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## **Monitoring viral contamination in shellfish growing areas**

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### **Abstract:**

Human and animal fecal wastes and urine contain a large number of different viruses that can enter the environment through the discharge of waste materials from infected individuals. Despite the high diversity of viruses that are introduced into the environment by human fecal pollution, only a few have been recognized to cause disease in association with consumption of contaminated shellfish. Viruses are present in shellfish in very low numbers. Nevertheless, they are present in sufficient quantities to pose a health risk as presented above. This low level of contamination has made it necessary to develop highly sensitive viral extraction methods to ensure virus recovery from shellfish tissues. The most common route for accidental contamination is after heavy rainfall, leading to overflow and release of untreated sewage into the aquatic environment. To limit shellfish contamination the most desirable and effective option is to reduce the viral input.

**Keywords:** human enteric viruses, shellfish, sewage, persistence, flux

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Shellfish have been identified as a vector for human enteric pathogens for more than 150 years. Shellfish filter large volumes of water during their feeding, and in the process they concentrate small particles containing micro-algae and microorganisms. The practice of consuming either raw or undercooked shellfish can lead to transmission of disease caused by human pathogens present in the shellfish. During the 1800s, outbreaks of typhoid fever and cholera were associated with shellfish consumption (Richards, 1985). Contamination of shellfish-growing waters with human sewage was recognized as a contributing cause of the outbreaks, leading to the development of bacteriologic criteria to assess the impact of sewage on shellfish and shellfish-growing waters.

Most countries have endorsed sanitary controls on live bivalve shellfish. In the European Union, these are covered by Council Directive 91/492/EEC (Anonymous, 1991) and in the United States, by interstate trading agreements set out in the Federal Drug Administration National Shellfish Sanitation Program Manual of Operations (Anonymous, 1993). These regulations cover similar ground on the requirements for harvesting area classification, depuration, relaying, analytical methods, and provisions for suspension of harvesting from classified areas following a pollution or public health emergency. The legislation also requires that third country imports into the EU and US have to be produced under the same standard as domestic products. Exporting nations therefore have developed programs for compliance with the regulations of their target export markets.

A major weakness of these controls is the use of traditional bacterial indicators of fecal contamination, such as the fecal coliforms or *E. coli*, to assess contamination and hence implement the appropriate control measures. Fecal indicators are either measured in the shellfish themselves (EU requirement) or in the shellfish growing waters (US FDA requirement). Levels of *E. coli* are used to categorise harvesting areas and prescribe levels of treatment required before they can be sold to consumers. These controls led to a significant decrease in the number of shellfish-associated outbreaks of bacterial infection, but a new problem emerged. Outbreaks of nonbacterial gastroenteritis and infectious hepatitis were recognized to be associated described in association with shellfish consumption (Richards, 1987). Several reports described a lack of correlation between bacterial indicator microorganisms and viruses, and pathogenic viruses may be detected in shellfish from areas classified as suitable for commercial exploitation using fecal coliform criteria (Abad et al., 1997; Bosch et al., 2001; Butt et al. 2004; Le Guyader et al., 2006, 2008). A number of examples of trans-national outbreaks have recently been reported following trade between EU Member States (Le Guyader et al. 2006), and importation of shellfish from third countries into the EU and the US (Bosch et al., 2001; Butt et al., 2004). In addition, the practice of depuration, a process by which shellfish “purify” themselves of enteric bacteria by filtering clean waters, failed to eliminate the risk of viral mediated disease (Richards et al. 1988). Given the failure of the current arrangements to fully protect public health there is a clear need to develop better approaches to controlling this problem.

European shellfish trade totals 460 M€ per year, and increases by approximately 7 % each year. The European production represents more than a third of the worldwide shellfish production (i.e., in 1991, 180 000 tons of live weight: 72 % of farmed bivalves, 28 % wild - Eurostat data) and 8 500 companies currently employ around 23 000 workers. This activity is one of the major sources of employment in coastal areas (Ireland, France, Spain, The Netherlands). The costs of outbreaks of shellfish-associated viral disease have not been clearly defined, but they are likely to be substantial. In the USA, foodborne diseases are a major cause of morbidity and hospitalization, with about 325000 hospitalizations and 5000 deaths per year (Butt et al., 2004). Among the 76 million estimated cases of foodborne disease, 10-19% of those for which a vehicle of transmission is identified involve seafood; half of these are caused by viruses, and half of the illnesses are associated with shellfish consumption (Mead et al., 1999; Butt et al., 2004). In countries with higher seafood consumption, or where seafood is traditionally eaten raw, a larger percentage of foodborne illnesses are due to seafood consumption. For example, in Japan as much as 70% of foodborne illness is associated with seafood consumption (Butt et al., 2004).

## 1. Source of pollution

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### 1.1 Human enteric viruses.

Human and animal fecal wastes and urine contain a large number of different viruses that can enter the environment through the discharge of waste materials from infected individuals. These enteric viruses cause a wide spectrum of illnesses in man including hepatitis, gastroenteritis, meningitis, fever, rash, and conjunctivitis. A brief description of the principal viruses that have been characterized either in outbreaks or in field studies is given below and their different characteristics are described Table 1.

#### *Hepatitis A virus (HAV)*

Infectious hepatitis, caused by the hepatitis A virus, is one of the most serious illnesses transmitted by shellfish. The hepatitis A virus belongs to the genus *Hepatovirus* of the family *Picornaviridae*, and is very stable in the environment, remaining viable for up to several weeks in water or on fomites (Abad *et al.*, 1994; Arnal *et al.*, 1998; Hollinger and Emerson 2007). Hepatitis A virus infection has a long incubation period and is generally asymptomatic or associated with a mild illness in young children, while in older children and adults the illness is characterized by jaundice in more than 70% of individuals (CDC, 2006). There is only a single serotype, and an effective vaccine is available for prevention of infection (CDC, 2006).

#### Noroviruses

Noroviruses are the most common infection currently associated with shellfish consumption. *Norovirus* is a genus in the family *Caliciviridae*, and the genus is divided into 5 genogroups (Zheng *et al.*, 2006). Genogroups I, II and IV contain human strains, and the genogroups are further subdivided into genotypes based upon analyses of the amino acid sequence of the major capsid protein, VP1. Norovirus infection causes gastroenteritis characterized by the symptoms of vomiting and diarrhoea (Atmar and Estes, 2006). The prevalence of vomiting along with the short incubation period (1-2 days) and short clinical illness (1-3 days) has been used epidemiologically to identify probable outbreaks of norovirus-associated gastroenteritis (Kaplan *et al.*, 1982; Turcios *et al.* 2006). The virus is stable in the environment, and the infectious dose is estimated to be less than 20 virions (Teunis *et al.* 2008).

Noroviruses are the major cause of epidemic non bacterial gastroenteritis worldwide and have been identified as the cause of 73% to more than 95% of outbreaks (Atmar and Estes, 2006). Mead and colleagues estimated that there are 23 million norovirus infections per year in the United States, and these viruses constitute 60% of the illness burden caused by known enteric pathogens (Mead *et al.* 1999). Although some studies provide a good indication of the substantial illness burden that results from NoV infection, the true extent of disease may still not be fully known (Patel *et al.* 2008).

#### *Rotavirus*

Rotaviruses are the main etiological agent of viral gastroenteritis in infants and young children. They constitute a genus in the *Reoviridae* family (Estes and Kapikian, 2007). In developing countries the burden of rotavirus disease in children under 5 years of age has been estimated to be over 125 million cases annually, of which 18 million are severe cases (nearly half a million deaths) (Oh *et al.*, 2003; Parashar *et al.*, 1998). In the developed world, it remains an important cause of morbidity and of hospitalization in young children and they are also increasingly recognized as a cause of infectious diarrhea in adults as well (Anderson and Weber, 2004).

#### *Astrovirus.*

Astroviruses classified in genus *Mamastrovirus* (human and animal strains) within the family *Astroviridae* (Mendez and Arias, 2007). In most species astrovirus are found in association

with gastroenteritis, although other manifestations have been described in avian species (Mendez and Arias, 2007).

#### *Enterovirus.*

Human enteroviruses belong to the genus *Enterovirus* in the *Picornaviridae* family. Poliovirus is a species within the Enterovirus genus and its three serotypes each can cause a devastating neurological disease for which, despite vaccination campaigns, the goal of global eradication has not yet been completed. Other enterovirus species and serotypes cause a variety of other clinical syndromes, including respiratory infections, haemorrhagic conjunctivitis, and myocarditis (Pallansch and Roos, 2007).

#### *Aichi virus.*

Aichi virus belongs to the *Kobuvirus* genus in the *Picornaviridae* family. Aichi virus was identified in several other outbreaks in Japan as a cause of gastroenteritis associated with shellfish consumption (Yamashita et al., 2000) and was recently recognized in an oyster-related outbreak in Europe in 2006 (Le Guyader et al. online first).

#### *Adenovirus.*

Human adenoviruses belong to the genus *Mastadenovirus* in the *Adenoviridae* family (Wold and Horwitz, 2007). There are 6 species of human adenoviruses, and members of species F (formerly called subgroup F), consist of two serotypes, Ad40 and Ad41 causing diarrhoea and also referred to as enteric adenoviruses.

#### *Hepatitis E virus*

Hepatitis E virus, a member of the *Hepeviridae* family. It is the primary cause of enterically transmitted non-A non-B hepatitis in tropical and subtropical developing countries, and it has an associated mortality rate of up to 20% in pregnant women (Lu et al. 2006).

### 1.2 Animal viruses, potential zoonotic viruses.

A number of enteric viruses inducing gastroenteritis in humans have also been identified in animals. The predominant animals involved are porcine and bovine species. These observations raise the possibility that zoonotic transmission may occur. Such transmission has been best demonstrated to occur among rotaviruses. Bovine-human and porcine-human group A rotavirus reassortants have been identified in India, Italy, Slovenia, and Brazil, and a porcine group C rotavirus was identified in a child in Brazil (Estes and Kapikian 2007; Martella et al. 2008; Gabbay et al. 2008; Steyer et al. 2008).

There is also the potential for zoonotic transmission of noroviruses. Several porcine genogroup II norovirus strains have been characterized, and based upon the sequences of their major capsid protein (VP1) they have been classified in genotypes that are distinct from those of human strains (Zheng et al. 2006; Wang et al. 2005). Bovine strains are even less related to human strains and have been classified in a separate genogroup (genogroup III) (Oliver et al. 2003). However, the ability of human strains to replicate in pigs and cattle (Cheetham et al. 2006; Souza et al. 2008) has been demonstrated experimentally, and a recent study from Canada reported the presence of human-like GII.4 norovirus strains in pig feces and retail beef (Mattison et al. 2007). To date, no human infections with animal norovirus strains has been reported, but the simultaneous detection of human and animal enteric calciviruses in oysters samples collected from the markets suggests the potential for such infections to occur if humans can be infected with animal strains (Costantini et al., 2006; Symes et al., 2007). The significance of such an infection is the potential for the emergence of new strains through recombination events, as such events appear to be common among human strains (Bull et al. 2007).

Enteroviruses are also important pathogens for cattle and swine and high concentrations of these viruses may be detected in surface waters (Fong and Lipp, 2005). As for norovirus, shellfish contamination with animal and human enterovirus strains was demonstrated in oysters samples collected in an area impacted both with human and bovine sewage (Dubois et al., 2004). The stringency of the host specificity among human and bovine strains suggest that they can be good indicators for identifying human and non-human sources of fecal contamination of natural waters (Fong and Lipp, 2005).

Hepatitis E virus is a potential emerging pathogen, and evidence of its global spread as a cause of disease in man is increasing. Clear demonstration of sequence homology between strains detected among swine or other domestic animals have now been made in several countries (Rutjes et al. 2007, Renou et al. 2007, Ward et al. 2008, Kaci et al. 2008). However, transmission seems to occur primarily through direct contact with infected animal or food consumption (e.g., pig liver) (Renou et al. 2007, Lewis et al. 2008). Shellfish consumption has been reported as a risk factor for hepatitis E virus infection, but additional studies are needed to establish this link (Cacopardo *et al.*, 1997; Koizumi *et al.*, 2004).

### 1.3 Examples of outbreaks with a special emphasis on the source of virus contamination.

Despite the high diversity of viruses that are introduced into the environment by human fecal pollution, only a few have been recognized to cause disease in association with consumption of contaminated shellfish. Potential explanations, for this observation, include a lack of susceptibility of the persons consuming the shellfish to these viruses (*i.e.*, pre-existing immunity), a requirement for exposure to higher doses than are present in the shellfish to establish infection, and a lack of recognition of disease either through under-reporting or the unavailability of sensitive diagnostic assays.

The instigation of regulations to specify acceptable levels of bacterial enteric pathogens in shellfish tissues or in shellfish growing waters in Europe (European regulation, 91/492/EC) and the United States (National Shellfish Sanitation Program) and improvements in sewage waste treatment procedures were followed by the virtual elimination of shellfish-associated outbreaks of typhoid fever and cholera in the United States (Richards, 1985; Rippey, 1994). However, as shellfish-associated bacterial infection declined, outbreaks of nonbacterial gastroenteritis and infectious hepatitis were described in association with shellfish consumption (Butt et al., 2004). In many instances, the shellfish and shellfish-growing waters met regulatory criteria for fecal bacterial levels, suggesting an accidental contamination event rather than exposure to a continuous sewage discharge.

The most common route for accidental contamination is after heavy rainfall, leading to overflow and release of untreated sewage into the aquatic environment. As mentioned above, untreated sewage is likely to be heavily contaminated by enteric viruses. Frequently the source of accidental events for shellfish contamination cannot be traced, but a number of reports have been able to elucidate the cause of human fecal pollution. For example, several clusters of gastroenteritis occurred in six states in the USA and were linked to oyster consumption. The oysters implicated in the outbreaks were all traced back to a single harvest area, and the epidemiological investigation showed that the outbreak resulted from disposal of human diarrheal stool from a single ill individual directly into the waters over the shellfish bed (Kohn et al., 1995). Another example was a large oyster-associated gastroenteritis outbreak that affected approximately 2000 persons during the summer of 1978 in Australia (Murphy et al., 1979). This outbreak was linked to sewage contamination of the oyster harvesting area near Sydney following a heavy rainfall. Runoff from heavy spring rains were also suspected to be responsible for 103 clusters of norovirus gastroenteritis involving more than 1000 persons after clam or oyster consumption in New York State in 1982 (Morse et al., 1986). In the south of France, heavy rainfall and sewage treatment plant

failure were twice implicated as the cause of large gastroenteritis outbreaks due to consumption of oysters harvested from a single lagoon (Le Guyader et al., 2006a; 2008).

The long incubation period of hepatitis A complicates linkage of this agent to particular food exposure incidents. However, linkage is still possible during large incidents. In 1988 in Shanghai, China, almost 300,000 hepatitis A cases were traced to the consumption of clams harvested from a sewage-polluted area (Halliday et al., 1991). A sizeable hepatitis A outbreak in the USA in 1973 was linked to Louisiana oysters. The harvesting areas were flooded by the Mississippi River, and there was evidence of sewage contamination based upon elevated fecal coliform levels that led to closure of the oyster beds. Subsequently, the oyster beds were reopened to harvesting, but apparently the hepatitis A virus was retained in shellfish for at least 6 weeks following the contamination event. At the time of harvesting, oysters were fully compliant with the US sanitation program standard but still contaminated with the virus (Mackowiak et al., 1976). Many other hepatitis A outbreaks linked to bivalve shellfish have been reported but the initiating fecal contamination event has been generally difficult to identify due to the protracted incubation period for this disease (Bosch et al. 2001).

In summary, where a cause is ascribed, most contamination accidents are linked to failures or bypassing of treatment processes, often due to heavy rainfall (figure 1). The problem is that heavy rain causes the storage capacity of the sewage treatment plant to be exceeded. In combined sewer and rainfall systems this leads to storm spills. Such discharges result in the release of untreated effluent heavily contaminated with microorganisms. This may be particularly true during the 'first flush'. Other causes are flooding of harvest areas with contaminated river water and disposal of faeces from infected individuals on boats. It is interesting to note that although the efficiency of sewage treatment processes at fully removing viruses from effluents can be questioned, such effluents are not usually associated with bivalve mollusc disease incidents. However, because most enteric viruses retain their viability in the environment, they can persist for a much longer time in the marine environment than bacterial indicators – up to weeks or months (Wait et al., 2001; Lipp et al., 2002; Griffin et al., 2003). In addition, viral particles have been noted to persist for months in shellfish tissues, either via ionic binding or specific attachment (Burkhardt and Calci, 2000; Loisy et al. 2005a, Le Guyader et al. 2006b).

## 2. Methods

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### 2.1 Rapid review.

Viruses are present in shellfish in very low numbers. Nevertheless, they are present in sufficient quantities to pose a health risk as presented above. This low level of contamination has made it necessary to develop highly sensitive viral extraction methods to ensure virus recovery from shellfish tissues. The observation that viruses are concentrated in digestive diverticulum tissues led to the development of a method that represented a major step in the improvement of extraction methodologies (Metcalf *et al.*, 1980; Atmar et al., 1995). This observation was subsequently confirmed by detection of hepatitis A virus (HAV) (Romalde et al., 1994) as well as through the tissue-specific quantification of infectious enteric adenoviruses and rotaviruses in mussels previously contaminated by bioaccumulation of such viruses and similarly of Norwalk virus in oysters and clams (Abad et al., 1997; Schwab et al., 1998). Analysis of digestive tissues provides several advantages, including increased sensitivity, decreased processing time and decreased interference with RT-PCR by inhibitory substances (Atmar et al., 1995). Focusing the analysis of shellfish on the digestive tissues enhances assay performance by eliminating tissues (i.e. adductor muscle) that are rich in inhibitors (Atmar et al. 1995). The digestive tissues represent about one tenth of the total animal weight for oysters and mussels. With the exception of small species, such as clams or

cockles, in which dissection may be technically difficult, most of recent methods are based on dissected tissues and thus will be discussed here.

Extraction of enteric viruses from shellfish is based on several steps: virus elution from shellfish tissues, recovery of viral particles, and then virus concentration. The weight analyzed generally ranges from 1.5 to 2 g of digestive tissues. Some recent methods propose larger weights for the first step but thereafter analyze only a fraction of the extracts (Boxman et al., 2006). Viruses are eluted from shellfish digestive tissues using various buffers (i.e. chloroform-butanol or glycine) before being concentrated either by polyethylene glycol precipitation or ultracentrifugation (Atmar et al., 1995; Nishida et al., 2003; Myrmel et al. 2004; Milne et al. 2007). Direct lysis of virus particles has also been used, including methods utilizing proteinase K or Trizol to destroy shellfish tissues or Zirconia beads and a denaturing buffer for virus and/or nucleic acid elution (Lodder-Verschoor et al., 2005; Jothikumar et al., 2005; Kittigul et al. 2008; Umesha et al. 2008; Lowther et al. 2008).

In addition to the in-house protocols that have been used for nucleic acids extraction and purification (le Guyader and Atmar, 2007), a number of commercial kits can also be successfully applied to accomplish this task. Advantages of the commercial kits used for nucleic acid purification include their reliability, reproducibility and ease of use. Most of these kits are based on guanidium lysis followed by capture of nucleic acids on columns, beads or silica (Nishida et al. 2003; Lodder-Verschoor et al. 2005; Costafreda et al., 2006; de Roda-Husmann et al., 2007; Kingsley 2007; Milne et al., 2007; Fukuda et al., 2008; Nenonen et al. 2008; Umesha et al., 2008).

One of the goals of extraction methods is to remove inhibitors of the RT and PCR reactions sufficiently to allow detection of viral nucleic acids. Polysaccharides present in shellfish tissue are at least one substance that can inhibit the PCR reaction (Atmar et al., 1993). Elimination of inhibitors is difficult to evaluate and depending on the time of the year and shellfish life, different compounds may be present (Di Girolamo et al. 1977; Burkhardt and Calci, 2000). Internal amplification control standards are used to detect the presence of significant sample inhibition, and the amount and frequency of sample inhibition has varied depending upon the shellfish tissue being analyzed (Atmar et al., 1995; Schwab et al., 1998; Le Guyader et al., 2000). Recent advances in food virology re-enforce the need of harmonization of methods as well as addressing quality assurance and quality control (Pinto and Bosch, 2008).

The addition of an external virus to a shellfish sample has been proposed as a control to evaluate the extraction efficiency of molecular virus detection methods (Costafreda et al., 2006; Nishida et al., 2007; Lowther et al., 2008). An ideal candidate would have the following properties: (1) it would be an encapsidated RNA virus with properties similar to the enteric viruses contaminating shellfish, (2) it would normally not be present in field samples (thus RNA phages may be problematic), and (3) it would be non-pathogenic. Based on these considerations Costafreda et al. (2006), proposed to use a mengovirus strain MC<sub>0</sub> as a control for extraction efficiency. Mengovirus, a *Picornaviridae* family member, was initially proposed as a control in validation studies of HAV removal in blood products manufacturing by several agencies such as the European Agency for the Evaluation of Medicinal products or the American Food and Drug Administration (Pinto and Bosch, 2008). Advantages of mengovirus are that it is unlikely to naturally contaminate shellfish, it is non-pathogenic for humans and it can be grown in cell culture. The use of a single extraction control for different enteric viruses that may be detected in shellfish or other type of food is also considered to be important for method standardization (European working group CEN/Tag4) and for comparisons between different laboratories.

Since the most important shellfish-borne viral pathogens (enteric hepatitis viruses A and E and noroviruses) are either non-culturable or grow only poorly in cell culture, RT-PCR and real-time RT-PCR have become the methods of choice for their detection. In addition to the problems posed by the presence of inhibitory substances in samples, there are other

difficulties encountered when molecular analyses are performed for the detection of viruses in shellfish samples. These include low virus concentrations in the sample and genomic diversity of the contaminating viruses. The extraction-concentration procedure is not virus-specific, allowing the nucleic acid of several viruses to be extracted simultaneously. RT-PCR must be performed under stringent conditions and confirmed by hybridization. A number of reviews on RT-PCR methods are now available that address issues related to these methods, including assay specificity and sensitivity (Wyn-Jones, 2007; Le Guyader and Atmar, 2007).

Real-time PCR assays allow the combination of RT, PCR and confirmatory hybridization assays in a single well, and these assays are now being used to detect enteric viruses in shellfish (Nishida et al., 2003; Loisy et al., 2005b; Jothikumar et al., 2005; Costafreda et al., 2006; Lowther et al. 2008). This technology significantly shortens the time needed for virus detection by removing the need for gel electrophoresis and the additional hybridization step. When extraction, RT and PCR efficiencies are measured, virus quantification in the sample can be estimated (Costafreda et al., 2006). The efficiency of the virus nucleic acids extraction is evaluated through the use of a model virus (as described above) while the efficiency of the RT-PCR reaction must be tracked by amplification of a RNA standard using the same combination of primers and probe used for virus detection (Pinto and Bosch, 2008). Such internal RNA controls have also been used for the detection of amplification inhibitors in qualitative assays (Schwab et al., 1998; Le Guyader et al., 2003).

## 2.2 Quantification.

The development of quantitative molecular assays for the analysis of shellfish has allowed estimates of the level of virus in naturally contaminated shellfish. However, relatively few data are currently available in this area. One report estimated the amount of HAV in coquina clams implicated in an outbreak to be between  $7.5 \times 10^3$  to  $7.9 \times 10^5$  genome copies per g of digestive tissues (Coastafreda et al., 2006). Another recent report used a semi-quantitative approach for norovirus detection to compare the levels of virus contamination between sites without describing the amount of shellfish analyzed (Lowther et al., 2008). Japanese investigators estimated the levels of norovirus contamination in oysters collected from two areas range from  $<100$  copies to  $7.9 \times 10^3$  genome copies per g of digestive tissues in two consecutive studies (Nishida et al. 2003, 2007). These estimates did not include an adjustment for extraction efficiency, as proposed by Costafreda et al. (2006). Similar data have been obtained for the levels of norovirus contamination in oysters from France (Le Guyader et al., 2008). Quantitative estimates of norovirus contamination in shellfish implicated in outbreaks are even more uncommon. By using MPN PCR or real-time RT-PCR, about 100 copies of genome/g of digestive tissues were found in oysters in France (Le Guyader et al., 2003, 2006a, 2008). This level of contamination is sufficient to cause human infection based upon the low infectious dose of the Norwalk virus, prototype for norovirus, in a volunteer study (Teunis et al., 2008). The availability of quantitative assays offers the potential to perform risk assessments associated with the consumption of virus-contaminated shellfish.

## 3. Input and flux

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### 3.1 Seasonal outbreaks.

The regular and predictable pattern of seasonal outbreaks dominates the epidemiology of many exclusively human pathogens (Dowell, 2001). The seasonal infection may vary between different pathogens, but the timing and characteristics of the annual outbreak of a single pathogen are remarkably consistent from year to year. It was shown that latitude has a clear influence on the timing and magnitude of outbreaks of rotavirus infections and poliomyelitis (Dowell, 2001). Data collected from different studies worldwide have also shown

a clear seasonality for norovirus outbreaks. A clear peak of norovirus outbreaks occurs during cold weather months on several continents, with lack of UV, cold temperature, frequent run-off being some of the possible explanations of extensive transmission (Mounts et al., 2000). However, noroviruses continue to circulate endemically throughout the year, and although there is the theoretic possibility of zoonotic spread, currently there is no direct evidence of the existence of a reservoir for re-introduction into the human population (Lopman et al., 2008). Gastroenteritis from all causes predominates during colder months of the year but it does not disappear during summer (Dowell 2001; Lopman et al., 2008). It is now evident that some viruses may be detected all year long, either in sporadic cases of illness or in untreated sewage (da Silva et al., 2007, Patel et al., 2008). The amount of virus shed by ill people may be very high (Atmar et al., 2008) (Table 2). Importantly post-symptomatic virus shedding may continue for some time, as demonstrated for enterovirus, hepatitis A, and norovirus (Pallansch and Roos, 2007; Hollinger and 2007; Atmar et al., 2008). For example, norovirus shedding in an experimental human infection model lasted a median of 28 days, with a range from 13 to 56 days, and most subjects were no longer symptomatic by day 4 (Atmar et al., 2008). These data suggest that the impact of continued virus shedding from ill and post-symptomatic patient on sewage may be very significant.

Enterically-transmitted hepatitis viruses are distributed worldwide with no clear season pattern. The endemicity in different parts of the world is closely linked to the level of socioeconomic development. In developing countries with poor sanitary and living conditions, transmission rates of HAV are high with a large number of subclinical cases shedding high concentration of viruses into the environment (Aggarwal and Naik, 2008). In contrast, the levels of HAV endemicity and transmission rates are much lower in developed countries, and detection of viruses in the environment is less common so that introduction into the environment may be limited in time and localization (Pinto and Saiz, 2007).

### 3.2 Animal output.

As noted earlier, some enteric viruses can also be detected in animals. These animal infections can lead to contamination of the environment and can confound the identification of human strains, depending on the detection methods that are used. For example, rotavirus is a pathogen for young calves and pigs. Calves can excrete very high levels of rotavirus (up to  $10^{11}$  viral particles/g of stool) for a week or longer. Most of the strains implicated in bovine disease belong to group A rotavirus, the most common cause of human disease (Dhama et al. 2008). Group A rotaviruses have also been detected in equine, swine, ovines, and caprines, and pet dogs and cats may also be infected with these viruses (Dhama et al., 2008).

Norovirus also infect other species including swine and cattle. The prevalence of norovirus infection in these animals has varied in studies from different countries. One in 17 porcine fecal samples from Hungary was found positive for norovirus (Reuter et al., 2006) while in Italy none of the 209 stool was positive for norovirus (Martella et al., 2008). In contrast, 2% of pigs stool samples in The Netherlands were NoV-positive (van der Poel et al., 2000), and the prevalence of porcine norovirus on farms in the United States was reported to range between 3 and 40% (Wang et al., 2006). In Canada, about 25% of swine fecal samples were found positive while only 1.6% of bovine fecal samples contained noroviruses (Mattison et al., 2007). Bovine norovirus have also been detected in 9.1% of diarrheic calves in Korea (Park et al. 2008). Other studies have identified bovine noroviruses in 72% of veal calves from the US (Smiley et al., 2003), in approximately a third of veal calf farm samples from The Netherlands (van der Poel et al., 2000) and in 8% of calf diarrhoea samples from the United Kingdom (Oliver et al., 2003).

### 3.3 Enteric viruses in sewage and rivers.

A review of available information in the literature on the fluxes of human enteric viruses discharged into the environment clearly shows that the presence of pathogens in waters

reflects the epidemiology of these viruses in the human population (Rohayem et al., 2006; Myrmel et al., 2006; da Silva et al., 2007; Schawb, 2007). The lack of reliable quantification methods (such as cell culture) for many of the enteric viruses has precluded accurate quantitative analyses of these viruses from environmental samples. Although the development of quantitative molecular assays now offers the promise to generate such data, they can be impacted by many factors such as virus concentration efficiency and amplification efficiency which many investigators fail to consider. In addition, molecular assays cannot evaluate virus infectivity, since they detect both live and inactivated virions. Consequently the molecular assay may lead to an overestimate of the amount of infectious virus present in a sample (Richards, 1999; Gerba, 2007).

The concentration of noroviruses measured in natural waters and raw sewage has shown both regional and seasonal variability. In The Netherlands, noroviruses were detected on average at  $2 \times 10^5$  PCR detectable units (PDU) /liter of raw sewage, with only one sample being negative (September) (Lodder and de Roda Husman, 2005). In the UK norovirus concentrations ranged from zero to  $1.7 \times 10^7$  cDNA copies/L and in Germany approximately  $10^6$  copies/L were reported (Laverick et al., 2004; Pusch et al., 2005). Haramoto et al. (2006) described seasonal differences in norovirus concentrations in untreated sewage, from a peak of  $1.9 \times 10^6$  RNA copies/L in December to a low of  $2.4 \times 10^3$  copies/L in September. Similarly, da Silva et al. (2007) and Katayama et al. (2008) reported higher noroviruses concentrations (about  $10^5$  genomes copies/L for both studies) in untreated sewage during winter months compared to spring or summer. All of these studies found both genogroup I and genogroup II norovirus strains. Genogroup II strains cause the large majority (80 to 90+% in most studies) of clinical cases seen by physicians (Atmar and Estes, 2006; Lopman et al. 2008). The consistent detection of genogroup I strains in raw sewage genogroup I demonstrates that these viruses are circulating in the population (Haramoto et al., 2006; van der Berg et al., 2005; da Silva et al., 2007; Katayama et al., 2008).

Rotaviruses and enteroviruses are also detected in raw sewage in approximately the same range of concentrations (Pusch et al., 2005; Lodder and de Roda Husman, 2005). Astrovirus may be detected as higher concentrations (up to  $10^8$  genome copies/l) (Pusch et al., 2005; Le Cann et al., 2004). The data for wastewater samples may reflect the presence of strains that circulate more widely in the population and as such may be a powerful and useful tool for public health surveillance (Hovi, 2006).

Despite treatment of sewage to remove bacterial and viral pathogens, treated wastewater contains enteric viruses that can potentially contaminate the environment (Table 3). Concentrations of hundred to thousands genome copies per liter of treated wastewater can be detected, and seasonal variability occurs similar to that seen for untreated sewage. Several studies have observed a higher frequency of genogroup I (GI) norovirus strains in treated effluent compared to genogroup II strains (van der Berg et al., 2005; Myrmel et al., 2006; La Rosa et al., 2007; da Silva et al., 2007). Haramoto et al. (2006) even reported that all treated sewage samples were positive for GI. The reason why GI noroviruses are more resistant to inactivation and removal during the sewage treatment process is not clear and should be studied in the future. Some differences in capsid proteins or binding properties may be one interesting hypothesis to analyze. That GI noroviruses are more often implicated in shellfish- and water-related outbreaks than GII may also be evidence in favor of higher resistance to inactivation in the environment (Lopman et al., 2004; Blanton et al., 2006). Overall noroviruses are found in concentrations ranging from undetectable to  $10^6$  genome copies/l of treated wastewater (Table 3).

The efficacy of different wastewater treatments for norovirus removal from wastewaters has varied from 0.5 to 4  $\log_{10}$  (Table 4). One of the most promising approaches utilizes ultrafiltration, such as MBR technology operating with biological treatment as well as physical separation of particles. Sano et al. (2006) reported complete elimination of noroviruses from

sewage sludge and treated water, with greater than 4 log<sub>10</sub> reduction values by ultrafiltration. The use of microfilters with pore sizes as large as 0.45 microns also effectively removed virus, with log<sub>10</sub> reduction values of more than 3.9 for sewage sludge and 2.9 for treated water. The microfilter membranes have permeability that is much larger than the diameter of viruses but can be effective since the viruses are embedded in or adsorbed to solids. Nevertheless, viral particles may be detected in effluents after treatment with microfilters with larger pore sized, implying that such membranes are not an absolute barrier for the passage of viruses (Ueki et al., 2005; da Sylva et al., 2007).

Another approach is to utilize techniques that enhance the effectiveness of ultraviolet disinfection. Norovirus appears to be quite resistant to UV radiation (Ottoson et al., 2006; Hijnen et al. 2006) but genome destruction may be achieved using a highly photocatalytic material TiO<sub>2</sub> (Titanium dioxide), in conjunction with UV (Kato et al., 2005). Addition of low concentration of TiO<sub>2</sub>U particles also increased the rate of inactivation of phage MS2, although the effect on murine norovirus (a surrogate for human noroviruses) was less (Lee et al. 2008).

### 3.4 Flux calculation from sewage.

Information on virus sources and virus levels in raw or treated wastewaters can be used to estimate the quantities of virus discharged into the environment. Relatively few data are available to allow the calculation of “virus base-flow” or “event-flow” discharges in river or in estuaries. Nevertheless recent data suggest that during non-epidemic periods less than 10<sup>3</sup>-10<sup>4</sup> genome copies/L of norovirus are present in treated wastewaters. During the epidemic period (winter) the concentration is probably 100- to 1000-fold higher. The rate of reduction of virus concentrations through sewage treatment processes is almost constant and independent of the concentration of viruses of the effluent (Myrmel et al., 2006; daSilva et al. 2007; Katayama et al., 2008). These data imply that viruses are discharged into environmental waters with a seasonal profile and raises questions about the frequency and duration of such peaks and the importance and impact of storm events that result in failure in wastewater treatment during a high flow and epidemic period. In the absence of precise information, calculations from epidemiological data suggest that 10<sup>6</sup> norovirus fluxes can be expected from a town of 60,000 population-equivalent when winter outbreaks are occurring (Pommepuy et al., 2004). Many environmental factors can have an impact, including currents, estuaries, and tide (Pommepuy et al. 2005). The persistence of viruses in the environment must be considered even in nonepidemic periods when risks of infection associated with shellfish consumption are estimated (Riou et al., 2007).

### 3.5 Surface water.

Viruses may be discharged into surface water from different sources, such as septic tank or sewer lines leaking, wastewater irrigation of sludge disposal. In a recent review, Gerba (2007) identified studies describing the occurrence of enteric viruses in surface freshwater and demonstrated that in all countries a number of different viruses can be detected. However it is clear that data are still limited and information on virus concentration and infectivity are often lacking.

Noroviruses are relatively resistant to inactivation compared to other enteric viruses, possibly explaining their predominant role in food-related outbreaks. Detection of infectious norovirus is not possible due to the lack of availability of an *in vitro* cell culture system or small animal model. Other culturable virus surrogates have been proposed to model virus persistence in environmental samples. Bae and Schwab (2008) used a murine norovirus strain to evaluate virus persistence in different types of surface waters compared with MS2 phage, poliovirus, and feline calicivirus in an infectivity assay. The murine norovirus infectivity persisted longer than feline calicivirus and as long as the other human norovirus surrogates. In addition, reduction of murine norovirus nucleic acids as measured by real-time RT-PCR was similar to that of a human norovirus strain in surface waters (0.08 and 0.09 log<sub>10</sub>/day, respectively) and

in groundwaters (0.01 and 0.00 log<sub>10</sub>/day, respectively) (Bae and Schwab, 2008). Norovirus are detected in surface waters less frequently than in wastewaters, probably due in part to dilution or sedimentation mechanisms during transport in the rivers. The reported frequencies of norovirus detection has varied between studies from 5.8% in a Brazilian river to 53% in Japan (Haramoto et al., 2005; Miagostovich et al., 2008). Peak norovirus concentrations of up to 10<sup>4</sup> genome copies/liter of surface waters have been reported, suggesting that the risk of infection can vary quantitatively as well as qualitatively (Lodder and de Roda Husman, 2005; Westrell et al., 2006). The other human enteric viruses can also be detected to various concentrations as noted in a recent review Gerba (2007). For example, hepatitis A virus is endemic in some Italian areas, and viral RNA has been detected at concentrations ranging from 75 to 730 genomes/L in Venetian canals (Rose et al., 2006).

Fluctuations in virus concentrations may result from a combination of different factors, such as sewer overflows leading to untreated water being discharged in rivers and run-off from pastures. Detection of short-term fluctuations is important to predict the risk for water contamination, but few data are available. A two-year study on the Meuse river in The Netherlands, found mean norovirus values to range from 4 to 4,900 genome copies/L (Westrell et al., 2006). Within the winter seasonal peak, quantitative and qualitative fluctuations of norovirus presence in the river waters occurred over varying lengths of time, and the highest concentrations could lead to significant contamination of drinking waters if water treatment measures failed. There are models to study the impact of river and wastewater discharges on coastal waters and shellfish quality (Pommepuy et al., 2005; Riou et al., 2007). However, these models, even though they appear to be useful, can be improved with additional information, including a larger input database, data on short-term fluctuation frequencies, and a better understanding of viral behavior and persistence in the environment (e.g., in sediments and shellfish).

### 3.6 Potential indicators: phages, animal markers.

The current regulatory controls in most countries rely on fecal pollution indicator organisms, such as *E.coli*, to assess microbiological hazards. Methods to detect *E.coli* are inexpensive, standardized and widely available (European regulation, 91/492/EC or United States National Shellfish Sanitation Program). However, it is clear now that fecal coliforms inadequately reflect the presence of human enteric viral contaminants (Butt et al., 2004). As no standardized methods are yet available for human enteric viruses in shellfish, a number of workers have proposed alternative indicators for better assessment of viral contamination. Various species of bacteriophages are some of the most frequently proposed indicators because of their physical and genomic similarity to human enteric viruses, their abundance in sewage effluents and the availability of simple methods for their detection. Male-specific RNA (F-RNA) bacteriophages have been proposed as a candidate indicator for water pollution (Havelaar et al., 1993). However, although F-RNA bacteriophages share physical and genomic properties with the human enteric viruses, their distribution (similar to fecal coliform bacteria) is not restricted to human effluents. Studies related to bivalve shellfish contamination by human enteric viruses and phages have shown variable results (Table 5). The F-RNA bacteriophage appears to perform best in sites that are consistently polluted. A number of studies have shown that F-RNA bacteriophages were not detected when human enteric viruses were present. For example, data collected in an European survey demonstrated that shellfish collected from southern Europe were negative for phages but contained human viruses (Formiga-Cruz et al., 2003). Similarly, a study from France reported that the detection of FRNA bacteriophages failed to predict the presence of human viruses (Miossec et al., 2001). A correlation was found between noroviruses and phage contamination of mussels in cold seawater, but more than half of the norovirus-positive samples were negative for FRNA phages and a positive FRNA phage result was less than twice as common in samples with norovirus than in these without norovirus, raising the question to use FRNA as an indicator (Myrmel et al., 2004). A one-year study in the Netherlands found phages were present in 67% of oyster samples analysed, but no pathogenic viruses such as norovirus or hepatitis A virus were identified in the shellfish

(Lodder-Verschoor et al., 2005). A discrepancy level of 55% was observed between the detection of hepatitis A virus and FRNA phages in association with a large outbreak of hepatitis A (184 human cases) from imported shellfish in the East of Spain in 1999 (Bosch et al., 2001). Phages may be used to classify areas for sanitary safety, but they have been unreliable indicators of virus-contamination of shellfish (Hernroth et al., 2002).

## **4. Strategies for reducing contamination**

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### **4.1 Resistance of virus.**

Many human enteric viruses are resistant to inactivation or removal from the environment. Nenonen et al. (2008) traced norovirus strains in the environment and were able to identify outbreak strains in mussels exposed to sewage three months after the outbreak. Similarly, Ueki et al. (2005) were able to follow clinical strains from stool to sewage, to river waters and ultimately to oyster samples. Similar results were reported more than 25 years ago for a number of picornaviruses (enterovirus, poliovirus and hepatitis A virus) (Metcalf, 1982). A characteristic of these enteric viruses is their prolonged survival in the environment. Temperature appears to be the most important factor in virus survival, with low temperatures being associated with increases in virus persistence. Virus survival for many months has been observed at freezing or near-freezing temperatures (Gerba, 2007). UV light may damage the nucleic acid causing dimer formation. Double stranded DNA viruses like adenoviruses are more resistant to UV light inactivation than are enteroviruses (Gerba, 2007). In addition to these two principal environmental factors, other physical-chemical parameters such as pH, salts, organic matter may impact virus survival and distribution, but generally this is only in a limited manner (Gerba, 2007). Few data are available on the behavior of norovirus since most studies have only reported qualitative virus detection. Real-time RT-PCR allows a quantification approach as does the use of cultivable animal norovirus strains as surrogates, and these approaches can be used to evaluate virus resistance to different environmental factors such as temperature or UV light (Duizer et al., 2004; Bae and Schwab, 2008).

### **4.2 Persistence of viruses in shellfish tissues.**

It was generally thought that oysters act as mere filters or ionic traps, passively concentrating particles such as bacteria or virus. However, unlike enteric bacterial species, enteric viruses persist in shellfish for an extended period of time and it is this persistence that appears to result in its significant impact on public health. Viruses are principally concentrated in the pancreatic tissue, also called digestive diverticula. A number of different mechanisms have been suggested to explain differences between in virus accumulation between different oyster species, including mechanical entrapment and ionic bonding (di Girolamo et al., 1977; Metcalf, 1982; Schwab et al., 1998; Burkhardt and Calci, 2000). Virus accumulation in oysters can also depend on factors such as water temperature, mucus production, glycogen content of the connective tissue, and gonadal development. The importance of secreted acid mucopolysaccharides in the concentration of poliovirus was first demonstrated 30 years ago (di Girolamo et al., 1977). Mucus present on gills was also suspected to be important for concentration of reovirus by oysters (Bedford et al., 1978). Later hepatitis A virus was demonstrated to persist for several weeks after bio-accumulation, with infectious virus being detectable after three weeks and viral RNA still being detectable after six weeks using molecular assays (Kingsley and Richards, 2003). Infectious adenovirus was still detected in mussels for three weeks following bioaccumulation and in oysters for six weeks (Hernroth and Allard, 2007).

Virus-like particles (VLPs) have also been used to study virus persistence in shellfish. Loisy et al. (2005a) used rotavirus VLPs in oyster bioaccumulation studies, and viral particles persisted in oyster tissues for one to three months depending on the initial input concentrations (Loisy et al., 2005a). We used VLPs of the prototype genogroup I Norwalk

virus (rNV VLP) and native Norwalk virus for bioaccumulation of oysters (*Crassostrea gigas*) to study norovirus persistence. We observed no differences in virus distribution between the native Norwalk virus and the VLPs, confirming that VLPs are good surrogates of infectious virions for this type of study. Interestingly, virus particle and VLP binding was to specific cell types, and some viral particles were detected in phagocytes located either in the epithelium or in the connective tissue (Le Guyader et al., 2006b). This observation might reflect the process of virus elimination or of normal digestion, but it is unclear if the immunoreactive material detected in phagocytes corresponds to particles being degraded and digested or whether particles are able to escape digestion. Specific binding was observed to the main ducts in the digestive tract and may be a mechanism for many viral particles to avoid entering in the food circulation and thus being degraded (Le Guyader et al., 2006b). The existence of a specific attachment to oyster cells and the internalization into phagocytes could explain the difficulty in using depuration to rid oysters of virus. In our work, we observed no differences in the tissue distribution of VLP binding between samples collected in March or October, although additional studies during other seasons are needed.

Human susceptibility to norovirus infection depends upon the presence or absence of certain carbohydrates of the ABH, secretor and Lewis histo-blood group families (Tan and Jiang, 2007). Using tissues section of oyster bodies, we observed that recognition of oyster digestive epithelial cells by rNV VLPs also involves carbohydrates. Similar to what was observed with human histo-blood group structures, the use of human saliva to inhibit VLP attachment to oyster tissues or the use of mutant VLPs that abolish VLP binding to histo-blood group antigens (alanine substitution at positions H329A and W375A) prevent binding to oyster tissue. Additional studies showed that the oyster ligands are similar to histo-blood group A. Thus, NV binds to oyster tissues through an A-like carbohydrate structure, a binding site also used for attachment to carbohydrate on human epithelial cells (Tan and Jiang, 2007). Norovirus VLPs can also specifically bind to tissues of other oyster species (*Crassostrea virginica*, *Crassostrea sikamea*) or clams (*Venerupis virginica*) or mussels (*Mytilus edulis*) (Tian et al., 2007).

Distinct norovirus strains belonging to both genogroup I and II exhibit various binding patterns with different carbohydrate structures of the histo-blood group family, suggesting a possible coevolution of this group of viruses and their host or carrier vector. The importance of these observations and the interaction with specific carbohydrates in shellfish is of primary importance to protect the consumer, and more research needs to be conducted on this subject. For example it could be interesting to see if some bivalve mollusks less sensitive to contamination may be selected or if we can prevent the binding. The identification of the receptor may help to improve depuration.

#### 4.3 Depuration.

Depuration processes are intended to eliminate or to purge live bivalves of the microbial contamination and is usually performed in tanks with clean sea-water. European legislation does not stipulate a minimum length of time over which depuration should be performed, but approximately two days is probably the most widely used depuration time in Europe (Richards 1988). Two days of depuration with clean sea-water will efficiently eliminate contaminating enteric bacteria, but as mentioned above, this is an insufficient length of time to efficiently remove enteric viral pathogens. Depurated shellfish have been implicated as the cause of a number of virus illness outbreaks (Gill et al., 1983; Heller et al., 1986; Le Guyader et al., 2006a, 2008).

Low levels of enterovirus contamination in soft shell clams following bioaccumulation were reported as often eliminated following depuration in early studies (Metcalf et al., 1979). However, more recent data obtained using bioaccumulated shellfish have shown that although depuration may decrease virus concentration in shellfish tissues it has usually been insufficient to completely remove viruses (Abad et al., 1997; Schwab et al., 1998; Kingsley and

Richards, 2003; Pommepeuy et al., 2003; Loisy et al., 2005a). Different factors influence depuration efficiency. Increasing water temperatures can enhance virus removal, although an increase in depuration temperature may also result in increased shellfish mortality and induce some bacterial growth (Dore and Lees, 1995; Pommepeuy et al., 2003). Poor depuration of enteric viruses can still occur at higher depuration temperatures as was reported in studies of Norwalk virus where only 7% of Norwalk virus was depurated compared to a 95% reduction in bacterial levels at a temperature of 22°C (Schwab et al., 1998). Feeding naturally contaminated oysters at 22°C increased depuration in a semi-professional size depuration tanks compared to fed oysters at lower temperatures (Pommepeuy et al., 2003). The large tank allowed depuration of up to 900kg of oysters and showed the feasibility of performing this procedure in the setting of a shellfish farm using producer conditions.

Phages have also been suggested as indicators of viral depuration as they are depurated less efficiently than *E.coli* (Doré et al., 2000). Feline calicivirus, another proposed surrogate, failed to be a good indicator of norovirus depuration from oysters (Ueki et al., 2007). Considering the differences in depuration observed among various species of human enteric viruses (Abad et al., 1997) and differences in virus binding observed to shellfish tissues, extrapolating data from a phage to a human pathogen may be hazardous. The employment of appropriate surrogates remains a crucial issue in the evaluation of shellfish depuration.

#### 4.4 Other issues.

Other approaches have been proposed to decrease the risk of shellfish for human consumption (Table 6). Cooking shellfish is one such approach, but failures of this strategy have also been reported. Following a large outbreak of viral gastroenteritis with cockles collected from the Thames estuary (England), a 'double cooking' was recommended (after an initial brief boil or steam treatment necessary for shell removal, cockle meats were to be boiled for a further 4 min). However gastroenteritis and hepatitis A linked to cockle or mussel consumption were still reported (Millard et al. 1987). Frozen coquinas clams imported from Peru and served cooked in paella, were responsible for hundred of cases of hepatitis A in Spain (Bosch et al. 2001). Croci et al. (2005) explored different approaches to kill the virus after bioaccumulation of hepatitis A virus in mussels, and these investigators showed that extended cooking times were needed to completely inactivate the virus (Table 6). Failures of cooking may be due to the inability to attain sufficiently high temperatures to inactivate the virus or to the relative resistance of some enteric viruses to heat inactivation and their low infectious dose (Koff and Sear, 1967; McDonnell et al., 1997). In food, several factors may influence viral behavior (Le Guyader and Atmar, 2008).

High-hydrostatic pressure processing (HHP) has emerged as a promising technology for virus inactivation. HHP inactivates enteric viruses suspended in buffer but the inactivation rates are affected by treatment temperature and virus strain (Chen et al., 2005; Kingsley et al., 2007). It has been applied to shellfish that have bioaccumulated HAV or a murine norovirus, and greater than 1000-fold reductions in viral titer was achieved with a treatment of  $\leq 400$  Megapascal (MPa) for 5 min at 5°C (Calci et al., 2005; Kingsley et al., 2007). A potential disadvantage of this method is that changes in the character of the shellfish have been demonstrated in organoleptic studies, and some consumers prefer to eat live oysters (Cruz-Romero et al., 2004).

The effect of marination (immersion in an acetic acid-based mixture) has been evaluated in mussels contaminated with norovirus or HAV. The commercial marination process is a two-stage procedure including a preliminary heat treatment (immersion in boiling water or steaming for 3 min) and then marination for several weeks. After 4 weeks of marination, the infectious titer for HAV decreased approximately 50-fold, and human norovirus RNA was still detected by real-time RT-PCR (Hewitt and Greening, 2004). These data suggest that marination alone is not sufficient to inactivate enteric viral pathogens in shellfish.

## 5. Perspectives

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Although seafood can generally be regarded as a wholesome, safe, and nutritious food, it may occasionally pose consumer risks. Regulations are currently based on routinely monitoring shellfish for fecal bacteria to determine their sanitary quality. However, viral contamination of bivalve molluscs is currently recognised as one of the major causes of illness associated with seafood. Monitoring viral contamination is complex and must take into account different factors such as detection methods, technology, social demands and the sustainable development of aquaculture. Recent advances in technology, especially in developing molecular tools, make it possible to look for pathogens directly in shellfish implicated in outbreaks. Evaluation of seafood for the presence of norovirus and hepatitis A virus for regulatory purposes has been recommended by experts involved in EU, FAO and WHO working groups. Mandatory surveillance for virus may be implemented in the near future but additional information including level of exposure and virus infectious dose are still needed to assess risks.

Different actions may be considered to lower the contamination of shellfish bed in coastal area:

*Reduction of fecal input in coastal areas.* Water quality studies have demonstrated the feasibility of determining the main sources of fecal contamination and to identify the critical points in the catchments. Hydrodynamical models when applied to these contaminants, even if they need further development and have to be validated by data bases, could lead to rational bases for the choice of treatment levels based on results from screening purposes to limit the contamination.

*Implementation of warning systems:* in developed countries, some data are already available on outbreaks occurring in the population, so, associated with forecast information, salinity, STP failure and other, a warning system could lead to real-time assessments of water quality in bathing or harvesting areas. The development of new tools for rapid pathogen detection (for example micro-array) may help to collect additional information on the presence of pathogens in sewage or waters.

*To limit shellfish contamination,* the most desirable and effective option is to reduce the viral input. Villages, small towns and dwellings must be equipped with small individual treatment tanks to comply with the regulations. New technologies may be needed to improve removal of viruses from sewage effluents (Shannon et al., 2008). Another solution could be the relocation of shellfish aquaculture away from the contamination sources.

Other factors will also need to be considered to protect the consumer and to provide safe shellfish on the market. An important aspect of monitoring concerns the sustainable development of aquaculture. This development is closely linked to environmental quality in shellfish breeding areas. The nested-regulations set up for water quality, bathing areas and shellfish growing areas, if well applied, should provide the guarantees and the management tools. There are promising examples of coastal management designed to reduce the fecal load which could make recovery of water quality feasible. Used in association with early warning systems, they could help ensure shellfish quality and increase consumer confidence and thus, would greatly contribute to sustainable development of aquaculture. Shellfish have long been recognized as being beneficial to human health and this benefit should also be taken into consideration in managing the coastal areas and preserving the water quality.

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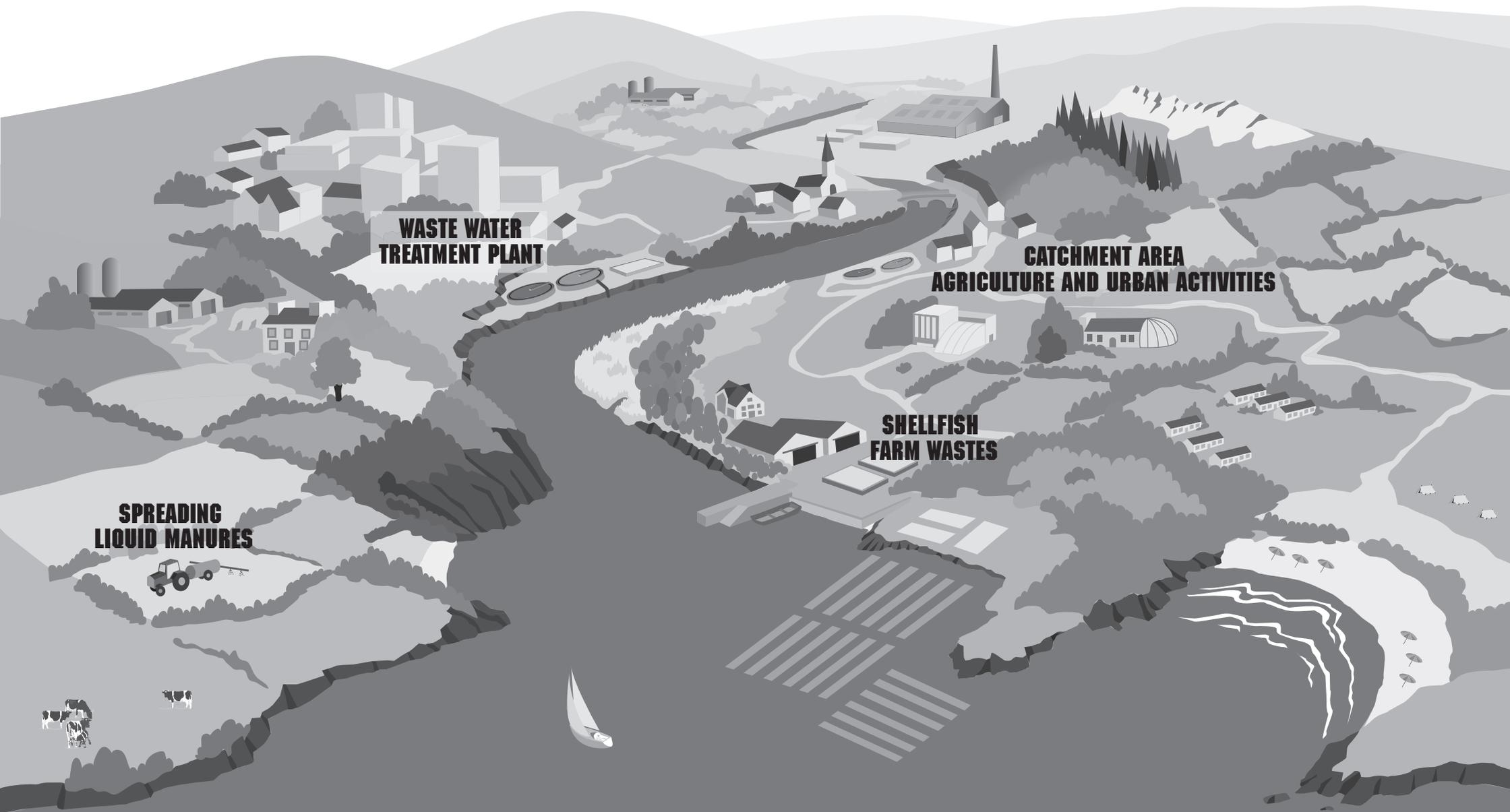


**WASTE WATER  
TREATMENT PLANT**

**CATCHMENT AREA  
AGRICULTURE AND URBAN ACTIVITIES**

**SHELLFISH FARM WASTES**

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Table 1: Characteristics of the the main enteric viruses.

name	Adenovirus	Aichi virus	Astrovirus	calicivirus	enterovirus	rotavirus	Hepatitis A virus
Size	70 nm	27-32 nm	27-32 nm	27-32 nm	20-30 nm	70nm	27-32nm
Capside	Complexe	icosahedral	icosahedral	icosahedral	icosahedral	Triple layer icosahedral	icosahedral
Genome	dsDNA	ssRNA	ssRNA	ssRNA	ssRNA	dsRNA	ssRNA
Size	35,900 bases	8,251 bases	6,797 bases	7,642 bases	7,200 bases	11 genes (3302 to 667 bp)	7478 bases
genome pair							
incubation	3-10 days	1-2 days	3-5 days	2-3 days	7-30 days	3 days	Up to 6 weeks
illness	gastroenteritis	gastroenteritis	gastroenteritis	gastroenteritis	diverse	gastroenteritis	hepatitis
season	All yar	All year	winter	winter	summer	winter	All year
age	Young children	Young adults	children	All ages	All ages	Young children	All ages

Table 2: Titers of enteric viruses in stool specimens

Virus	Range of virus concentrations (RNA copies/g)		References
	minimal	maximal	
HAV	$3.4 \times 10^5$	$5.6 \times 10^{11}$	Costafreda et al., 2006
HEV	$<10^2$	$10^7$	Takahashi et al., 2007
Rotavirus	$10^3$	$10^{10}$	Freeman et al., 2008
NoV	$1.4 \times 10^7$	$4.2 \times 10^9$	Ludwig et al., 2008
	$10^8$	$10^{12}$	Lee et al., 2007
	$2.2 \times 10^4$	$10^{11}$	Chan et al., 2006
	$9 \times 10^7$	$6 \times 10^{10}$	Kageyama et al., 2003
	$5 \times 10^8$	$1.6 \times 10^{12}$	Atmar et al., 2008
SaV	$1.3 \times 10^5$	$2.1 \times 10^{10}$	Oka et al., 2006

Table 3: Average concentration of human enteric viruses detected in treated wastewater samples

Virus	positive sample (%)	Average concentration		reference
		PCR units/L	PFU/L	
HAV	100	9x 10 <sup>1</sup> -3.5x 10 <sup>3</sup>		Brooks et al., 2005
NoV	93	<10 <sup>1</sup> -3x 10 <sup>4</sup>		Van der Berg et al., 2005
	7	0 – 1.6x10 <sup>5</sup>		Laverick et al., 2004
	53	1.8x 10 <sup>4</sup> -9.7x 10 <sup>5</sup>		Pusch et al., 2005
	100	8.7x 10 <sup>1</sup> -2.9x 10 <sup>3</sup>		Katayama et al., 2008
	100	6x 10 <sup>2</sup> -2.4x 10 <sup>4</sup>		Lodder and de Roda-Husman, 2005
	38	2.1x 10 <sup>2</sup> -6x 10 <sup>6</sup>		Da Silva et al., 2007
	100	6- 6.4x10 <sup>3</sup>		Haramoto et al., 2006
SaV	58	0.5-1.8x 10 <sup>2</sup>		Haramoto et al., 2008
AV	40	3,7x 10 <sup>3</sup> -1.2x 10 <sup>5</sup>		Pusch et al., 2005
	50	1.1x 10 <sup>3</sup> -6.2x 10 <sup>5</sup>		El-Senousy et al, 2007
	82	8x 10 <sup>2</sup> -5x 10 <sup>5</sup>		Le Cann et al., 2004
EV	90	10 <sup>1</sup> -5.4x 10 <sup>4</sup>		Schvoerer et al., 2001
	100			Lodder and de Roda-Husman, 2005
	100	4x 10 <sup>0</sup> -1.7x 10 <sup>2</sup>	5-92	Katayama et al. 2008
AdV	100	9x 10 <sup>2</sup> -5x 10 <sup>4</sup>		Katayama et al., 2008
Reo	90			1-233 (MPN) Sedmak et al., 2005

Table 4: Efficiency of different waste water treatment plant to remove norovirus.

<b>Treatment</b>	<b>genogroups</b>	<b>log<sub>10</sub> reduction value.</b>	<b>References</b>
Physical treatment	GI	1.82 (±0.61)	Haramoto et al., 2006.
	GII	2.74 (±1.10)	
Chemical treatment (chlorination)	GI	0.45 (±0.49)	Haramoto et al., 2006.
	GII	0.95 (±1.80)	
Tertiary filtration	GI & GII	0.50 (±0.84)	Ottoson et al., 2006
Membrane Bioreactor	GI & GII	1.14 (±0.88)	Ottoson et al., 2006
Activated sludge treatment	GI & GII	2 –2.7	van der Berg et al., 2005
Wastewater Stabilized Ponds	GI	0-3*	Da Sylva et al., 2007
	GII	0-5*	
Membrane separation	GI & GII	2.9-3.5	Sano et al., 2006

\* depending on the residence time

Table 5: human enteric viruses and phages detection in shellfish samples.

Country	Shellfish	class	# of sample	Phages	NoV	HAV	EV	Reference
UK	oysters	A	134	50	62	-	-	Dore et al., 2000
Italy	mussels	A	36	8	-	14	36	Croci et al., 2000
Norway	mussels	A	681	23.8	6.8			Myrmel et al., 2004
Spain	mussels	A	7	14	-	0	0	Munian-Mujika et al., 2003
		B	36	22	-	22	25	
Netherlands	Oysters	B	11	36	-	18	9	Lodder-Verschoor et al., 2005
	Oysters	A	22	100	0	0	27	
India	Oysters	B	100	57	0	0	37	Umesha et al., 2008
	clams	B	74	73	0	0	45	

Phages and viruses expressed in %

Table 6: Effects of different physical factors on virus inactivation in shellfish.

Treatment	Shellfish	Recipe	Virus	contamination	Persistence of virus	Reference		
Heat	Oysters	Stewed in milk	poliovirus	Seeded	10% survival after 8 min	Di Girolamo et al., 1970		
		Fried/oil 177°C	poliovirus	Seeded	10% survival after 8 min.	Di Girolamo et al., 1970		
		Baked /oven 121°C	poliovirus	Seeded	13% survival after 20 min.	Di Girolamo et al., 1970		
		Steamed	poliovirus	Bioaccumulated	7% survival after 30 min.	Di Girolamo et al., 1970		
	Mussels	Boiling		HAV	Seeded	No survival after 3 min for HAV	Hewit and Greening, 2006	
				norovirus		100% persistence for NoV RNA		
		Steamed		HAV	Bioaccumulated	0.1% survival after 5 min.	Abad et al. 1997	
				rotavirus		0.1% survival after 5 min.		
		Steamed		HAV	Seeded	No survival after 3 min for HAV	Hewit and Greening, 2006	
				norovirus		100% persistence for NoV RNA		
			in hors d'oeuvre		HAV	Bioaccumulated	Infectious viruses detected after 9 min*	Croci et al., 2005
			au gratin		HAV	Bioaccumulated	Infectious viruses detected after 5 min*	Croci et al., 2005
			in tomato sauce		HAV	Bioaccumulated	No survival after 8 min.	Croci et al., 2005
		Cockles	Boiling		poliovirus	Bioaccumulated	No survival after 3 min.	Millard et al. 1987
	HAV				No survival after 2 min.			

Cold	Oyster	kept at 5°C	poliovirus	Bioaccumulated	13% survival after 1 month	Di Giralimo et al., 1970
		Frozen o (-17.5°C)	poliovirus	Bioaccumulated	10% survival after 12 weeks	Di Giralimo et al., 1970
Marinade	Mussels	Steamed and marination (pH 3,75)	HAV norovirus	Seeded	3% survival after 4 weeks for HAV 100% persistence for NoV RNA	Hewit and Greening, 2004
High pressure	oysters		Murine Nrovirus	Bioaccumulated	No survival after 5 min at 5° at 325 MPa	Kingsley et al., 2002
	oysters		HAV	Bioaccumulated	0.1% survival after 1 min at 400 MPa	Calci et al., 2005

\*: infectious virus too low for quantification