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Review

Bacteriophages as enteric viral indicators in bivalve mollusc management

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ABSTRACT

Human enteric viruses, such as norovirus and hepatitis A virus, are spread by a variety of routes including faecal-oral transmission. Contaminated bivalve shellfish are regularly implicated in foodborne viral disease outbreaks internationally. Traditionally indicator bacteria, the coliforms and *Escherichia coli*, have been used to detect faecal pollution in growing waters and shellfish. However, studies have established that they are inadequate as indicators of the risk of human enteric viruses. Bacteriophages have been identified as potential indicators or surrogates for human enteric viruses due to their similarities in morphology, behaviour in water environments and resistance to disinfectant treatments. The somatic coliphages, male-specific RNA coliphages (FRNA coliphages) and the bacteriophages of *Bacteroides* are the groups recognised as most suitable for water and shellfish testing. In this review, we discuss the rationale and supporting evidence for the application of bacteriophages as surrogates for human enteric viruses in shellfish under a variety of conditions. There is some evidence to support the validity of using bacteriophage levels to indicate viral risk in shellfish in highly contaminated sites and following adverse sewage events.

Contents

1. Introduction

The human health risks associated with consumption of raw or

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lightly cooked shellfish containing human enteric viruses are well recognised with numerous foodborne outbreaks documented. Internationally, there were 368 foodborne viral outbreaks associated with shellfish reported in the scientific literature between 1980 and 2012 (Bellou et al., 2013). The most common viral pathogens involved were norovirus (NoV) (83.7%) and hepatitis A virus (HAV) (12.8%) with oysters (58.4%) the most frequent shellfish implicated in outbreaks.

The major source of viruses in bivalve molluscs is contamination of growing waters with human sewage pre-harvest (FAO/WHO, 2012). Both NoV and HAV are shed at high levels in the faeces of infected individuals (10^4-10^{11} viral genomic copies/g) (Atmar et al., 2008; Chan et al., 2006; Fiore, 2004). If this faecal matter reaches the growing waters, enteric micro-organisms can be bio-accumulated in shellfish tissues through filter feeding, at rates dependent on shellfish species, environmental conditions, season and type of microorganism (Burkhardt and Calci, 2000; Polo et al., 2014; Ropert and Goulletquer, 2000). Oysters can selectively retain NoV strains through specific binding to carbohydrate ligands within their tissues (Le Guyader et al., 2012).

The median infectious dose (ID_{50}) for NoV is only 18 infectious particles (Baert et al., 2011; Teunis et al., 2008) and for HAV is 10–100 virus particles (Atmar, 2010; Yezli and Otter, 2011). The ability of bivalve shellfish to accumulate virus particles, combined with the low infectious dose, contributes to the high risk of illness if shellfish are harvested from contaminated waters. Furthermore, NoV and HAV have been recognised to remain infectious in shellfish for days to weeks following contamination (Lee et al., 2015; Maalouf et al., 2010a).

Currently there are no effective options to eliminate viral contamination of bivalve molluscs prior to consumption without changing the desired sensory characteristics of the shellfish. Therefore, effective risk management strategies need to focus on prevention of contamination. In the case of shellfish, prevention has to occur primarily at the pre-harvest level (FAO/WHO, 2008).

2. Indicator organisms and hydrodynamic modelling for water quality

The potential presence of faecal pathogenic micro-organisms in water and shellfish can be identified through the detection of appropriate indicator organisms. Bacteria have been used as indicators of water quality since the late 19th century (WHO, 2001). Indicator organisms should be readily detected, not present in the absence of contamination and present in relatively large numbers when pathogens from similar origins are present. They should also display similar survival times and sensitivities to disinfection and treatment processes as pathogens. No single organism has been identified that fulfils all these qualities, therefore multiple indicators are often preferred, with specific indicators being more suited for certain situations (NHMRC, 2011).

The validity of an indicator is affected by the relative rates of removal of the indicator versus the potential pathogen. Hence, differences in environmental resistance or even the ability to multiply in the environment influence their application. Thermotolerant coliforms, including *Escherichia coli* (*E. coli*), indicate the presence of recent faecal contamination and have been widely applied as useful indicators for this purpose, despite reports that some may multiply in tropical waters (Byappanahalli et al., 2012). Although on an individual sample basis *E. coli* is a poor predictor of NoV risk, on a site-specific basis average *E. coli* levels have been shown to correlate with average NoV levels in the United Kingdom (UK) during the winter season (Lowther et al., 2012). Faecal enterococci, from the *Streptococcus* and *Enterococcus* genera, exist in high numbers in the faeces of humans and other warm-blooded animals, do not multiply in the environment, are absent from pristine waters and are found in faecally polluted water. Enterococci do not persist for long in water, although longer than *E. coli*, therefore are also useful for detection of recent faecal pollution (Leclerc et al., 1996; NHMRC, 2011). The coliforms are a heterogeneous group of bacteria, they are not exclusively faecal in origin with some occurring in the environment. Hence, they are unsuitable as absolute indicators of faecal pollution (Ashbolt et al., 2001).

Risk management for bivalve shellfish destined for human consumption relies on the use of enteric bacteria as indicators of faecal contamination. International regulations have been developed to specify the acceptable levels of enteric bacterial pathogens in shellfish tissues or in waters where shellfish are grown. These have led to the classification of production areas for shellfish harvest fit for human consumption. The United States (US) shellfish safety program utilises water based sampling developed around thermotolerant coliform indicators, including *E. coli*. In contrast, the European Union (EU) shellfish safety sampling program is based on *E. coli* levels in shellfish. Australia has flexibility to utilise either system. The Codex Alimentarius Commission has published guidelines to minimise the presence of human enteric viruses in foods (FAO/WHO, 2012). This document includes an annex on the control of HAV and NoV in bivalve molluscs with specific recommendations covering primary production with water or shellfish monitoring based on *E. coli* and/or coliform data.

Despite the universal use of coliforms and *E. coli* as indicators to predict the risk of exposure to pathogens of faecal origin in water and shellfish, bacteria have been shown to be poor indicators of human enteric viral contamination (Doré and Lees, 1995; Flannery et al., 2009). Structurally viruses are diverse and are quite distinct from bacterial cells. They also display significantly different resistance and susceptibility responses to environmental conditions such as desiccation, UV irradiation, and water and sewage treatment processes (Blatchley et al., 2007; Stewart et al., 2008). Bacteriophages (phages) have been proposed as indicators or surrogates for human enteric viruses due to similarities in morphology and survival dynamics (Havelaar et al., 1986).

Hydrodynamic modelling is an approach with potential for improved shellfish risk management through the prediction of water and shellfish contamination levels with associated flushing and depuration times after adverse events. Riou et al. (2007) developed a two-dimensional hydrodynamic model simulating the impact of rainfall events on water quality and shellfish in an area in Normandy, France (Riou et al., 2007.). The hydrodynamic model incorporated features specific for the study location, with inputs including water surface elevation, velocity, water column height and turbulent viscosity. The microbiological components included storm water input and fluxes for the selected indicator microorganisms, *E. coli*, Astrovirus (AstV) and FRNA phages, to model the subsequent decay rate of the microorganisms. This approach may contribute to the identification of periods of viral risk associated with shellfish.

3. Direct detection of human enteric viruses

Polymerase chain reaction (PCR) detection is currently the best methodology available for foodborne virus detection yet it is not conducive for routine end product monitoring in shellfish as it requires significant technical expertise, time and expense. Furthermore, whilst PCR methods are sensitive, the number of viral copies detected does not directly relate to infectivity (Liu et al., 2011; Stals et al., 2012). PCR will also detect naked non-encapsulated or degraded viral RNA and viruses with damaged capsids that cannot initiate infection but which contain a viral RNA genome (EFSA, 2011). This could lead to an over-estimate of the actual infective

viral particles present (EFSA, 2011; Flannery et al., 2013), although studies investigating the recovery of a process control exogenous virus during the virus extraction procedure have indicated significant losses of virus may also occur (Costafreda et al., 2006). Virus extraction efficiency is deemed acceptable if >1%, as determined by the recovery of the process control virus added to the oyster digestive tissue (ISO/CEN, 2013). This is likely an under-estimate of the actual number of genome copies in the original sample. Therefore, it is difficult to determine the infectious risk associated with NoV and HAV detected in environmental or shellfish samples using PCR based methodologies.

4. Phage biology

Phages are viruses that specifically infect bacteria. They are ubiquitous in the environment and are the most abundant biological entities on Earth (Kutter and Sulakvelidze, 2005). Phages are non-pathogenic to other organisms and can remain infective in the environment without a suitable host for long periods of time. They co-exist and co-evolve with bacteria in a dynamic predator-prey relationship (Brüssow et al., 2004). They infect and replicate in the bacterial host and are generally specific to species level, although there are some phages with a broader host range (Hyman and Abedon, 2010). Phages that infect *E. coli* and closely related coliforms are called coliphages.

The three main groups of phages of interest for water quality testing and as indicators of viral pollution in shellfish are the somatic coliphages, male-specific RNA coliphages (FRNA phages) and the phages of *Bacteroides* (WHO, 2001).

4.1. Somatic phages

The somatic phage group comprises a large number of lytic phages of the *Myoviridae*, *Siphoviridae*, *Podoviridae* and *Microviridae* families with diverse morphologies and single stranded or double stranded DNA (ss or dsDNA) genomes (Ackermann, 2009). Somatic phages initiate infection by attaching to specific receptors on the cell wall of the bacterial coliform host. They can replicate in the gastrointestinal tract of warm-blooded animals. There is potential for further replication of somatic phages outside the gut in water environments, as some of the host bacteria have been shown to metabolise and multiply in water (Legnani et al., 1998; Muniesa and Jofre, 2004).

4.2. Male-specific RNA phages (FRNA phages)

FRNA phages, also referred to as male-specific phages (MSC) or F-specific phages, have been the most extensively studied due to their similarity to pathogenic human enteric viruses in particular enteroviruses (EV), caliciviruses, adenovirus (ADV), HAV and hepatitis E virus (Jofre, 2007). They are a restricted group of closely related phages of the *Leviviridae* family with similar size, shape and single-stranded RNA (ssRNA) genome to human enteric viruses. They infect the host cell via attachment to the fertility (F) fimbriae on the *E. coli* host (Leclerc et al., 2000). The F-fimbriae can only be produced by cells carrying a plasmid that encodes for this factor and only when the cells are in the logarithmic growth phase at temperatures above 30 °C. FRNA phages replicate in the gastrointestinal tract of mammals, but are unlikely to multiply in water environments where the conditions are unsuitable (Havelaar et al., 1986). FRNA phages can be divided into four genogroups which are useful for identification of the source of faecal contamination, that is, whether primarily of animal or human origin.

4.3. Phages of bacteroides

Bacteria of the genus *Bacteroides* are among the most abundant group found within the gastrointestinal tract of mammals, and display significant host specificity enabling differentiation between livestock and human faecal sources (Layton et al., 2006; Madigan, 2006). The *Bacteroides* infecting phages that have been studied belong to the *Siphoviridae* family and have long contractile tails and contain dsDNA genomes. They have not been reported to replicate outside the gastrointestinal tract due to the specific nutritional requirements of the host and its anaerobic nature. Phages to *B. fragilis* HSP40 are human specific, however, those to other strains such as *B. fragilis* RYC2056 are more numerous and not human specific. There also appear to be significant differences in numbers recovered in different areas of the world dependent on the host strains (Puig et al., 1999).

5. Phages as potential indicators of human enteric viruses

All three groups of phages are routinely isolated from the gastrointestinal tract of mammals and sewage. The phages of *Bacteroides* generally have narrow host ranges leading to differences in phage numbers depending on the host strain and require more costly, complex methods of detection. The somatic phages are usually in higher numbers which is an advantage when assessing water quality. However, the fact that FRNA phages are unlikely to multiply in the environment and have the most similar morphological structure to human enteric viruses, both containing ssRNA genomes, makes them more appropriate indicators of human enteric viral contamination.

Historically, data on the incidence of phages in water has been inconsistent for a number of reasons including variables such as temperature, pH and rainfall, all of which have a significant impact on the survival and replication of the host bacteria and their phages (Pereira et al., 2015; Reyes and Jiang, 2010). Comparison of studies is also compromised by differences in sample preparation, recovery of phages from the sample and methodology including the host bacteria used for phage enumeration (Leclerc et al., 2000; Schaper and Jofre, 2000). The most traditional and useful method for enumeration of phages is the plaque assay resulting in a count of infective phages or plaque forming units (pfu). The selection of a permissive host is critical for successful assays. Phages vary in their host specificity; assaying a sample against different host strains will often result in different phage isolates and titres (Hyman and Abedon, 2010).

The development of international standard methods such as ISO 10705 for the detection and enumeration of FRNA phages has improved the situation for water quality testing (ISO, 1995). An adaptation of the ISO method for shellfish has been published by the UK Centre for Environment, Fisheries and Aquaculture Science (Cefas) (CEFAS, 2007). The host bacteria commonly used in standard methods are *E. coli* CN-13 or *E. coli* Famp K-12 ATCC 700891 (EPA, 2001a, b; FDA, 2015) and *Salmonella typhimurium* strain WG49 phage type 3 Nal^r (F′42 lac:Tn5) NCTC 12484 or ATCC 700730 (CEFAS, 2007; ISO, 1995). The *Salmonella* WG49 strain carries a plasmid encoding F-pili production enabling the detection of FRNA phages and removing potential interference from somatic phages. MS2 phage is the type species of the *Levivirus* genus. It is the positive control FRNA phage for the US Environmental Protection Authority (EPA) and ISO 10705-1 standard methods for water quality (EPA, 2001a, b; ISO, 1995), and the US Food and Drug Authority (FDA) and Cefas methods for enumeration of phages in bivalve shellfish (CEFAS, 2007; FDA, 2015). To date, the US is the only country that uses phage detection and enumeration as a regulatory tool in shellfish production. The Interstate Shellfish Sanitation

Conference (ISSC) in 2009 officially recognised the application of phages as indicators of viral contamination of bivalve shellfish from sewage (FDA, 2009).

The detection and genotyping of FRNA phages based on PCR, serotyping and F+ phage latex agglutination and typing has also been widely used as a tool for microbial source tracking (Long et al., 2005; Love and Sobsey, 2007; Mieszkin et al., 2013; Osawa et al., 1981). This enables determination as to whether the contamination source is predominantly animal or human. Data consistently, although not absolutely, indicates that the majority of genogroups I and IV FRNA phages are associated with animal sources, and genogroups II and III FRNA phages are associated with human or wastewater sources (Long et al., 2005). FRNA phage GA is a human specific strain of the *Levivirus* genogroup II and can be used for direct comparison between phage concentrations determined by quantitative reverse transcriptase-PCR (qRT-PCR) (total infectious and non-infectious phages) and plaque assay (infectious phages) (Flannery et al., 2013). This FRNA phage genogroup is the most resistant to water treatment processes and potentially a better and more conservative surrogate for waterborne viruses than the widely used MS2 phage (Boudaud et al., 2012).

6. Effectiveness of phages as indicators of enteric viruses

6.1. Early development

Havelaar and Hogeboom developed a method for the enumeration of FRNA phages in sewage and compared the methodology with detection in human and animal faeces with somatic phages and other bacterial indicators (Havelaar et al., 1986; Havelaar and Hogeboom, 1984). The authors noted that phage titres were unrelated to bacterial counts. Somatic phages, thermotolerant coliforms, faecal streptococci and sulphite-reducing clostridial spores were identified in all samples. FRNA phages were detected in relatively high numbers in faeces from pigs, broiler chickens, sheep and calves. These samples also yielded high numbers of somatic phages. FRNA phages were not detected or detected in very low numbers in faecal samples from dogs, cows, horses and humans. The FRNA phage serotyping results indicated that the phages from hospital wastewater were not of animal origin, although their relatively high numbers in sewage did not correlate with the low numbers detected in human faecal samples. All animal samples were from subgroup I or IV and hospital wastewater from subgroup II or III as determined by serology.

A further review by Havelaar et al. (1993) summarised data from studies investigating faecal indicator bacteria (thermotolerant coliforms and faecal streptococci), human enteric viruses (EV and reoviruses) and FRNA phages from various fresh water environments and treatment processes including river water, bank infiltration, coagulation, flocculation, filtration, secondary effluent, chlorination, UV irradiation and other surface waters (Havelaar et al., 1993). FRNA phages were found to be effective indicators of human enteric virus levels in the majority of environments and treatment processes investigated. The exceptions were raw and biologically treated sewage and some unpolluted environments. Concentrations of human enteric viruses in sewage are impacted by active virus carriers in the population excreting high levels of virus leading to sporadic and variable titres. The thermotolerant coliforms and faecal streptococci had more variable relationships with the human enteric viruses than the FRNA phages, possibly due to the contribution of bacteria from non-human faecal sources. Despite these factors the FRNA phages appeared to be adequate indicators for human enteric viruses in fresh water (Havelaar et al., 1993). The conclusion was that more data was needed to assess the suitability of FRNA phages as indicators of human enteric viruses due to the limited geographical coverage of samples and that only fresh waters were tested.

6.2. Bioaccumulation and depuration of enteric micro-organisms in shellfish

The bioaccumulation factors (mean ratio of the concentration of organism in shellfish compared to water) for faecal coliforms, *E. coli*, *Clostridium perfringens* and FRNA phages have been investigated in Eastern oysters (*Crassostrea virginica*) grown in estuarine waters in the Gulf Coast of the US (Burkhardt and Calci, 2000). The mean bioaccumulation factor observed over a year varied depending on the organism investigated and was 4.4, 3.8, 59.5 and 19 for faecal coliforms, *E. coli*, *C. perfringens* and FRNA phages, respectively. Unlike the other organisms investigated, the bioaccumulation of FRNA phages was inversely related to water temperature and a period of hyper-accumulation was observed from October to January when the mean bioaccumulation factor was 49.9, and significantly higher than the mean bioaccumulation factor of 2.9 observed during the rest of the year. The period of hyper-accumulation of FRNA phages corresponded with the period when the majority of oyster related NoV illnesses were reported in the community.

Depuration is the controlled cleansing of shellfish in clean seawater under conditions which facilitate the natural filtering activity resulting in the expulsion of contaminants from bivalves (FAO, 2008). It is commonly applied to reduce bacterial contamination in shellfish, however, it is less effective in reducing the viral load (FAO, 2008; Formiga-Cruz et al., 2003; Love et al., 2010). Monitoring pathogen levels during depuration has been dependent on *E. coli*, faecal coliform or total coliform counts which have been shown to be inadequate for the determination of risk associated with potential viral contamination. Various studies have indicated that depuration rapidly reduces *E. coli* levels in shellfish to those equivalent to category A areas according to the EU regulation: European Community (EC) Directive 91/492 (<230 *E. coli*/ 100 gm shellfish flesh) with category A, suitable for direct sale and category B, requiring depuration before sale. In contrast, enteric viruses and phages are removed more slowly and by different mechanisms (Humphrey and Martin, 1993; Muniain-Mujika et al., 2002). The elimination of viruses during depuration depends on the species of shellfish, depuration parameters, virus species and concentration of virus bound to shellfish digestive tissue (Nappier et al., 2008; Olalemi et al., 2016; Polo et al., 2014).

Doré and Lees (1995) monitored the differential rates of reduction in *E. coli* and FRNA phages in depurating Pacific oysters (*Crassostrea gigas*) and mussels (*Mytilus edulis*) following artificial contamination (Doré and Lees, 1995). The shellfish accumulated *E. coli* and FRNA phages rapidly, within 24 h, and reached an equilibrium with the majority of both detected in the digestive gland and intestine (*E. coli* 90.7% and FRNA phages 87.5%). *E. coli* levels were reduced during depuration by 90% in 6.5 h or less in contrast to the FRNA phages where 90% reduction ranged from 41.3 to 60.8 h depending on the time of exposure to sewage and species of shellfish. The data indicated that the FRNA phages were not sequestered into other tissues and were retained only in the digestive gland. Similarly, specific binding of NoV to oyster digestive tissue through an A-like carbohydrate structure, very similar to human histo-blood group A antigen, has been demonstrated (Le Guyader et al., 2006; Maalouf et al., 2010b).

6.3. Phage studies in shellfish

Table 1 summarises published studies covering the use of phages as enteric virus indicators in bivalve shellfish. A

Table 1
Investigations of bacteriophage occurrence with human enteric viruses in bivalve shellfish from surveys and contamination events.

Reference	Phage method	*E. coli* method	Human virus method	No. of samples and time frame of study	Source of samples	Contamination event
Lewis 1995	FRNA phages: Host: *S. typhimurium* WG49	NT[1]	BGM cell culture for Enteroviruses	Pooled sewage (n = 38), effluent (n = 17), shellfish (n = 38), sediment (n = 23), water (n = 8) over 22 months	NZ Treatment plants, receiving waters, environmental samples	NA[2]
Doré et al. 2000	FRNA phages Host: *S. typhimurium* WG49	MPN[3]	Nested RT-PCR for NoV	Oysters (n = 134 total) periodically over 2-years (1995–1997)	4 UK commercial production sites: Site 1: Category A Site 2: reclassified Category B Sites 3 and 4: Category B	NA
Croci et al., 2000	FRNA phages: Host: *E. coli* HS(pFamp)R Bacteroides phages: Host: B. fragilis HSP40 Somatic phages: Host: not specified	MPN	BGM cell culture for enteroviruses and HAV then RT-PCR	Mussels (n = 36) from 3 areas of the Adriatic Sea monthly for 1 yr	All 3 sites: Category B No specific contamination event or source	NA
Miossec et al., 2001	FRNA phages: Host: *S. typhimurium* WG49	Conductance	RT-PCR	Mussels (n = 47) and oysters (n = 48) over 37 months	2 French coastal areas Site 1: Category A (oysters) Site 2: highly contaminated non-commercial (mussels)	NA
Muniain-Mujika et al., 2003	FRNA phages Host: *S. typhimurium* WG49 Bacteroides phages: Host: *B. fragilis* RYC2056	MPN	EV and HAV by RT-PCR and nested PCR ADV by nested PCR	Mussels (n = 44) and oysters (n = 16) in 3 months in 1998 and 6 months in 1999	Spain: Site 1: Category A (oysters and mussels) Site 2: Category B (oysters and mussels) Site 3: highly contaminated unclassified (mussels)	NA
Formiga-Cruz et al., 2003	FRNA phages: Host: *S. typhimurium* WG49 Somatic phages: Host: *B. fragilis* RYC2056 Host: *E. coli* WG5	MPN	Nested PCR	Oysters and mussels (n = 475 total) from 4 countries over 18 months	Greece: 4 Category A (mussels) and 2 Category B (oysters) Spain: Category A (oysters and mussels), Category B (oysters and mussels), non-classified (oysters) Sweden: Category A, B and non-classified (mussels) UK: Category A (oyster), Category B (oyster and mussels), Category C (oysters and mussels), prohibited (oysters and mussel)	NA
Shieh et al., 2003	FRNA phages: Host: *E. coli* Famp	MPN	RT-PCR	Oysters (n = 18) over 18 months from Nov 1999 to Dec 2001.	US: contaminated site (downstream from effluent discharge point)	NA
Myrmel et al., 2004	FRNA phages: Host: *S. typhimurium* WG49 and *E. coli* HS(pFamp)R	NT	qRT-PCR for NoV, AV, CV	Mussels (n = 681) Oyster (n = 15) Sampled every 2–4 weeks over 3–36 months.	Norway: commercial production sites (Category A), non-commercial harvest sites, polluted sites.	NA
Greening and Lewis 2007	FRNA phages: Host: *S. typhimurium* WG49	MPN	ADV: Nested PCR 2 step RT-PCR then semi-nested PCR Real-time PCR for ADVEV and NoV	Oysters, pipi, cockles, and mussels (n = 360 total) from Jan 2004 to Feb 2006	28 NZ sites likely to be contaminated (harvesting sites and several downstream from a sewage outfall). A further 12 sites sampled occasionally over the period.	Sewage outfall
Lowther et al., 2008	FRNA phages: Host: *S. typhimurium* WG49	MPN	5′ fluorogenic nuclease assay (TaqMan) NoV GI and GII	Oysters (n = 237 total) from Jan 2004 to July 2006	2 UK commercial production sites: Category B	NA
Flannery et al., 2009	FRNA phages: Host: *S. typhimurium* WG49	MPN	5′ fluorogenic nuclease assay (TaqMan) NoV GII	Oysters (n = 167 total) from Aug 2005 to Oct 2007	Ireland: 17commercial sites: Category A and B 4 non-commercial sites: Category B	NA
Scholes et al., 2009	Semi-quantitative multiplex real-time RT-PCR	MPN	Real-time PCR for ADV and NoV	Oysters, pipi, cockles, and horse mussels (n = 72 total) monthly for 1 yr (2008)	7 NZ sites within 2 estuarine areas. Comprehensive sampling also after 2 adverse pollution events: sewage overfall and significant rainfall.	Sewage overflow and Rainfall event
Goblick et al., 2011	FRNA phages: Host *E. coli* Famp	MPN	RT-PCR for NoV GI and GII	Oysters (n = 32 total) during March 2008	4 US WWTP sentinel stations sampled after 6 weeks and then monthly for 7 months.	NA
Flannery et al., 2013	FRNA phages: Host: *S. typhimurium* WG49 and qRT-PCR for FRNA phage GA and *in-situ* probe hybridisation	MPN	qRT-PCR for NoV	Oysters (n = 25 total) weekly for 6 months from Jan 2011 to June 2011	Ireland: WWTP newly commissioned.	Combined sewer overflows
Hartard et al., 2016	FRNA phages: Host: *S. typhimurium* WG49 and qRT-PCR for FRNA phage	Direct impedance measurement	qRT-PCR for NoV	Oysters (n = 8) and mussels (n = 8) from Jan to April 2014 then Oct 2014 to Jan 2015 and Oysters (n = 28) from Jan to March 2016	France: harvest area, Category B and Harvest areas: France, Ireland and Portugal	NA

[1]NT not tested, [2]NA not applicable, [3]MPN most probable number.

conservative indicator should be detected in the presence of enteric viruses in a qualitative analysis and may or may not be present in the absence of the enteric viruses. In the UK, the application of FRNA phages as indicators of human enteric viruses such as NoV and HAV in shellfish was investigated over a two year period, at point of sale and at harvest (Doré et al., 2000). Market ready oysters from the UK were sourced from category A and category B commercial sites representing varying degrees of sewage pollution. In market ready oysters, no *E. coli*, NoV or FRNA phages were detected in samples taken from the category A site (FRNA phage 0/13 samples, NoV 0/8 samples), although FRNA phage and NoV were detected in some depurated samples harvested from polluted category B sites. In summer months FRNA phages were detected in less than 25% of category B samples and NoV was not detected. The percentage of category B samples collected in winter positive for FRNA phages ranged from 64% to 100% and NoV was detected in 62% of samples from one site and not detected in samples from two sites. The presence of FRNA phages was not definitive for the presence of human enteric viruses; it indicated the potential for viral contamination. The frequency and degree of FRNA phage detection showed significant seasonal trends with an increase in winter, was correlated with human enteric disease incidence and more accurately reflected the risk to consumers than *E. coli*. Importantly when no FRNA phages were detected, there were no virus detections and no shellfish related outbreaks, i.e. the absence of FRNA phages appeared to be a reliable indicator of the absence of human enteric viruses (Doré et al., 2000).

An 18-month survey conducted by Shieh et al. (2003) examined the prevalence of human enteric viruses in shellfish in the Gulf of Mexico (Shieh et al., 2003). Oysters were harvested (30 individual shellfish every 4 weeks) from a conditionally approved production area in open status, depurated, then relocated for 2 weeks to a coastal site 30 m downstream from the effluent discharge point of a municipal sewage treatment plant. The shellfish were then analysed for EV, NoV, FRNA phages, faecal coliforms and *E coli*. Human enteric viruses were detected in 14 of the 18 samples; EV only in 11% (2/18 oysters), NoV only in 27.8% (5/18 oysters) and both EV and NoV in 38.9% (7/18 oysters). FRNA phages were detected in all samples. The four oyster samples negative for enteric viruses had been collected when mean water temperatures were at their highest (22.8–30.4 °C). The FRNA phage bioaccumulation ratios and levels for two of these four samples were the lowest for the survey period. The absence of enteric viruses during the period of warm water correlates with the trend for outbreaks of gastrointestinal illness in the community in the winter months.

In a study by Flannery et al. (2009), 167 oyster samples were collected from 17 category A and B harvesting areas in Ireland between August 2005 and October 2007 (Flannery et al., 2009). The NoV levels correlated with FRNA phage levels, showing typical seasonal trends with higher concentrations in the northern hemisphere winter. No seasonal differences were found for *E. coli*. Of the samples positive for NoV, 96% (59/62 samples) also contained FRNA phages. The investigators also reported that 31% of compliant oysters harvested from category A Irish waters were positive for NoV. By reanalysing shellfish based on three categories determined using FRNA phage titres of <1000, 1000 to 10,000 and > 10,000 pfu/mL, it was shown that the frequency and level of NoV significantly increased with increasing levels of FRNA phages. The study confirmed that monitoring of *E. coli* in shellfish was unable to accurately predict the risk of enteric virus contamination. Samples compliant with the EU regulatory levels for *E. coli* in category A and B areas showed no significant difference in mean NoV levels. The authors concluded that FRNA phages were better indicators of NoV levels in shellfish than *E. coli*.

In the US, state shellfish authorities establish prohibitive closure zones associated with wastewater treatment plant (WWTP) discharges with the aim of minimising hazards associated with contamination of molluscan shellfish with human pathogens. The US FDA recommends that shellfish growing areas should not be placed within a minimum dilution of estuarine water to treated wastewater effluent of 1000:1 (FDA, 2015). The recommendation has been in place since 1995 and was based on current relevant scientific literature. More recently, the US FDA has evaluated data from various State Shellfish Control Authorities and industry collaborative studies from the period 2008–2015 (FDA, 2015). These studies included diverse meteorological, seasonal and hydrological conditions plus various types of wastewater treatment, disinfection and shellfish species. In total, 161 shellfish samples were analysed for NoV and FRNA phages. Of the 66 NoV positive samples, 94% (n = 62) were also positive for FRNA phages and 85% (n = 53) had levels >50 pfu/100 g. NoV was detected in the absence of FRNA phages in four samples although three were near the limit of detection (LOD) for NoV.

Goblick et al. (2011) undertook further studies to assess the efficacy of the 1000:1 dilution recommendation by evaluating the impact of wastewater effluent discharge from a municipal WWTP in Mobile Bay Alabama over a two year period (Goblick et al., 2011). The 1000:1 dilution of effluent was determined by hydrographic studies using rhodamine WT tracer dye. Microbiological impacts on molluscan shellfish were assessed by testing caged oysters at four sentinel stations along the predicted path of the effluent. The parameters assessed were levels of faecal coliforms, *E. coli*, FRNA phages and NoV GI and GII. The indicator microorganisms and viral pathogen levels correlated inversely with increased dilution of the effluent. NoV GII was detected 5.74 km from discharge where the dilution level was estimated to be close to 1000:1. The monthly survey results for FRNA phages were similar to NoV GII. For example, two of the three times FRNA phage was detected at the furthest sentinel station from the initial discharge NoV GII was also detected. Furthermore, the relative abundance of FRNA phages and NoV GII were comparable and consistent. Faecal coliform and *E. coli* levels were noted to be poor predictors of the risk of enteric virus accumulation in shellfish. In conclusion, the authors noted that the FRNA phages correlate well with the human viruses in shellfish. Notably NoV was not detected in the absence of FRNA phages.

A study by Flannery et al. (2013) used qRT-PCR to determine the concentrations of NoV and FRNA phage GA over a six month period in influent, secondary treated and UV treated effluent at a newly commissioned WWTP and oysters from a commercial harvest area 380 m from point of effluent discharge (Flannery et al., 2013). *E. coli* and FRNA phages were detected in all water and effluent samples with detection of NoV GI in 61% and NoV GII in 95% of influent waters. Concentrations of infectious (plaque assay) and total FRNA phage GA (qRT-PCR) were not significantly different in influent waters (5.26 $\log_{10}$ pfu/100 g and 5.11 $\log_{10}$ genome copies/100 g, respectively) indicating that most were likely to be infective phages. However, in secondary and UV treated effluent the total FRNA phage levels were greater than infective levels by more than 1 $\log_{10}$ concentration. The authors concluded that qRT-PCR significantly underestimated the reduction of infectious viruses during wastewater treatment and that the plaque assay may have provided a more accurate estimate of infectious virus reduction during treatment. In oysters, the levels of infectious FRNA phage and NoV GII were similar (3.14 $\log_{10}$ pfu100 g and 3.04 $\log_{10}$ genome copies/100 g respectively).

In a New Zealand study, the validity of using FRNA phages as indicators of the presence of EV was investigated in the marine environment, sewage wastes and shellfish tissue (Lewis, 1995). The phages and cultivable EV showed similar survival characteristics during the sewage treatment processes and infectivity losses.

Samples of water, sediments and shellfish collected from areas likely to be contaminated with faecal wastes often contained FRNA phages, yet EV were usually absent. EV were never detected in the absence of FRNA phages. The data indicated that the phages displayed similar environmental survival patterns to EV. Lewis concluded that FRNA phages fulfilled the indicator criteria of being present when the viral pathogen was also present, with higher numbers than the pathogen and persisting in the environment for similar or longer times than the pathogen. However, the low numbers of phages made detection difficult and the author was not confident that they were from the same source as the pathogen (Lewis, 1995).

A study by Miossec et al. (2001) sampled shellfish (n = 47 oysters and n = 48 mussels) over 37 months (1995–1998) in two coastal areas subject to different sewage contamination levels (Miossec et al., 2001). The study evaluated the sensitivity and specificity of *E. coli*, enteric viruses (EV, HAV, AstV, rotavirus and NoV) and FRNA phage indicators based on a screening procedure used in clinical experiments. The authors demonstrated the inadequacy of *E. coli* standards for protection of public health as it was the least sensitive and specific for identification of samples contaminated with pathogenic human enteric viruses. The ratio of the number of shellfish samples positive for FRNA phages in the presence of NoV or HAV to the number of shellfish samples positive for FRNA phages was 1:1, as phages were always present when NoV or HAV were present. FRNA phages were conservative indicators as the ratio of true negatives (negative phage detection in the absence of virus) to true negative plus false positives (phage detection in the absence of virus) was 0.37:1. The FRNA phages were more sensitive and better indicators for samples from the more contaminated site than from the less contaminated site. No indicator organism was highly correlated in samples from the less contaminated areas, although the FRNA phage was better than other indicators in that it was conservative when used to predict the risk related to consumption of shellfish.

A six month study undertaken by Flannery et al. (2013) included an investigation on the impact of combined sewer overflow (CSO) discharge on NoV GII, total and infectious FRNA phage GA concentrations in oysters (Flannery et al., 2013). CSO events are the discharge of untreated effluent due to periods of extreme or prolonged rainfall resulting in volumes of wastewater that exceed the capacity of holding tanks. These events have been associated with increased levels of human enteric viruses in shellfish and numerous outbreaks of gastroenteritis (Grodzki et al., 2012; Le Guyader et al., 2008; Maalouf et al., 2010a). The analysis of four CSO samples for FRNA phages confirmed that the level of infectious virus was greater than the level in treated wastewater effluent. One oyster sample was taken approximately 3 h before a CSO event. The sample was analysed for NoV GII by qRT-PCR and determined to be below the LOD. The infectious FRNA phage GA concentration using *in-situ* probe hybridisation was <LOD and the total FRNA phage GA concentration determined by qRT-PCR was 1130 genome copies/100 g. These were likely inactivated non-infectious virus particles. Twelve h post-CSO discharge, the concentrations of NoV GII in oysters had increased to 3150 genome copies/100 g and the concentrations of infectious FRNA phage GA increased to levels comparable with total FRNA phage GA. Oysters continued to be sampled for 72 h (n = 4) and were determined to be positive for NoV GII, total and infectious phage GA with no notable trend in concentrations evident over that period.

Hartard et al. (2016) investigated FRNA phage levels in shellfish and water to evaluate their potential as faecal and viral indicators and for microbial source tracking (Hartard et al., 2016). Shellfish were collected over a 12 month period from a French category B harvest area known to be impacted by human pollution and other European harvest areas over 3 months with lower expected faecal contamination. Samples were analysed for *E. coli*, infectious FRNA phages and the genomes of NoV GI, NoV GII and FRNA phages. The FRNA phage plaque assay was performed on extracted digestive tissue rather than whole shellfish. In the French category B harvest area, infectious FRNA phages were detected in all shellfish samples (2–566 pfu/g digestive tissue) with levels higher than surrounding waters indicating bioaccumulation. Greater concentrations were found during winter months. NoV genomes were detected in 87.5% of samples (3/4 oysters and 4/4 mussels) in the first winter period, but were not detected in the second winter period (n = 8). Genogroups I and II FRNA phages were detected in shellfish with genogroup II predominant in the first winter period (6/8 samples). This corresponded to epidemiological data when the gastroenteritis incidence rate was higher than the national epidemic threshold. It is difficult to compare this study to others as the methodology for infectious phages is more sensitive due to the use of digestive tissue only.

In the other European harvesting areas examined in the Hartard et al. (2016) study, a significant relationship was observed between the presence of NoV and genogroup II FRNA phage genomes with only NoV GII (12/28 samples) and genogroup II FRNA phages (11/28 samples) detected. The authors concluded that FRNA phages were useful as faecal pollution indicators in shellfish and recommended further investigations into the relationship between infectious genogroup II FRNA phages and infectious NoV in shellfish.

6.4. Qualified or non-supportive evidence

Formiga-Cruz et al. (2003) evaluated levels of *E. coli*, FRNA phages, somatic phages and *B. fragilis* strain RYC2056 specific phages in 475 shellfish samples over 18 months in growing areas with different faecal pollution levels in Greece, Spain, Sweden and the UK (Formiga-Cruz et al., 2003). FRNA phages demonstrated the seasonal trend typical of NoV with higher numbers recorded in growing areas during the northern hemisphere winter months, although the actual NoV levels were not investigated in the study. They were also shown to have the most significant relationship with the presence of human enteric viruses that was strongest for NoV and weakest for HAV, EV and ADV. However, most of the samples collected in category A or cleaner category B areas with low levels of *E. coli* that were positive for human enteric viruses were negative for FRNA phages. The study also measured physicochemical properties in the growing waters; specifically temperature, salinity, pH and dissolved oxygen. Fluctuations in these environmental parameters, notably in shallow waters, may have contributed to low phage recovery as previous studies have reported that viral stability and viability are influenced by temperature and UV radiation (Girones et al., 1989; Sinton et al., 2002). The FRNA phage levels were often below the level of sensitivity for the assay (30 pfu/100 g) in samples from southern European waters. The authors concluded that quantification of FRNA phages could be useful and complementary to *E. coli* levels and improve microbiological control of shellfish. However, in category A and clean category B areas, especially in southern Europe, it would be unlikely to predict the presence of human viruses.

Muniain-Mujika et al. (2003) collected 60 shellfish samples (n = 16 oysters and n = 42 mussels) from three sites in Spain with different levels of faecal contamination, specifically, category A and B production areas and one highly polluted unclassified site (Muniain-Mujika et al., 2003). The shellfish were analysed for *E. coli*, total coliforms, *C. perfringens*, somatic coliphages, F-RNA phages, *B. fragilis* RYC2056 phages, human ADV, EV and HAV. Human ADV were detected in 47% of samples (3/7 category A, 20/47 category B, 6/7 unclassified), EV in 19% of samples (10/47 category

B, 1/7 unclassified) and HAV in 24% of samples (10/47 category B, 4/7 unclassified). *B. fragilis* RYC2056 phages were detected in higher frequencies than the FRNA phages in the category B samples in contrast to other studies (Croci et al., 2000; Formiga-Cruz et al., 2003). This may have been due to the host strain used, that sample preparation included an elution step at pH10 potentially inactivating the FRNA phages or geographical variances. Human enteric viruses were detected in 5 samples (3 category A, 1 category B, 1 unclassified) where both FRNA and *B. fragilis* phages were below the level of detection (0.78 log pfu/g shellfish flesh) and 10 category B samples with FRNA phages below the level of detection. The data indicated that *B. fragilis* RYC2056 phages were suitable indicators of human enteric viruses in shellfish, but methodology issues may have impacted the results with respect to FRNA phages.

The relationship between the occurrence of enteric viruses (ADV, EV, NoV) and FRNA phages was investigated over a two year period in New Zealand shellfish harvested from 28 sites where sewage contamination was likely, specifically areas downstream from a sewage outfall (Greening and Lewis, 2007). Human enteric viruses were detected in 48.3% of shellfish from all but two sites, while FRNA phages were detected in 66.3% of samples. No significant numerical correlation was noted between the occurrence of FRNA phage and viruses in shellfish harvested from growing areas impacted by sporadic faecal contamination from a mix of sources. Furthermore, no correlation was observed between the presence of viruses and *E. coli*. However, in an area where shellfish were growing in close proximity to a sewage outfall there was a correlation between the occurrence of enteric viruses and FRNA phages in shellfish (Greening and Lewis, 2007).

Lowther et al. (2008) collected 237 oyster samples from two category B commercial production areas in the UK over 31 months between January 2004 and July 2006 and analysed them for NoV contamination (Lowther et al., 2008). FRNA phage levels were determined for 168 samples, establishing a distinct correlation between the presence of FRNA phages and NoV detection. High levels of NoV were associated with high FRNA phage counts, specifically, in 70% of shellfish samples with >1000 pfu/100 g shellfish flesh (32/46 samples). These NoV levels were up to 40-fold the levels in samples with low FRNA phage counts (<100 pfu/100 g shellfish flesh). NoV was detected in 67% of samples with FRNA phage levels between 100 and 999, and 31% of samples (21/68 samples) with phage levels <100 pfu/100 g shellfish flesh, including some below the limit of detection (<30 pfu/100 g shellfish flesh). Despite this, the authors concluded that FRNA phage detection and enumeration could be valuable as an indicator of potential virus contamination improving risk management of shellfish production areas.

The 2007–2008 study by Scholes et al. (2009) in New Zealand included two suspected adverse pollution events that were investigated using increased sampling frequencies for *E. coli*, faecal coliforms, enterococci, FRNA phages and NoV GI and GII (Scholes et al., 2009). These were a sewage overflow from council reticulated wastewater system (>50 m^3) and a significant rainfall event increasing water flow to more than double the median. High levels of NoV were detected after the sewage pollution event in shellfish located 50 m from the source and remained detectable for up to 3 months. The authors concluded that indicator bacteria were not reliable indicators of viral contamination, as no distinct relationship was observed between the faecal bacterial indicators and viruses. Whilst the authors state there was good correlation between the presence of NoV and human-associated FRNA phages in shellfish after the sewage pollution events, they found that on 5 of 19 occasions, NoV was present but FRNA phages were not. The relationship between FRNA phage presence and viral presence after the rainfall events was poor.

Viral contamination of Norwegian shellfish was surveyed by sampling mussels (n = 681) and oysters (n = 15) from commercial production (category A), non-commercial harvest and sewage polluted sites in 2–4 week periods over 3–36 months (June 2000–June 2003) (Myrmel et al., 2004). The prevalence of NoV and FRNA phages were higher in winter typical of the northern hemisphere seasonal trend. NoV was detected in 6.8% (46/681 samples) of mussels and no oyster samples. FRNA phages were detected in 23.8% (162/681 samples) of mussels and 20% (3/15 samples) of oysters, but in only 43% (20/46 samples) of NoV positive mussel samples. Notably, 19 of the 20 NoV and FRNA phage positive mussels were sampled in winter, when the decrease in water temperature and biological activity favours viral stability and bioaccumulation of viruses.

Croci et al. (2000) determined the incidence of EV, HAV, *E. coli* and somatic and FRNA phages in mussels from the Adriatic Sea (Croci et al., 2000). The study was designed to evaluate the incidence of enteric viruses and investigate the potential of somatic and FRNA phages as indicators of viral contamination. Most samples were below the detection limits for *E. coli* of 230 MPN/100 g. HAV was detected in 13/36 samples and EV in 5/36 samples. Somatic phage determination was negative (<1 pfu/g) for all samples. FRNA phages were detected in 3/36 samples. The authors concluded that neither *E. coli* nor phages were appropriate indicators of viral contamination.

7. Conclusion

Risk management programs globally rely heavily on microbial indicators of faecal pollution (Lee and Reese, 2014), which are not satisfactory indicators of human enteric viral risk (Croci et al., 2000; Doré et al., 2000; Flannery et al., 2009; Formiga-Cruz et al., 2003; Goblick et al., 2011; Greening and Lewis, 2007; Love et al., 2010; Miossec et al., 2001; Muniain-Mujika et al., 2002; Scholes et al., 2009). Consequently, consumption of contaminated bivalve shellfish is a significant health risk which has led to numerous outbreaks of gastrointestinal illness. The current most sensitive and reliable method of detection and enumeration of enteric viruses is qRT-PCR which has limitations for monitoring and screening of shellfish. These include the expense of testing, the technical expertise required to undertake testing and the time lag between sampling and reporting. The most important limitation is the lack of relationship between detected viral genomes and infectious viral risk.

The Codex Alimentarius Commission has produced a guidance document on the risk management of viruses in food which includes an annex dedicated to shellfish (FAO/WHO, 2012). This annex does not contain any information regarding the potential use of phages for viral risk management in shellfish, reflecting the emerging nature of this research, and the current conflicting international opinion. To date, the US has been the only country employing phages in a regulatory sense for risk management in shellfish. The Interstate Shellfish Sanitation Conference (ISSC) in 2009 officially recognised the utility of FRNA phages as indicators of viral contamination, for example NoV and HAV, following sewage contamination events (FDA, 2011). The conference ratified that the normal obligatory three week closure following a sewage spill could be avoided if samples collected at least 7 days post potential contamination with raw untreated sewage discharge contained FRNA phage levels below the critical limit of 50 pfu/100 g shellfish, or below background FRNA phage levels (FDA, 2015). Recent changes to the Australian Shellfish Quality Assurance Operations Manual allow shellfish regulators to adopt a similar approach (ASQAAC, 2016).

A number of studies have been conducted on the use of phages as indicators of human enteric viruses with varying results. A

confounding factor in this debate is that phages are most often detected and enumerated using the plaque assay, resulting in an infectious virus titre. Thus, discussion of data from the detection and enumeration of enteric viruses based on molecular methods (genome copies) and phages based on infectivity (pfu) must be viewed with caution and the caveat that they are not directly comparable.

There is some supporting evidence from published data justifying the use FRNA phage indicators for contaminated sites (Doré et al., 2000; Formiga-Cruz et al., 2003; Greening and Lewis, 2007; Hartard et al., 2016; Lewis, 1995; Lowther et al., 2008; Miossec et al., 2001; Shieh et al., 2003) and post adverse events (Flannery et al., 2013; Scholes et al., 2009), as occurs in the US. Only one study was not supportive of phages as able to indicate contaminated sites (Myrmel et al., 2004).

The use of phages as a routine indicator of viral risk under normal growing area conditions is more complicated. Several studies support this concept (Doré et al., 2000; Flannery et al., 2009, 2013; Goblick et al., 2011; Hartard et al., 2016; Lowther et al., 2008; Muniain-Mujika et al., 2003), however, others do not (Croci et al., 2000; Formiga-Cruz et al., 2003; Greening and Lewis, 2007; Miossec et al., 2001; Myrmel et al., 2004; Scholes et al., 2009). Whilst the indicators *E. coli* and coliforms will continue to provide valuable information related to faecal pollution of water and shellfish, shellfish compliant with these standards can harbour infectious human enteric viruses. The addition of FRNA phage data may be useful as a more accurate indicator of viral risk associated with shellfish under certain circumstances, thus complementing the use of other indicators such as *E. coli* in a conservative approach to public health. Further studies detailing FRNA phage data following adverse events and shellfish related human enteric virus outbreaks would be valuable.

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