in regulated degradation of cells, and in eukaryotes it plays a role in the degradation of viruses. Autophagy is an evolutionarily conserved process across plants and animals, and recently it has been shown

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Table 3–Levels of surrogate viruses in oysters following depuration and relaying, and the number of days of purification estimated to achieve a 1-log reduction.

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

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

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that it protects Pacific oysters from certain bacterial and viral infections such as from *Vibrio aestuarianus* and ostreid herpesvirus 1, leading to oyster survival (Moreau and others 2015). Given the wider role of autophagy in viral degradation in animals, and indeed oysters, it will be interesting to evaluate its involvement in the removal of NoV and other enteric viruses.

Data gaps and limitations

Several information gaps and limitations have been identified through this literature review and are summarized below:

(1) Many studies have investigated the persistence of NoV in oysters following depuration and relaying. These have been conducted using RT-PCR methods, as no practical culture method exists for NoV in shellfish at this time. Thus, the rates at which infectivity of NoV in oysters declines under different depuration conditions (such as variable temperature, salinity, disinfection regimes, and so on) is not known and makes comparisons with surrogate viruses difficult.

(2) Until recent times, the quantitative approach used in many depuration studies (particularly historical older research) may not have been appropriate, due to the lack of viral reference standards to enable incorporation of standard curves in assays, and higher levels of accuracy. Further studies using the standard ISO method for quantitation, or new technologies such as digital PCR (Polo and others 2016), may improve our understanding of depuration rates and enable direct comparisons to be made between studies.

(3) Numerous illness outbreaks of NoV have occurred from the consumption of depurated oysters (Table 1). 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.

(4) Many studies have investigated the impact of depuration process parameters such as temperature, time, salinity, and the feeding and disinfection approach, through modulation of 1 factor at a time. Few studies, however, have sought to optimize the physiology of Pacific oysters and use a combination of conditions that promote optimal clearance rates. If further such studies are conducted, care must be taken that conditions do not favor proliferation of potentially harmful bacteria such as *Vibrio* spp.

(5) Potential differences in depuration rate for oysters that have bioaccumulated NoV in the natural environment (potentially overextended periods of time) compared with oysters that have been artificially contaminated in laboratory uptake studies are not well understood.

Part 3: Potential New Approaches for Viral Depuration

Since the inception of depuration over a century ago, it has become increasingly apparent that the traditional process does not adequately eliminate NoV from shellfish, and this poses a major food safety challenge. The on-going nature of NoV illness outbreaks highlights the need for improved water quality throughout the world, but also for new postharvest treatment processes to ensure the virological safety of shellfish. This section is focused on new potential approaches to depuration that may enhance NoV reduction.

Viral depuration has been described by several authors as being "two phase," with elimination in the 1st few days being more rapid than subsequent days (Love and others 2010; Provost and others 2011; Polo and others 2014b, 2015). The 1st rapid phase of viral depuration is likely related to extracellular digestion and purging of the digestive tract; the speed of purging is governed by physiological traits of the shellfish species concerned, including filtration and clearance rates, digestion rate, and enzymatic activity. Thus, optimizing the physiological state of shellfish through adjusting different parameters such as temperature and salinity contributes to maximizing NoV reductions in the 1st phase of depuration (that is, gut purging). However, the persistence of NoV in shellfish during the 2nd slower phase of elimination indicates that other properties are at play. Indeed, the binding of NoV to HBGA-like ligands present on oyster gastrointestinal cells, gills, and mantle represents a major barrier to enhancing depuration and will be a key point to address in future studies (Nappier and others 2008; Maalouf and others 2011; Provost and others 2011; Le Guyader and others 2012; Polo and others 2014a, b).

Enzymatic pretreatment

Considering the specific binding of NoV to ligands in oyster tissues, an approach that may enhance depuration is the application of enzymes to disrupt the ligands that are involved. Such enzymes could be diluted in seawater and the oysters immersed for a short period prior to depuration. This may lead to degradation of the ligands, and enhanced release of NoV from oyster tissues during the standard depuration process. As noted previously, Norwalk virus (prototype of GI.1) binding involves a type-A HBGA (Le Guyader and others 2006a; Tian and others 2006) and for GII strains (such as GII.4 and GII.3) binding involves both an A-like HBGA and a SA residue at the α2,3 linkage (Maalouf and others 2010, 2011). HBGAs are complex carbohydrates that contain structurally related saccharide moieties and there are different groups of enzymes and other compounds that could promote degradation of these ligands (Table 4).

Proteases, including those of animal (such as pronase, trypsine, α-chymotrypsine), plant (such as papain, ficin, bromelain), and fungal origin (such as proteinase K), cleave proteins at defined sites along peptide sequences. The broad activity of proteases (even in the presence of various salts) make them good candidates for targeting the protein structures involved in viral binding. Another group of enzymes that may show promise are the glycosidases, which break glycosidic bonds in complex sugars at specific locations. Glycosidases are more specific than proteases; however, several have potential to degrade the HBGAs involved in NoV binding. For example, α-amylase acts upon α(1,4)-D-glucosidic linkages, and β-galactosidase hydrolyzes the β-glycosidic bond formed between a galactose and its organic moiety, thus these glycosidases could favor the hydrolysis of type-A HBGAs. Sialidase and O-sialoglycoprotease release the sialyl groups from glycoconjugates and could degrade the SA ligands involved in the binding of NoV GII strains.

Bacteria

Another avenue worthy of further research is the potential for particular bacteria to produce compounds that have antiviral activity and promote viral elimination. Various bacteriocins with antiviral activity have already been reported against several viruses, including herpes simplex type 1 (Todorov and others 2010), influenza virus (Serkedjieva and others 2000), and New Castle disease virus (Saeed and others 2007). Lange-Starke and others (2014) also reported a 1.25-log reduction of MNV when lactic acid

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Table 4–Summary of compounds that may act to degrade HBGA (histo-blood group antigen)-like compounds 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	–	Ca^{2+} necessary for activity Also from plant, bacterial, and fungal origin
	Trypsine	Porcine/bovine pancreas	Serine Endoprotease	Peptides on the C-terminal side of Lys and Arg amino acid residues	7.0 to 9.0	Ca^{2+} retards the autolysis ability and maintains the stability in solution
	α-Chymotrypsine	Bovine pancreas	Serine Endoprotease	Peptide bonds on the C-terminal side of Tyr, Trp, Phe, Leu	–	Ca^{2+} activates and stabilizes the enzyme
	Pronase XIV (Pronase E)	*Streptomyces grisseus*	Endo-/exoproteases	Nonspecific protease	7.0 to 8.0	Ca^{2+} is recommended for protection from autolysis. Much more effective in digestion of casein than trypsin, chymotrypsin, 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	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 to 7.0	Much more effective than pancreatic proteases. Upon prolonged incubation further bonds are split. The addition of L-cystein (0.5% w/v; 5 mM) 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	–	
Fungal protease	Proteinase K	*Engyodontium album*	Serine Endoprotease	Peptide bonds adjacent to the carboxyl group of aliphatic and aromatic amino acids	7.5 to 12	Ca^{2+} necessary for activation (1 to 5 mM Ca^{2+}). Maximum activity at 37 °C; 80% loss of activity at 20 to 60 °C
Glycosidases	α-Amylase	Porcine/human pancreas	Endoglycosidase	$\alpha(1,4)$-D-glucosidic linkages in polysaccharides of ≥ 3 of $\alpha(1,4)$ 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*	Endoglycosidase	β-glycosidic bond formed between a galactose and its organic moiety	6.0 to 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*	Endoglycosidase	O-Glycans. N-acetylgalactosamine glycosidic linkage	6.0 to 8.0	
	Neuraminidase (sialidase)	*Vibrio cholerae*	Exoglycosidase	Preferentially $\alpha(2\rightarrow3)$, but also $\alpha(2\rightarrow6)$ and $\alpha(2\rightarrow8)$ linkages between neuraminic acid and galactose	5.0	Ca^{2+} necessary for activity.
	α-L Fucosidase	Bovine kidney	Exoglycosidase	$\alpha(1\rightarrow(2,3,4,6))$ linked fucose from N- and O linked glycans.	5.5 to 5.8	It cleaves α-1$\rightarrow$6 linked fucose on the trimannosyl core of N-linked glycans more efficiently than other α-fucose linkages.
Other compounds	$NaIO_4$		Sodium salt of periodic acid	Sialic acids and carbon–carbon bonds of a wide range of carbohydrates	–	More effective in dark conditions.

bacteria were applied; however, the active antiviral metabolite could not be identified.

Recently, *Enterococcus hirae* (designated as 3M21) isolated from mussels (*Mytilus galloprovincialis*) showed antibacterial activity against *Listeria monocytogenes*, *Listeria innocua*, and *Enterococcus faecalis*, the bacteria also showed antiviral activity against MNV-1 (Fajardo and others 2014). A proteinaceous active substance was identified, but its efficacy in reducing NoV titers during depuration or other postharvest treatments has not yet been reported.

Microencapsulation

A key issue with regard to the efficacy of enzymatic or bacterial-based treatments relates to ensuring that an adequate concentration is targeted at the sites of interest within the shellfish body, particularly the DT, which is the main site of viral accumulation. Microencapsulation techniques have been used in the aquaculture sector, initially to overcome marine larval feeding problems, but it has evolved to include the delivery of probiotic bacteria and bacterial substances, immunostimulants, vaccines, and enzymes to target species (Polk and others 1994; Skjermo and Vadstein 1999; Borgogna and others 2011; Rosas-Ledesma and others 2012; Darmody and others 2015). Three main polymers have been used for microencapsulation, chitosan, alginate, and polylactic-co-glycolic acid (Behera and others 2010; Luzardo-Alvarez and others 2010; Borgogna and others 2011; Plant and LaPatra 2011).

While microencapsulation techniques have been used in the aquaculture industry (Martínez Cruz and others 2012; Fajardo and others 2014; Darmody and others 2015; Prado-Alvarez and others 2015), the success of this approach in delivering substances to oysters to enhance NoV depuration has not yet been evaluated. However, Darmody and others (2015) 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 hemocytes. Similar results were also reported after the oral administration of alginate microcapsules containing immunostimulants in *O. edulis* against the protozoan parasite *Bonamia ostreae* (Prado-Alvarez and others 2015). The proteinaceous active substance isolated from the bacterium 3M21, which shows antiviral activity against MNV-1, was also successfully microencapsulated in alginate and able to be ingested by oysters (Darmody and others 2015; Prado-Alvarez and others 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 hemocytes (a potential virus repository), suggest that microencapsulation could represent a viable tool for the transport of antiviral substances directly to these areas.

Food safety considerations

To ensure oysters that have been treated with enzymes or bacteria are safe for human consumption, it will be necessary to undertake a risk assessment to evaluate the toxicology of the compounds involved, quantities that may be present in oysters following treatment and depuration, and the potential for acute and/or chronic impacts in consumers. 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.

Conclusions

The introduction of depuration was highly successful in reducing shellfish-related outbreaks of typhoid fever and cholera, but has failed to eliminate outbreaks of NoV-related gastroenteritis, which still regularly occur today. NoV outbreaks have occurred from the consumption of oysters that were depurated under a wide variety of conditions, suggesting that the modulation of depuration parameters, such as water temperature and UV, does not result in significant improvements. Consistent with this, there was no reported reduction of NoV in oysters in 50% of the depuration studies to date, and for those in which a reduction was demonstrated it took between 9 and 45.5 d to reduce levels by 1 log. The time frames required to achieve a 1-log reduction are clearly much longer than those used for commercial depuration, which is generally between 24 and 48 h. Furthermore, illness outbreaks commonly involved NoV concentrations of around 10^3 viral copies/g of DT. Thus, a 1-log reduction is unlikely to be sufficient to protect public health, and much more time would be required to reduce levels sufficiently. This information strongly suggests that depuration is not an appropriate postharvest control measure for NoV-contaminated oysters at this time. Despite this, some studies indicate that very small reductions in NoV are possible, which may be beneficial for oysters containing very low NoV concentrations; however, the impact of depuration on low levels of NoV ($\leq 10^2$ copies/g DT) has not been investigated to date. Given the foregoing, it is acknowledged that while depuration is not effective at completely preventing illness, it may play a small role in reducing illness levels.

In contrast to depuration, relaying oysters in areas with clean seawater over a 4-wk period appears to be successful in reducing NoV levels to around the LoD (100 genome/copies), and no illnesses have been reported to date following the consumption of such relayed oysters. While no illnesses have been reported, it is acknowledged that relaying may be less commonly practiced than depuration, which potentially contributes to the lack of reported outbreaks. Further, access to clean relaying waters may be a challenge in the future, with an increasing global population, and a 4-wk delay prior to marketing oysters may not be commercially viable for all food business operators. Nonetheless, relaying oysters into areas with clean seawater for 3 to 4 wk is a beneficial virus risk management strategy.

This review also shows that NoV is retained for longer periods in oysters than a variety of surrogate viruses. A comparison of the days required to achieve a 1-log reduction in studies to date (excluding those which show no reduction) shows that NoV takes an average of 19 d to reduce by 1 log, whereas surrogate viruses take 7.5 d. While this difference may be partly attributable to the use of different assays for surrogate viruses (culture based) and NoV (PCR), it is notable that PCR was used for the analysis of both NoV and surrogate viruses in several studies and large differences in reduction rates were still identified. This suggests that surrogate viruses are not suitable for assessing the virological safety of depurated oysters; the recent development of a culture-based NoV method (Ettayebi and others 2016) may allow small-scale studies to further investigate the relative depuration rates of infectious NoV and surrogates. The longer persistence of NoV compared to other viruses also highlights that special factors govern the retention of NoV in oysters. Indeed, the binding of GI and GII NoV to HBGA-like ligands in the digestive tract, mantle, and gills of oysters plays a significant role in the persistence of NoV. Furthermore, it has been demonstrated that hemocytes are repositories of

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viruses in oyster tissues, and the high acid resistance of NoV (unlike most surrogate viruses and bacteria) may also partially account for their persistence. In light of the special relationship between NoV and oysters, and the limited success of studies which have focused on optimizing operational parameters of the depuration process (such as temperature, salinity, feeding), it is suggested that the following topics be given priority when considering future research to support the production of virologically safe oysters:

- A detailed review of depuration undertaken by Richards in 1988 commented: "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 contamination of shellfish at the source. To this end, further collaboration between water companies, local authorities, and the shellfish industry should be prioritized to improve wastewater treatment, and also processes governing discharges and communication of these to all affected parties.
- Improvement of our understanding of the special virus–oyster relationship and binding interactions, and investigations into postharvest interventions that exploit the mechanisms by which NoV is retained (namely, binding to HBGAs).
- Limited information currently exists regarding the time over which NoV infectivity is retained in oysters during depuration and relaying. Recent advances have resulted in a cell culture method for NoV being developed (Ettayebi and others 2016). It is suggested that this method could be used for limited studies that investigate viral infectivity during depuration and relaying.

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Author Contributions

Catherine McLeod researched prior studies, interpreted results, compiled data, and drafted the manuscript. David Polo researched prior studies and drafted several sections of the manuscript. Jean-Claude Le Saux researched prior studies, reviewed the draft manuscript, and added additional text. Françoise S. Le Guyader researched prior studies, reviewed the draft manuscript, and added additional text.

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