Gilbert Sauvé
Editor

Molluscan Shellfish Safety

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The organizers and myself were very pleased to welcome you to the 8th edition of the International Conference on Molluscan Shellfish Safety, held in Charlottetown, Prince Edward Island, from June 12 to 17, 2011.

Despite the tough economic times, there was a good turnout with representatives from various fields. This allowed us to make this 8th edition one of reflection on the scientific basis of requirements imposed to those who wish to export molluscs, often developing countries, as well as those economically stronger countries that would like to import more. The former can produce more with limited technological support while the latter develop and require the use of sophisticated and expensive technologies for their internal production. Managing this dichotomy is an ongoing challenge and our risk management measures need to find a balance in effectively minimising risk without unrealistic costs.

It is no coincidence that the conference logo features a long bridge – symbolic of the way people reach one another. Let's try to keep those bridges open without requiring excessive tolls.

We especially wish to thank the Food and Agriculture Organization (FAO) for their help with more than ten participants from developing countries who were given the opportunity to present the results of their research at the conference.

As in previous years, the 2011 ICMSS developed a program centred on the key themes and made it possible for everyone to attend more than 50 oral presentations and view 55 poster presentations. A special thank you to all these experts for enlightening us and enabling meaningful discussions. What other reasons would there be for us to meet every two years but to learn more, create new relationships and encourage new partnerships?

An event such as the ICMSS can only happen with the support of strong organizations. Members of various organizing committees were recruited from the ranks of the Canadian Food Inspection Agency and the Department of Fisheries, Aquaculture and Rural Development within the province of Prince Edward Island. Members of the ICMSS International Advisory Committee cannot be forgotten for their support from the beginning.

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Bioaccumulation and Removal Dynamics of Murine Norovirus in Manila Clams (*Venerupis philippinarum*) and Mussels (*Mytilus galloprovincialis*)

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Introduction

Infectious diseases associated with shellfish consumption have been widely reported (Butt et al. 2004; Koopmans and Duizer 2004). Historically, the study of food-borne diseases associated with contaminated shellfish has mainly focused on bacterial pathogens. As a result, the development and design of shellfish sanitary controls and depuration practices has been strongly influenced and guided with the purpose to eliminate fecal coliforms. Compliance with the end-product fecal coliform standard is frequently mistakenly seen as a evidence of a satisfactory depuration process. However, due to their filter feeding nature, shellfish also provide a potential vehicle of transmission for a wide variety of others infectious agents, making the bivalve molluscs a high-risk food group (Butt et al. 2004; Polo et al. 2010; Vilariño et al. 2009). Human enteric viruses can be bioaccumulated and retained by shellfish. In fact, outbreaks after consumption of shellfish contaminated with enteric viruses like norovirus (NoV) and hepatitis A virus (HAV) have been widely reported (Koopmans and Duizer 2004; Lees 2000).

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resistance to sewage treatment, and persistence in the environment (Da Silva et al. 2007). Depuration allows the purging of gastrointestinal contents under controlled conditions in order to reduce the likelihood of transmitting infectious pathogens. The efficacy of depuration in the elimination of such agents is a critical issue for the development of improved shellfish sanitary controls. Although depuration can reduce bacterial levels from shellfish, the removal of viral particles is not as effective (Ueki et al. 2007) and therefore, depuration may not ensure the absence of viral contamination (Croci et al. 2007; Loisy et al. 2005; Schwabm et al. 1998). Periodic outbreaks of enteric diseases linked to shellfish which comply with legal standards and/or are subjected to depuration, indicate the inability of both bacterial indicators (Romalde et al. 2002; Umesha et al. 2008) and commercial depuration (Chalmers and McMillan1995; Heller et al. 1986; Le Guyader et al. 2003, 2006a) to predict the viral risk.

Depuration is a complex biological process, which varies according to bivalve species, pathogens (bacteria or virus) and possibly between viral species. Therefore, a better understanding of specific behavior of diverse viruses in different bivalve species is needed. Experimental depuration systems provide a useful tool to find the best operational parameters and to improve commercial depuration. This study evaluates and compares by reverse transcription-real time RT-PCR (qRT-PCR) the effectiveness of depuration in clams and mussels subjected to bioaccumulation with murine norovirus (MNV-1), as a surrogate of human norovirus (Wang et al. 2008) in an experimental depuration system.

Materials and Methods

Sample Collection and Depuration Facilities

A closed experimental depuration system (isothermal ASE M BINS system, 500 kg of capacity) (AdriaticSeaAquarium and Equipment SRL, San Clemente, Italy) with mechanical, biological and chemical static filter systems, thermal control and water sterilization by ozone and UV-C radiation was employed (Fig. 14.1). Ten depuration experiments were carried out with Manila clams (*Venerupis philippinarum*) and mussels (*Mytilus galloprovincialis*) (five with each species) after bioaccumulation with MNV-1. Each experiment was performed with 60 kg of mollusc.

Bioaccumulation of Virus by Molluscs

Molluscs were obtained from local producers, kept at 4 °C during shipment and arrived at the laboratory within the next 4 h. After 24 h of acclimatization in tanks with 100 l of seawater and continuous aeration, bioaccumulation was performed

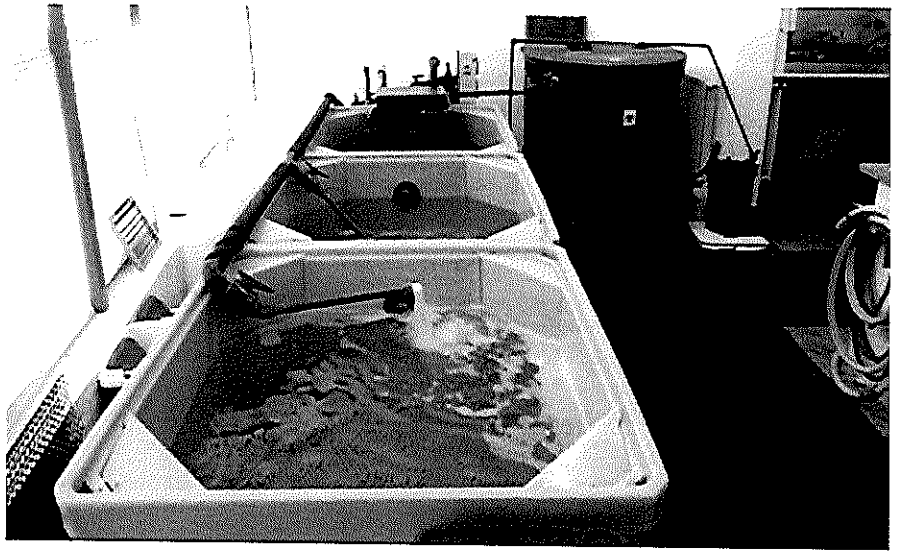

Fig. 14.1 Experimental depuration system

by adding 10^2 pfu/ml (final concentration) of MNV-1 to the tanks (1 pfu equals to approximately 10^2 RNA copies (Baert et al. 2008)). Moreover, 500 ml of two species of phytoplankton (*Isochrysis* sp. and *Nanocloropsis* sp., 1:1 v/v) were added in order to induce the clams to filter. Five independent trials were carried out with each mollusc species.

After 24 h the efficacy of bioaccumulation (T_0) was determined and the molluscs were relocated in the experimental depuration system during 7 day, under exhaustive control of the following depuration parameters: dissolved oxygen (O_2), pH, water temperature (T°), ammonia (NH_3/NH_4), nitrites (NO_2), nitrates (NO_3), conductivity and salinity. Sampling was performed every 24 h (T_1–T_7) in order to evaluate the removal kinetics of viral loads. Each sample was composed of 20 clams or 10 mussels for each analysis.

Cell Culture and Viral Stocks

A mutant non-virulent infective strain of mengovirus (vMC_0) was employed as extraction control as previously described (Costafreda et al. 2006). MNV-1 was employed as a surrogate for modeling human NoV. Stocks of $vMC_0$and MNV-1 were generated by inoculation onto confluent monolayers of HeLa (Costafreda et al. 2006) and RAW 267.4 (Wobus et al. 2006) respectively.

For virological analysis, molluscs were shucked and the digestive tissue (DT) removed and homogenized as previously described (Wang et al. 2008). Viral RNA was extracted in duplicate from DTusing Nucleospin RNA Virus Kit (Macherey-Nagel; Germany) according to the manufacturer's protocol.

Extraction and qRT-PCR Controls

Prior to the viral RNA extraction, known amounts of mengovirus clone vMC_0 were spiked in sample homogenates (10 µl of mengovirus stock, 10^3 pfu) to be employed as a control for the process of nucleic acid extraction (Costafreda et al. 2006). To test the presence of RT-PCR inhibitors and calculate the qRT-PCR efficiency, an external control (MNV-1 RNA) was included in each reaction. The cycle threshold (C_t) value of a sample extracted RNA (2.5 µl) mixed with MNV-1 external control (2.5 µl) was compared to the C_t value of the external control mixed only with RNA-free sterile water. These steps allowed the identification of samples that required re-extraction and provided assurance that the samples with no amplification signal were negative (values below the limit of detection for the assays) and not simply inhibited. Negative controls containing no nucleic acid as well as positive controls, containing RNA of MNV-1, were introduced in each run.

Primers and Probes

qRT-PCR for viral detection and quantification was carried out with TaqMan probes using the Platinum Quantitative RT-PCR Thermoscript One-step System (Invitrogen, France) following the manufacturer's instructions in 25 µl of a reaction mixture containing 5 µl of extracted RNA. For MNV-1 detection 0.2 µM of reverse and forward primers as well as probe were added. Primer sets and probes employed were Fw-ORF1/ORF2, Rv-ORF1/ORF2 and probeMGB-ORF1/ORF2 (Baert et al. 2008).

Amplification Conditions

A Mx3005P QPCR System (Stratagene, USA) thermocycler was employed following amplification conditions described by Baert et al. (2008) with minor modifications. Briefly, after a RT step at 45 °C for 1 h, PCR amplification was carried out employing the following cycling conditions: an initial denaturation at 95 °C for 5 min, and 50 cycles of amplification with a denaturation at 95 °C for 15 s and annealing-extension step at 60 °C for 1 min.

Quantification of MNV-1 was estimated by standard curves constructed with serial dilutions of viral RNA, plotting the number of genome copies against the C_t. This quantification was corrected with the extraction and qRT-PCR efficiencies and expressed as number of RNA viral genome copies per gram of digestive tissue (RNA/g DT). A sample with a $C_t \leq 41$, with no evidence of amplification in the negative controls, was considered as positive. The number of RNA copies present in each positive sample was estimated by comparing the C_t value of the sample to the standard curve. All samples were tested in duplicate and with a ten-fold dilution. The final concentration was then adjusted based on the dilution factor used.

Statistics

ANOVA analysis compared the results of the number of copies of RNA/g DT obtained among the different depuration times (T_0–T_7) and between the two species of molluscs. Moreover, *post-hoc* tests were–employed to determine the statistical significance of bioaccumulation and the viral reduction detected for each depuration using the Student-Newman-Keuls (SNK) and Dunnett's tests. All statistical analyses were permormed using the statistical package IBM SPSS v20.0.0 software.

Results

Physicochemical parameters of water did not significantly differ for each depuration cycle, and showed normal levels for the depuration process. The average values were: conductivity (mS/cm) 49; salinity 30.6‰; pH 8.1; water temperature 14.5 °C and dissolved oxygen 7.6 ppm. In addition to these parameters, the concentration of ammonia, nitrites and nitrates were evaluated, showing no differences among trials. Ammonia and nitrite concentrations ranged between 0 and 0.3 mg/l except in one of the experiments where nitrite values reached to 0.8 mg/l. Nitrate concentrations were between 0 and 50 mg/l.

Experimental depuration trials carried out with mussels, showed a quantification range at the initial stage (T_0) between 4.7and 5.9 logRNA copies/g DT. However, in one of them (trial 3) higher contamination levels were achieved at T_1 (24 h of depuration) coinciding with the highest contamination value reached in this trial (5.6 log RNA copies/g DT) (Table 14.1). All experiments with mussels showed some reduction in viral quantification. At the end of the depuration (T_7), results showed contamination values between 4.1 and 4.9 log RNA copies/g DT (Table 14.1, Fig. 14.2). The average reduction for these trials was 0.8 log units (74 % of reduction in RNA copies/g DT), being statistically significant ($p < 0.05$) from 72 h (T_3) until the end of the depuration period (T_7).

mussels contaminated with MNV-1

T_P	0	0	0	0	0	0 (±0.0)
T_0	5.44	5.88	4.66	5.72	5.11	5.36 (±0.5)
T_1	5.41	5.82	5.56	4.86	4.69	5.27 (±0.5)
T_2	4.85	5.22	5.01	5.94	4.57	5.12 (±0.5)
T_3	4.98	5.27	5.03	5.13	4.63	5.01 (±0.2)
T_4	4.54	5.15	4.93	4.20	4.32	4.63 (±0.4)
T_5	4.90	5.04	4.81	4.53	4.43	4.74 (±0.3)
T_6	5.02	4.83	4.59	4.86	3.86	4.63 (±0.5)
T_7	4.68	4.89	4.56	4.14	4.44	4.54 (±0.3)
vr[b]	0.76	0.99	0.10	1.59	0.67	0.82 (±0.54)

Results are as expressed as logRNA copies/g digestive tissues. T_P sampling at shellfish arrival, T_0 sampling at transfer of shellfish from bioaccumulation tanks to depuration system, T_1–T_7 day 1–7 samplings during purification process

[a]Average of the five-depuration trials and standard deviation

[b]Log virus reduction at the end of depuration

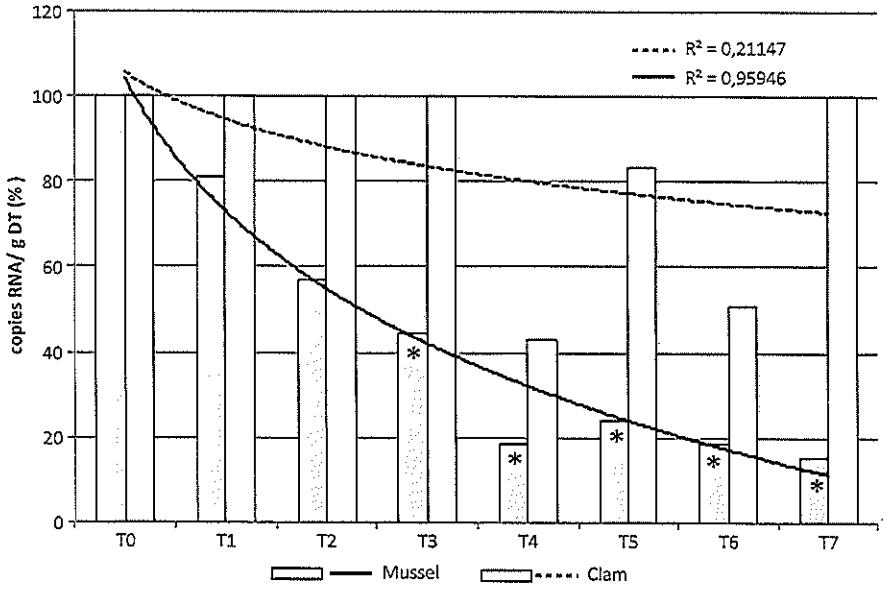

Fig. 14.2 Average removal kinetics for the five experimental depuration trials of mussels (*M. galloprovincialis*) and clams (*V. philippinarum*) artificially contaminated with MNV-1 (10^2 pfu/ml). Results are expressed as percentage (%) of RNA copies/g digestive tissues. T_P sampling at shellfish arrival, T_0 sampling at transfer of shellfish from bioaccumulation tanks to depuration system, T_1–T_7 day 1–7 samplings during purification process. *Significance at $p < 0.05$ with regard to the initial time of depuration

Trial	1	2	3	4	5	Av[a]
T_P	0.00	0.00	0.00	0.00	0.00	0.00 (±0)
T_0	6.49	5.54	6.27	6.04	6.57	6.18 (±0.4)
T_1	8.00	6.83	6.29	6.12	5.80	6.61 (±0.9)
T_2	7.63	7.24	5.27	5.51	5.77	6.28 (±1.1)
T_3	6.48	6.84	6.26	5.36	5.98	6.18 (±0.6)
T_4	6.07	5.81	5.85	5.65	5.70	5.82 (±0.2)
T_5	6.69	6.91	6.20	5.80	4.91	6.10 (±0.8)
T_6	6.41	6.94	5.54	5.52	5.03	5.89 (±0.8)
T_7	6.50	7.13	5.89	5.71	5.89	6.22 (±0.6)
vr[b]	−0.01[c]	−1.59[c]	0.38	0.33	0.68	−0.04 (±0.9)

Results are expressed as logRNA copies/g digestive tissues. T_P sampling at shellfish arrival, T_0 sampling at transfer of shellfish from the bioaccumulation tanks to the depuration system, T_1–T_7 day 1–7 samplings during purification process

[a]Average of the five-depuration trials and standard deviation

[b]Log units of viral removal at the end of the depuration

[c]Increase in the depuration rate between T_0 and T_7

In the five trials carried out with clams contaminated with MNV-1 (Table 14.2), quantification at T_0 ranged from 5.5 to 6.6 log RNA copies/g DT, however, in four out of five trials (1–4) the highest contamination levels were observed at T_1 (24 h of depuration), reaching values between 6.1 and 8.0 log RNA copies/g DT. In one of these trials (trial 2), the highest contamination value was reached at T_2 (7.2 log RNA copies/g DT). Only three out of five depuration trials (3, 4 and 5) showed some reduction in viral quantification (Table 14.2). The average reduction for these three experiments was 0.5 log units (41.4 % reduction in RNA copies/g DT). However, in trial 1 similar values were obtained at initial (T_0) and final (T_7) stage, and in trial 2 an increase in RNA copies/g DT of 1.6 log units was observed. At the end of the depuration (T_7), clams in the different experiments showed contamination values between 5.7 and 7.1 log RNA copies/g DT (Table 14.2). For the average of the five trials, no virus removal with regard to the initial depuration level (T_0) was achieved (Table 14.2, Fig. 14.2). No statistical significance was found for the average viral reduction among depuration times (T_0–T_7).

Significant differences ($p < 0.05$) in the viral uptake between molluscs were detected. Clams showed an average viral uptake of 5.4 log RNA copies/g DT, and mussels 6.2 log RNA copies/g DT. In addition, significant differences ($p < 0.05$) were also observed between clams and mussels for the average removal rate, being higher for mussels (74 % of reduction in RNA copies/g DT).

The main objective of this study was to obtain a broad picture of the MNV-1 removal dynamics, as a model of human norovirus behavior, in clams and mussels subjected to depuration processes. The inadequacy of current European regulations to assess the sanitary quality of shellfish and classification of harvesting areas to prevent viral contamination is well known (Anonymous 2004; Le Guyader et al. 2003; Romalde et al. 2002).In addition, enteric viruses concentrated by shellfish can persist under depuration conditions that are sufficient for bacteria removal. How infectious viruses can persist within the shellfish and their removal kinetics under depuration conditions are important questions to be addressed.

The results of this study showed differences in the viral uptake and the removal rate for viruses between clams and mussels subjected to an artificial bioaccumulation process with MNV-1. Fluctuations of the viral loads in consecutive depuration stages were detected, which can be attributed to the heterogeneity distribution of viral load. After 24 h of bioaccumulation the average viral uptake in clams was 73.8 % higher than in mussels. However, only three out of five depuration experiments with clams showed some reduction in viral quantification. The average reduction in these three experiments was 0.5 log units (41.4 % reduction in RNA copies/g DT). Mussels showed viral reduction in all the depuration trials with an average reduction of 0.8 log units (74 % reduction in RNA copies/g DT).

Bioaccumulation rates and removal dynamics of MNV-1 seem to be different in manila clams and mussels. Higher bioaccumulation levels were reached in clams, but higher depuration rates have been observed in mussels.

These results indicate a different behavior ofMNV-1 in these two bivalve species. Nappier et al. (2008), also reported a statistically higher bioaccumulation and retention rate of MNV, NoV and HAV in *Crassostrea ariakensis* than in *C. virginica*. Other studies carried out in our laboratory have shown a different bioaccumulation and removal pattern in Manila clams artificially contaminated with HAV and MNV-1 (unpublished data).

Although there are other studies in which viral bioaccumulation and subsequent depuration of enteric viruses in bivalve molluscs is determined, to our knowledge, this is the first to compare the rate of bioaccumulation and subsequent removal dynamics of murine norovirus by qRT-PCR in these two molluscs.

The observed differences in MNV-1 uptake and removal dynamics could be related to viral properties and/or to the existence of specific ligands. The specific binding of NoV strains to digestive tract of shellfish through an A-like human histo-blood group antigens (HBGAs) has been demonstrated (Le Guyader et al. 2006b; Tian et al. 2006, 2007). Bivalve shellfish could, therefore, specifically concentrate different viruses or specific strains on the basis of these receptors/ligands, while other virus could be accumulated to a lesser extent by nonspecific mechanisms of attachment, like mechanical entrapment and ionic bonding (Burkhardt and Calci 2000; Di Girolamo et al. 1977; Schwabm et al. 1998), and consequently, be more rapidly depurated.

tissue) (Le Guyader et al. 2006b; Mcleod et al. 2009; Wang et al. 2008). Viruses present in the digestive tract lumen could theoretically be removed relatively rapidly via defecation when shellfish are placed in clean water. The specific attachment and internalization of intact viral particles into gastrointestinal cells or captured by phagocytes in the main ducts could be a viral mechanism to avoid being degraded by the digestive system and may provide an explanation for the generally poor efficiency in removing viruses from shellfish (Le Guyader et al. 2006b; Mcleod et al. 2009). The relatively high levels of final viral loads in the depurated samples observed in this work support this hypothesis.

In terms of risk to the consumer, interpreting the real significance of these results is complicated. Although the final viral loads remain at relatively high levels in all samples, molecular techniques like qRT-PCR detect RNA copies rather than infective particles, and nucleic acids remain detectable for long periods. Assays are currently in progress to assess the infectivity of the virus detected after mollusc depuration.

Both the findings reported here, as in other studies mentioned above, suggest that virus concentration in shellfish is not a passive process. It may depend on many factors such as mucus production, glycogen content, water temperature, gonadal development (Maalouf et al. 2010), and possibly on the presence, amount and/or distribution of specific receptors in host tissues. In this way, not only a different virus or virus strain could behave differently in shellfish, but also one virus could behave differently in different bivalve species and/or in different stages of the mollusc life cycle.

Conclusions

Depuration can reduce viral levels in shellfish, but not sufficiently to consider them safe. The final viral load in samples remains in relatively high concentrations, and virus, unlike bacteria, can be infectious at very low doses. Thus, it is necessary to reduce virus to near negligible levels to improve the safety of shellfish but it is also necessary to understand that the depuration process is a method capable of reducing relatively low levels of contamination but not, at least for now, for highly contaminated shellfish. This study will provide the baseline for future studies focused on improving the efficacy of viral depuration of shellfish.

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