The application of phage-based faecal pollution markers to predict the concentration of adenoviruses in mussels (*Mytilus edulis*) and their overlying waters

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Running head: Bacteriophages as surrogates of viral pathogens

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Abstract

Aim: This study set out to determine whether phage-based indicators may provide a 'low-tech' alternative to existing approaches that might help maintain the microbial safety of shellfish and their overlying waters.

Methods and Results: Mussels and their overlying waters were collected biweekly from an estuary in southeast England over a two-year period (May 2013 - April 2015) (n= 48). Levels of bacterial indicators were determined using membrane filtration and most probable number methods and those of bacteriophages were determined by direct plaque assay. The detection of adenovirus was determined using real-time polymerase chain reaction. The results revealed that somatic coliphages demonstrated the most significant correlations with AdV F and G in mussels (rho=0.55) and overlying waters (rho=0.66), followed by GB124 phages (rho=0.43) while *E. coli* showed no correlation with AdV F and G in mussels.

Conclusion: This study demonstrates that the use of somatic coliphages and GB124 phages may provide a better indication of the risk of adenovirus contamination of mussels and their overlying waters than existing bacterial indicators.

Significance and impact of study: Phage-based detection may be particularly advantageous in low-resource settings where viral infectious disease presents a significant burden to human health.

Keywords: Adenoviruses, somatic coliphages, microbial indicators, human health, shellfish, surrogates, policy

Introduction

Adenoviruses are medium-sized (60-90 nm), non-enveloped icosahedral viruses containing linear, non-segmented double-stranded DNA, belonging to the family *Adenoviridae*. They have a worldwide distribution and they are ubiquitous in environments contaminated by human faeces or sewage. And as a result, they are most commonly transmitted through the faecal-oral route, i.e., through ingestion of faecally-contaminated food or water (Pond et al. 2005). They represent the largest non-enveloped viruses and are usually stable to chemical or physical agents and adverse pH conditions, allowing for prolonged survival in the natural environment, particularly water bodies (Thurston-Enriquez et al. 2003). Adenoviruses have also been proposed as viral faecal indicators because of their stability and prevalence throughout the year (Girones et al. 2013; Rodriguez-Manzano 2014).

One gram of faeces may contain ten million viruses, one million bacteria, 1,000 parasite cysts and 100 parasite eggs (UNICEF 2008) and faecally-polluted waters to which humans may come into contact and faecally-polluted shellfish that are consumed therefore present a hazard (potential risk) to human health. Many pathogens of faecal origin (and human health significance) may contaminate the environment and food chain unless barriers to their environmental transmission (such as water and wastewater treatment) are sufficiently robust. Several practices, such as sanitation, hygiene and surveillance activities to monitor the microbiological quality of water and food by environmental and public health protection agencies, support efforts to control the environmental transmission of human enteric diseases. Several terms have been used to describe the microbial tools used in these monitoring exercises, including ‘microbial indicator’, ‘index organism’, ‘faecal indicator’ and ‘surrogate organism’ (Ashbolt et al. 2001; Sinclair et al. 2012). *E. coli*, faecal coliforms and intestinal enterococci represent the classic bacterial indicators of the presence of faecal pollution (faecal indicator bacteria) in the environment.

There are, however, limitations to the use of traditional faecal indicator bacteria to predict the presence of enteric pathogens and they appear to have limited or no predictive value for many important human viral pathogens, such as enterovirus, norovirus, hepatitis A virus (Jofre 1992, Muniain-Mujika et al. 2003) and parasite cysts. Critically, Dore et al. (2003) and Griffin et al. (2003) suggested that shellfish may meet *E. coli* standards for human consumption but still contain infectious doses of human enteric viruses that can cause gastroenteritis.

Human enteric viruses are found in the gastrointestinal tract and the group that causes gastroenteritis infects the epithelial cells (DiCaprio et al. 2013). Large numbers of these viruses are excreted in the faeces and urine of infected humans (Melnick and Gerba 1980). Indiscriminate discharge of sewage into surface waters is the most conspicuous channel through which these viruses contaminate the environment (Pouillot et al. 2015), where their low infectious dose may present a human health hazard (Fong and Lipp 2005). Their commonest route of transmission is faecal-oral route, principally through the consumption of contaminated drinking water and
shellfish (Lipp and Rose 1997). Symptoms of viral gastroenteritis include diarrhoea, vomiting, abdominal pains and nausea (Bosch 2010). Some infected individuals fail to show any symptoms but nevertheless constantly shed the virus in their faeces and urine and the existence of these asymptomatic carriers can complicate epidemiological studies of human disease transmission (Pina et al. 1998).

Advances in culture-independent molecular-based approaches to monitoring the sanitary quality of shellfish, through amplification and quantification of viral nucleic acids by real-time polymerase chain reaction (qPCR) or reverse transcription polymerase chain reaction (RT-qPCR), have aided the rapid detection of pathogenic human viruses (Campos and Lees 2014). These molecular methods demonstrate high levels of sensitivity and specificity (Pina et al. 1998) but remain relatively expensive option, thus, less likely to be taken up in low-resource settings. The methods are also unable to distinguish readily between infective and non-infective viral particles (Bosch 2010).

Bacteriophages, such as somatic coliphages, F-RNA coliphages and phages infecting Bacteroides fragilis have been suggested as potentially useful indicators of viral contamination (IAWPRC 1991; Pina et al. 1998; Hernroth et al. 2002; Ebdon et al. 2012; Jofre et al. 2014, Pouillot et al. 2015). This is because they have several characteristics in common with enteric viruses. For instance, they are found in high concentrations in sewage, they are non-latent and their cell sizes, shapes, morphology, physiochemistry and surface chemistry are all similar to those of enteric viruses (IAWPRC 1991). They also exhibit similar patterns of persistence and do not replicate in the environment except where there is a suitable bacterial host at sufficient concentration. Phages are easier and less expensive to enumerate in the laboratory and they pose little or no risk to the health of higher organisms (Tufenkji and Emelko 2011). Phages of host-specific strains of enteric bacteria may also represent useful targets for microbial source tracking (MST) and have successfully been used to particularly identify human sources of faecal pollution (Ebdon et al. 2007).

Fifty seven immunologically distinct human adenovirus serotypes have been identified and have been divided into seven groups (A–G), based on their physical, chemical and biological properties (Jones et al. 2007). Some reported cases of human adenoviral infection caused by various serotypes include conjunctivitis, pharyngitis, pneumonia, acute and chronic appendicitis, bronchiolitis, haemorrhagic cystitis, nephritis, acute respiratory diseases and keratitis. Only human adenovirus F serotypes 40 and 41, and adenovirus G serotypes 52, however, have been strongly associated with gastroenteritis (Pond 2005). Furthermore, adenovirus F serotypes 40 and 41 have been associated with acute gastroenteritis and responsible for up to 20 % of the cases of diarrheal disease globally in children (Dey et al. 2011). Infections are usually mild or self-limiting but can be fatal in immunocompromised individuals (Jiang 2006).

The aim of this study was to examine the distribution pattern of faecal indicator bacteria, bacteriophages and AdV F and G in M. edulis and their overlying waters harvested from a site that could best be described as a ‘Class B’ harvesting area according to the EU shellfish hygiene classification criteria (Regulation (EC) No. 854/2004) (CEU 2004). This may be considered an essential first ‘hazard evaluation’ step in the chain of effective human health risk management practices of shellfish harvesting areas. The objective of this study was to examine the applicability of phage-based pathogen surrogates to predict the concentration of AdV F and G in M. edulis and their overlying waters. This objective was based on the widely-accepted principle that phages are (and will for some time be) easier and cheaper to detect and enumerate than viral pathogens (Hernroth et al. 2002; Ebdon et al. 2012; Jofre et al. 2014) and the proposition that using phages to predict the risk of AdV F and G in M. edulis and their overlying waters could be of immense benefit to human health protection.

Materials and methods

Sampling site and collection of samples

The sampling site was situated in the village of Piddinghoe, on the estuary of the River Ouse, which is the second largest river in the county of East Sussex in southern England, draining an area of 396 km² to its tidal limit. At mid-tide, the river has a depth of approximately three to four metres at its deepest point, about eight metres at high tide and less than one metre at low tide with a fluctuating salinity of between 0.6 and 16 ppt. This estuarine site is
affected by fluctuating faecal input from biologically treated municipal wastewater (e.g., at Scaynes Hill, about 24.32 km upstream from the sampling site) and by diffuse pollution from animal rearing (mainly cattle and sheep).

Sampling activities were carried out every two weeks over a consecutive period of twenty-four months. On each sampling occasion, a grab sample of approximately one litre of overlying water was collected in a pre-sterilised plastic bottle in accordance with standard protocol (Anon. 2012). Mussels with an approximate length (six to ten centimetre) and weight (35 to 41 g) were collected by hand-picking into transparent polyethylene bags. Approximately 1800 mussels were sampled in total (i.e., 30 – 40 mussels were analysed on each occasion depending on the size of individual mussel). Mussels and overlying water samples were transported to the laboratory in the dark at approximately 4 °C and processed immediately (within less than one hour).

**Enumeration of faecal indicator bacteria**

The concentrations of *E. coli*, faecal coliforms and intestinal enterococci in overlying waters were determined using the membrane filtration method (ISO 9308-1, ISO 7899-2) (Anon. 2000b; 2000c). The membrane filters were placed on freshly prepared selective media (m-FC, m-Ent, MLSA and TBX). Agar plates were incubated at 37 °C for 48 hours (m-Ent), 37 °C for 24 hours (MLSA) and 44 °C for 24 hours (m-FC, TBX), and colonies were counted, calculated and expressed as colony-forming units (CFU) 100 ml⁻¹ of water. Similarly, the concentrations of *E. coli*, faecal coliforms and intestinal enterococci in *M. edulis* were determined using the most probable number method (MPN) (ISO 16649-3) (CEFAS 2011). Briefly, ten to fifteen *M. edulis* were rinsed in cold water, then cut open to remove their flesh and intravalvular fluids, 0.1 % peptone solution (one ratio two) was added and homogenised using a stomacher (400 Lab System) and shaker (Stuart Scientific Ltd). A master mix (M) was prepared by adding 30 ml shellfish homogenate to 70 ml 0.1 % peptone solution and a dilution (N) was then made from the master mix. Five test tubes containing ten ml each of double-strength Mineral Modified Glutamate Broth (dsMMGB) and ten test tubes containing ten ml each of single-strength Mineral Modified Glutamate Broth (ssMMGB) were prepared. Ten millilitres of M (equivalent to one gram of tissue per tube and 10⁹ dilutions) was inoculated into the five test tubes containing ten ml dsMMGB. One millilitre of M (equivalent to 0.1 g of tissue per tube and 10⁷ dilutions) were inoculated into the five test tubes containing ten ml ssMMGB. One millilitre of N (equivalent to 0.01 g of tissue per tube and 10⁵ dilutions) was inoculated into the remaining five test tubes containing ten ml ssMMGB. Inoculated test tubes were incubated at 37 °C for 24 hours and were then examined for acid production. Test tubes with yellow colouration throughout the medium were sub-cultured onto freshly prepared selective media (m-FC, m-Ent and TBX). Agar plates were incubated at 37 °C for 24 hours (m-Ent) and 44 °C for 24 hours (m-FC, TBX), and colonies were counted, and expressed as most probable number (MPN) 100 g⁻¹ of shellfish flesh and intravalvular fluid.

**Enumeration of bacteriophages**

The concentrations of somatic coliphages, F-RNA coliphages and bacteriophages infecting *B. fragilis* GB124 in *M. edulis* and their overlying waters were determined by direct plaque assay using a standardised double-agar method (ISO 10705) (Anon. 2000a; 2001a; 2001b). Briefly, approximately 50 ml of overlying waters were filtered through a 0.22 µm cellulose nitrate filter (Millipore). Similarly, on each occasion, ten to fifteen *M. edulis* were rinsed in cold distilled water and then cut open to remove their flesh and intravalvular fluids. Their digestive glands were separated from the flesh, and chopped finely to ensure proper mixing. 0.25 mol L⁻¹ glycine (pH 9.5) was then added to two grams of the digestive gland (one ratio five). The mixture was placed on a shaker (Stuart Scientific Ltd) for approximately 20 mins, and subsequently centrifuged (Heraeus megafuge 16R) at 2000 g, 4 °C for 15 mins. The supernatant was filtered through a 0.22 µm cellulose nitrate filter (Millipore). The required media (MSA, TYGA, BPRMA), semi-solid agar (ssMSA, ssTYGA, ssBPRMA) and broth (MSB, TYGB, BPRMB) were prepared according to the manufacturer’s specifications. Bacterial host strains (*E. coli* WG5, *Salmonella typhimurium* WG49, and *B. fragilis* GB124) were grown to ensure confluent lawns for phage detection. One millilitre of sample (filtered water or shellfish glands) was added to one millilitre of bacterial host strain and 2.5 ml of semi-solid agar in a 10 ml vial (Sterilin). The mixture was vortexed and poured onto a solid agar plate, swirled gently for even distribution and allowed to solidify. All inoculated plates were incubated appropriately and plaques (clear zones of lysis) were counted and expressed as plaque-forming units (PFU) 100 ml⁻¹ of water or 100 g⁻¹ of shellfish digestive gland.
Concentration of viral particles and nucleic acids extraction

The basic steps adopted for quantification of viral pathogens in *M. edulis* and their overlying waters were sample concentration, nucleic acid extraction and molecular detection, as described by Harwood et al. (2013). AdV F and G were concentrated from 300 ml of overlying river water filtered through 0.45 µm membrane cellulose nitrate filters. Magnesium chloride (five mol L⁻¹ MgCl₂) was prepared by dissolving 475 g in 1000 ml of distilled water, then filter-sterilised through 0.22 µm membrane cellulose nitrate filters. Prepared MgCl₂ was added to the water samples in the filter unit before filtration to increase viral recovery by facilitating and enhancing virus attachment to the filters (Mendez et al. 2004). In brief, six ml of five mol L⁻¹ MgCl₂ was added aseptically to 300 ml of sample to achieve a final concentration of 0.1 mol L⁻¹ MgCl₂ before filtration. The filters were stored at -80 °C until nucleic acid extraction. Again, ten to fifteen mussels to be analysed were cleaned with sterile running water and placed in a stainless steel shucking tray. Using a shucking knife and wearing a chain-mail shucking glove, *M. edulis* were removed from their shells. The digestive glands were separated from the flesh and intravalvular fluid.

The digestive glands have been shown to bio-accumulate viruses more effectively than other parts of the organism, as demonstrated in numerous studies (Jothikumar et al. 2005; Le Guyader et al. 2009; Pinto et al. 2009; Westrell et al. 2010; Iizuka et al. 2010; Baker et al. 2011; Bosch et al. 2011; Lowther et al. 2012; Trajano Gomes Da Silva 2013). Digestive glands were therefore chopped finely to expose the content and stored in centrifuge tubes at -80 °C until nucleic acid extraction.

The frozen filters were equilibrated at room temperature and the surface was scraped using a fresh scalpel blade (Fisher Scientific) into a 1.5 ml microcentrifuge tube. Viral nucleic acids were extracted using the QIAamp MinElute Virus Spin Kit (Qiagen GmbH) according to the manufacturer’s instruction. The nucleic acid extracts were stored at -80°C until quantification. Similarly, the frozen glands were equilibrated at room temperature and 200 µl of the finely chopped glands was placed in a 1.5 ml microcentrifuge tube. Viral nucleic acids were extracted using the QIAamp MinElute Virus Spin Kit (Qiagen GmbH) according to the manufacturer’s instruction. The nucleic acid extracts were stored at -80°C until quantification.

Detection of AdV F and G by qPCR

AdV F and G were enumerated by quantitative real-time polymerase chain reaction (qPCR) (Ogorzaly et al. 2009) on QIAGEN Rotor-Gene® Q thermocycler using PrimerDesign™ genesig AdV F and G Hexon gene Advanced kit (Primerdesign UK) following the manufacturer’s protocol. The components of the kit were reconstituted in RNase/DNase-free water, and then vortexed to ensure complete resuspension. AdV F and G had a sequence accession number of KC632648.1, anchor nucleotide position of 790 and context sequence length of 190. Thermocycling steps were as follows: enzyme activation (95 °C for two minutes), 50 cycles of denaturation (95 °C for ten seconds) and data collection (60 °C for 60 seconds). Data were analysed using Rotor-Gene 2.1.0.9 software with a threshold fluorescence value of 1.000. Standards were prepared, serially diluted and quantified to make standard curves following the manufacturer’s protocol. The highest concentration of AdV F and G standard was 2 × 10⁵ copies µl⁻¹. Standard curve was run in triplicate and the ‘pooled’ standard curve was then used to relate quantification cycles to copy numbers and quantity of AdV F and G in samples. Again, all data were analysed with the comprehensive Rotor-Gene® Q software, which enables quantification and enhances data security.

Statistical analysis

Data were transformed to log₁₀, then examined using general descriptive statistics and checked for normality using the skewness and kurtosis statistic, Shapiro–Wilk normality test and corresponding normal probability plots. Further analyses were undertaken using Statistical Package for Social Sciences (SPSS) Version 20.0, and all data were subjected to the Spearman’s rank non-parametric two-tailed correlation analysis (with test of significance at 0.01 and 0.05) to determine whether there were positive correlations between the concentration of AdV F and G and those of bacterial and viral indicators.
Results

Detection of faecal indicator bacteria

The concentration of E. coli in M. edulis ranged from 2.43 to 4.27 log_{10} MPN 100 g⁻¹ of shellfish flesh and intravalvular fluid and in overlying waters the concentration ranged from 1.55 to 4.00 log_{10} CFU 100 ml⁻¹. The concentration of faecal coliforms in M. edulis ranged from 2.52 to 4.30 log_{10} MPN 100 g⁻¹ of shellfish flesh and intravalvular fluid and in overlying waters the concentration ranged from 1.56 to 4.16 log_{10} CFU 100 ml⁻¹. Similarly, concentration of intestinal enterococci in M. edulis ranged from 2.23 to 3.97 log_{10} MPN 100 g⁻¹ of shellfish flesh and intravalvular fluid and in overlying waters ranged from 0.99 to 3.50 log_{10} CFU 100 ml⁻¹ (Table 1).

Detection of bacteriophages

The concentration of somatic coliphages in M. edulis ranged from 3.43 to 5.36 log_{10} PFU 100 g⁻¹ of digestive gland (mean = 4.60 ± 0.52 log_{10} PFU 100 g⁻¹) and 2.00 to 4.02 log_{10} PFU 100 ml⁻¹ in overlying waters (mean = 3.01 ± 0.54 log_{10} PFU 100 ml⁻¹). The concentration of F-RNA coliphages in M. edulis ranged from 0 to 3.82 log_{10} PFU 100 g⁻¹ of digestive gland (mean = 1.84 ± 1.62 log_{10} PFU 100 g⁻¹) and 0 to 2.30 log_{10} PFU 100 ml⁻¹ in overlying waters (mean = 0.60 ± 0.95 log_{10} PFU 100 ml⁻¹). The concentration of bacteriophages infecting Bacteroides fragilis (GB124) in M. edulis ranged from 0 to 5.29 log_{10} PFU 100 g⁻¹ of digestive gland (mean = 1.79 ± 1.64 log_{10} PFU 100 g⁻¹) and 0 to 3.35 log_{10} PFU 100 ml⁻¹ in overlying waters (mean = 0.67 ± 1.13 log_{10} PFU 100 ml⁻¹). Fourteen shellfish samples (31 %) and seven overlying water samples (16 %) presented positive for both F-RNA coliphages and bacteriophages infecting Bacteroides fragilis (GB124). However, all the samples (100 %) of shellfish and overlying water were positive for somatic coliphages (Table 1). Statistically significant elevated levels of somatic coliphages, F-RNA coliphages and bacteriophages infecting Bacteroides fragilis (GB124) in M. edulis compared with their overlying waters.

Detection of AdV F and G

Concentration of AdV F and G in M. edulis ranged from 0 to 2.94 log_{10} genome copies 100 g⁻¹ of digestive gland (mean = 0.43 ± 0.85 log_{10} genome copies 100 g⁻¹) and in overlying waters ranged from 0 to 1.34 log_{10} genome copies 100 ml⁻¹ (mean = 0.22 ± 0.35 log_{10} genome copies 100 ml⁻¹). The limit of detection for AdV F and G was 10 detectable virus genome copies 100 ml⁻¹ and 100 g⁻¹ for overlying waters and shellfish samples, respectively. Nine shellfish samples (27 %) and eleven overlying water samples (24 %) presented positive for AdV F and G (Table 1). There were statistically significant elevated levels of AdV F and G in M. edulis compared with levels recorded in their overlying waters.

The relationship between bacteriophages and AdV F and G

The relationships between the concentrations of bacteriophages and those of AdV F and G in M. edulis and overlying waters were analysed using a two-tailed Spearman’s rank correlation at two levels of significance (P < 0.01 and P < 0.05) representing 99 % and 95 % confidence interval, respectively (Table 2). Levels of AdV F and G in overlying waters showed a positive correlation with those in M. edulis (rho = 0.58, P < 0.01). Similarly, levels of AdV F and G in overlying waters showed positive correlations with levels of somatic coliphages in both overlying waters (rho = 0.66, P < 0.01) and M. edulis (rho = 0.48, P < 0.05). Importantly, levels of AdV F and G in M. edulis showed a positive correlation with levels of somatic coliphages in M. edulis (rho = 0.55, P < 0.01). Levels of somatic coliphages in overlying waters showed a positive correlation with those in M. edulis (rho = 0.64, P < 0.01). Levels of AdV F and G in overlying waters also showed positive correlations with levels of bacteriophages infecting B. fragilis (GB124) in both overlying waters (rho = 0.43, P < 0.05) and M. edulis (rho = 0.49, P < 0.05).

Levels of somatic coliphages in overlying waters, furthermore, showed positive correlations with levels of bacteriophages infecting B. fragilis GB124 in M. edulis (rho = 0.41, P < 0.05). Levels of somatic coliphages in M. edulis showed a positive correlation with levels of bacteriophages infecting B. fragilis GB124 in M. edulis (rho = 0.53, P < 0.01). Levels of F-RNA coliphages in overlying waters showed a positive correlation with those in M. edulis (rho = 0.52, P < 0.01). Levels of bacteriophages infecting B. fragilis GB124 in overlying waters showed a positive correlation with levels of AdV F and G in M. edulis (rho = 0.53, P < 0.01).
Discussion

This study investigated the distribution pattern of faecal indicator bacteria, bacteriophages and AdV F and G in M. edulis and their overlying waters, and examined whether the enumeration of bacteriophages may offer a way to predict, manage and prevent gastroenteritis caused by adenovirus contamination of mussels consumed by humans. The Shapiro-Wilk’s normality test (P > 0.05) (Shapiro and Wilk 1965; Razali and Wah 2011) and a visual inspection of histograms, normal Q-Q plots and box plots showed that levels of E. coli, faecal coliforms, intestinal enterococci and somatic coliphages in both overlying waters and M. edulis were approximately normally distributed, whereas the levels of F-RNA coliphages, GB124 phages and AdV F and G appear not to have been normally distributed (Cramer, 1998; Cramer and Howitt 2004; Doane and Seward 2011) (Table 3). Evidently, the normality tests demonstrated a variable pattern of occurrence and distribution of faecal indicator bacteria, bacteriophages and AdV F and G in M. edulis and their overlying waters during the study. The difference in distribution patterns were clearly influenced by the non-detection of F-RNA coliphages, GB124 phages and AdV F and G on several sampling occasions. It is worthy of note that the statistical dataset on the varying levels of faecal indicator bacteria and bacteriophages (against which the levels AdV F and G were compared) were in broad agreement with the findings of Wyer et al. (2012). These authors suggested that statistical models based on adequate goodness of fit may be used to predict the probability of the presence of human adenovirus from routine surveillance of European waters for faecal coliforms.

In this study, the Spearman’s rank correlation co-efficient demonstrated the bacterial indicators: E. coli (rho = -0.11), faecal coliforms (rho = 0.07), and intestinal enterococci (rho = 0.03) to have far less predictive value for the presence of AdV F and G in M. edulis (Table 4) compared with somatic coliphages (Table 2). Although, correlation analysis revealed that E. coli (rho = 0.57, P < 0.01), faecal coliforms (rho = 0.53, P < 0.01) and intestinal enterococci (rho = 0.45, P < 0.05) presented positive correlations with AdV F and G in overlying waters, these were lower than those observed using somatic coliphages (rho = 0.66, P < 0.01). It is interesting to note that the findings from this study contrasted with those observed by Ogorzaly et al. (2009) who examined the relationship between F-specific RNA phage genogroups, faecal pollution indicators and human adenoviruses in river water (that was prone to faecal pollution from anthropogenic activities that included animal rearing activities involving approximately 112,000 cows, 7,500 sheep and about 4,100 pigs) in the northeast of France, and demonstrated positive correlations between the concentration of human adenovirus and E. coli (rho = 0.513), enterococci (0.616), somatic coliphages (rho = 0.593) and F-RNA phage genogroup II (rho = 0.493) (Ogorzaly et al. 2009). This observed difference may be the result of differences in the source of the faecal pollution and the distance from the source. The difference may also be as a result of geographical variation, stability of the indicator organisms, physiochemical, hydrological and meteorological factors etc. (Formiga-Cruz et al. 2003).

AdV F and G were detected on several sampling occasions in overlying waters and M. edulis during the study period. GB124 phages were not detected on ten sampling occasions in overlying waters and on two sampling occasions in M. edulis. F-RNA coliphages were not detected on twelve sampling occasions in overlying waters and on six sampling occasions in M. edulis, whereas somatic coliphages were detected in all cases. In the samples that tested positive for AdV F and G, all investigated phages were also consistently detected at varying concentrations. However, these phages were also detected in some samples that tested negative for AdV F and G (Figure 1). These findings are consistent with other studies (Haramoto et al. 2007; Ogorzaly et al. 2009) that have demonstrated the wide spatial distribution of human adenovirus genome in faecally-impacted aquatic environments.

Phages were also detected in overlying waters and in M. edulis that presented negative results for AdV F and G (i.e., were below the detection limit of 1 PFU ml⁻¹ or g⁻¹). Total concentrations of 32.01 log₁₀PFU 100 ml⁻¹ somatic coliphages, 2.00 log₁₀PFU 100 ml⁻¹ GB124 phages, and 4.30 log₁₀PFU 100 ml⁻¹ F-RNA coliphages were detected in overlying waters presenting negative results for AdV F and G. Again, 52.05 log₁₀PFU 100 g⁻¹ somatic coliphages, 12.00 log₁₀PFU 100 g⁻¹ GB124 phages, and 17.68 log₁₀PFU 100 g⁻¹ F-RNA coliphages were detected in M. edulis presenting negative results for AdV F and G. Again, a number of these samples may reasonably be described as ‘false negatives’, because, of all the AdV F and G negative samples, GB124 phages were detected on two sampling occasions in overlying waters and on eight sampling occasions in M. edulis, F-RNA coliphages
were detected on four sampling occasions in overlying waters and on 12 sampling occasions in *M. edulis*, whereas somatic coliphages were detected in all cases. This shows the volume of samples which are positive for phages but negative for AdV F and G. In general, somatic coliphages were detected at significantly higher levels than other groups of phages (i.e., GB124 phages and F-RNA coliphages) in overlying waters and *M. edulis* presenting both positive and negative results for AdV F and G. Also, bacteriophages (especially somatic coliphages) and faecal indicator bacteria were detected at significantly higher levels in AdV F and G positive samples compared with samples that were negative for AdV F and G (Figure 1). These findings further demonstrate the abundance of somatic coliphages in faecally-polluted environments (Grabow 2004).

This work is applicable to other types of shellfish species, though different shellfish species may demonstrate varying abilities to bioaccumulate microbial indicators or pathogens. This may be the result of differences in the physiology of the shellfish or the pathogen, the rate of metabolism in the shellfish, the duration of exposure of the shellfish to the pathogen or its source, the exposure dose and other factors, such as salinity and temperature which all serve to influence filtration rates within the shellfish (Olalemi et al. 2016).

The current hygiene classifications for shellfisheries, which are based on levels of faecal indicator bacteria, do not appear to offer an accurate indication of risk of viral contamination. There is a clear need to target alternative indicators to maintain and improve the microbial safety of shellfish that are harvested for human consumption. In this study, the enumeration of bacteriophages by a simple phage-lysis method was demonstrated to provide a better indication of the risk of adenovirus contamination in *M. edulis* and their overlying waters than is provided by the faecal indicator bacteria prescribed in EU Directives. Some studies have demonstrated the ability of bacteriophages infecting *Bacteroides fragilis* GB124 to predict the presence of norovirus in mussels (Trajan da Silva 2013), and mussels and oysters (Olalemi 2015). Despite the fact that enumeration of somatic coliphages better predicted the risk of viral pathogens than GB124 phages, this study demonstrates for the first time the ability of the human-specific *Bacteroides fragilis* GB124 phages to predict the presence of human AdV F and G in *M. edulis*. These findings support the use of bacteriophages as alternative faecal indicators and effective surrogates of AdV F and G in *M. edulis* and their overlying waters. This is consistent with the findings of other recent studies that have demonstrated the usefulness of bacteriophages as effective surrogates of enteric viral contamination. Incorporating this relatively simple tool into shellfish safety management and planning, especially as a component of routine monitoring of shellfish and their overlying waters would provide improved human health protection.

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Conflict of interest

No conflict of interest declared.

References:


**Table 1: Mean concentration of AdV F and G, bacteriophages and faecal indicator bacteria in *M. edulis* and overlying waters**

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th><em>M. edulis</em></th>
<th>Overlying waters</th>
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<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range (% +)</td>
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<tr>
<td>Adenovirus F and G</td>
<td>0.43 ± 0.85</td>
<td>0.00-2.94 (27%)</td>
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<td><strong>Somatic coliphages</strong></td>
<td><strong>4.60 ± 0.52</strong></td>
<td><strong>3.43-5.36 (100%)</strong></td>
</tr>
<tr>
<td>F-RNA coliphages</td>
<td>1.84 ± 1.62</td>
<td>0.00-3.82 (31%)</td>
</tr>
<tr>
<td>GB124 phages</td>
<td>1.79 ± 1.64</td>
<td>0.00-5.29 (31%)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>3.37 ± 0.51</td>
<td>2.43-4.27 (100%)</td>
</tr>
<tr>
<td>Faecal coliforms</td>
<td>3.60 ± 0.50</td>
<td>2.52-4.30 (100%)</td>
</tr>
<tr>
<td>Intestinal enterococci</td>
<td>3.10 ± 0.47</td>
<td>2.23-3.97 (100%)</td>
</tr>
</tbody>
</table>

**Key:** Enteric viruses mean Log_{10} genome copies 100 ml^{-1} or 100 g^{-1} digestive gland ± standard deviation; Phages mean log_{10} PFU 100 ml^{-1} or 100 g^{-1} digestive gland ± standard deviation; Faecal bacteria mean log_{10} MPN 100g^{-1} shellfish flesh and intravalvular fluid or CFU 100 ml^{-1} of overlying waters ± standard deviation; Range (Minimum-Maximum); (%) – Percentage of occurrence. Figure in **bold** – Most abundant parameter in both *M. edulis* and overlying waters.
Table 3: Skewness and Kurtosis, significance values for the Shapiro-Wilk normality tests based on log_{10} transformed concentration values in *M. edulis* and their overlying waters.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Overlying waters</th>
<th>M. edulis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Skewness</td>
<td>Kurtosis</td>
</tr>
<tr>
<td></td>
<td>(SE=0.472)</td>
<td>(SE=0.918)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.146</td>
<td>-1.001</td>
</tr>
<tr>
<td>Faecal coliforms</td>
<td>-0.238</td>
<td>-1.073</td>
</tr>
<tr>
<td>Intestinal enterococci</td>
<td>-0.521</td>
<td>-0.859</td>
</tr>
<tr>
<td>Somatic coliphages</td>
<td>-0.242</td>
<td>-0.834</td>
</tr>
<tr>
<td>F-RNA coliphages</td>
<td>1.013</td>
<td>-1.015</td>
</tr>
<tr>
<td>GB124 phages</td>
<td>1.393</td>
<td>0.630</td>
</tr>
<tr>
<td>Adenovirus F and G</td>
<td>2.005</td>
<td>3.860</td>
</tr>
</tbody>
</table>

Key: SE = Standard error
Table 2: Significant Spearman’s rank correlation between bacteriophages and AdV F and G in *M. edulis* and overlying waters (n=48)

<table>
<thead>
<tr>
<th></th>
<th>Water AdV</th>
<th>Water SomC</th>
<th>Water F-RNA</th>
<th>Water GB124</th>
<th>Mussel AdV</th>
<th>Mussel SomC</th>
<th>Mussel F-RNA</th>
<th>Mussel GB124</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water AdV</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water SomC</td>
<td>0.66**</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water F-RNA</td>
<td>0.17</td>
<td>0.19</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water GB124</td>
<td>0.43*</td>
<td>0.25</td>
<td>0.18</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mussel AdV</td>
<td>0.58**</td>
<td>0.40</td>
<td>-0.04</td>
<td>0.53**</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mussel SomC</td>
<td>0.48*</td>
<td>0.64**</td>
<td>0.20</td>
<td>0.32</td>
<td>0.55**</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mussel F-RNA</td>
<td>0.11</td>
<td>0.03</td>
<td>0.52**</td>
<td>0.27</td>
<td>0.05</td>
<td>0.09</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Mussel GB124</td>
<td>0.49*</td>
<td>0.41*</td>
<td>-0.18</td>
<td>0.36</td>
<td>0.34</td>
<td>0.53**</td>
<td>-0.04</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Key: SomC – Somatic coliphages; F-RNA – F-RNA coliphages; GB124 – GB124 phages; AdV – Adenovirus F and G; ** – Correlation is significant at the 0.01 level (2-tailed); * – Correlation is significant at the 0.05 level (2-tailed)

Table 4: Significant Spearman’s rank correlation between faecal indicator bacteria and AdV F and G in *M. edulis* and overlying waters (n=48)

<table>
<thead>
<tr>
<th></th>
<th>Water AdV</th>
<th>Water E. coli</th>
<th>Water FC</th>
<th>Water Ent.</th>
<th>Mussel AdV</th>
<th>Mussel E. coli</th>
<th>Mussel FC</th>
<th>Mussel Ent.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water AdV</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water E. coli</td>
<td>0.57**</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water FC</td>
<td>0.53**</td>
<td>0.97**</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water Ent.</td>
<td>0.45*</td>
<td>0.71**</td>
<td>0.73**</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mussel AdV</td>
<td>0.58**</td>
<td>0.10</td>
<td>0.07</td>
<td>0.12</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mussel E. coli</td>
<td>0.33</td>
<td>0.75**</td>
<td>0.72**</td>
<td>0.55**</td>
<td>-0.11</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mussel FC</td>
<td>0.48**</td>
<td>0.84**</td>
<td>0.84**</td>
<td>0.65**</td>
<td>0.07</td>
<td>0.90**</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Mussel Ent.</td>
<td>0.16</td>
<td>0.38</td>
<td>0.38</td>
<td>0.39</td>
<td>0.03</td>
<td>0.60**</td>
<td>0.59**</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Key: FC – Faecal coliforms; Ent. – Intestinal enterococci; AdV – AdV F and G; ** – Correlation is significant at the 0.01 level (2-tailed); * – Correlation is significant at the 0.05 level (2-tailed)