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Viral elimination during commercial depuration of shellfish

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ABSTRACT

The effectiveness of depuration for the removal of hepatitis A virus (HAV), norovirus (NoV) genogroups I (GI) and II (GII), and F+RNA bacteriophage (F+RNA) was evaluated for pullet carpet shell clams (*Venerupis pullastra*) and Mediterranean mussels (*Mytilus galloprovincialis*). The objective was to compare the behaviour of the different pathogens under commercial depuration conditions during 7 days in an authorized plant. Standard double agar overlay method (ISO 10705-1) was employed for F+RNA quantification. Recently developed ISO/TS 15216:2013 standard method, based on RT-real time PCR, were employed for the quantification of HAV and NoV. The reduction of F+RNA showed a two-phase depuration kinetic. The average reduction rates were 1-log units for clams and 2-log units for mussels, with residual levels after the process of 6.3×10^3 and 8.3×10^1 F+RNA/100 g, respectively. HAV, NoV GI and GII were detected intermittently throughout the entire process, ranging mostly from 10^3 to 10^5 RNA copies/g digestive tissue (DT). NoV GI showed the higher viral levels followed by NoV GII and HAV. All of them were detected in clams after seven days of depuration, however, in mussels only NoV GI was detected after the process. Generally, clams showed slower depuration rates and higher contamination levels for all viruses analysed.

1. Introduction

Shellfish are one of the most commonly transmission vector of different human enteric pathogens. Among them, the enteric viruses are the most frequently involved in foodborne outbreaks, and Norovirus (NoV) and hepatitis A virus (HAV) the leading etiological agents of food-borne illness (including shellfish), attending to the number of cases and to the illness severity, respectively (Koopmans & Duizer, 2004; Lees, 2000).

NoV, member of the family *Caliciviridae*, is the most important human pathogen of diarrhoea worldwide, being the main cause of food-borne outbreaks of acute gastroenteritis and sporadic infectious gastroenteritis (Atmar & Estes, 2006). HAV, member of the family Picornaviridae, displays a high degree of genetic conservation throughout the genome and is less common in countries with a high standard of hygiene. However, it may cause infection later in life, with the risk of a more severe disease outcome (Hollinger & Emerson, 2001).

The inability of sewage treatments to completely remove or inactivate viruses is well known (Da Silva et al., 2007; Iwai et al. 2009). According to their prevalence in the community, enteric viruses are discharged from sewage outfalls into fresh, marine and estuarine waters, and may subsequently be concentrated by shellfish, due to their filter-feeding activity. Globalization of food production and shellfish imports favours the transmission and dissemination of viruses around the world (Polo, Vilariño, Manso, & Romalde, 2010). Furthermore, the traditional way of consuming shellfish, raw or slightly cooked, and whole, including digestive tissues (where viruses are mainly bio-accumulated), increases the infection risk and makes the bivalves a high-risk food group (Romalde et al., 1994).

Shellfish depuration is a commercial compulsory process required in many countries for the fresh shellfish commercialization. The natural pumping activity of the bivalves is used in the process to purge the contaminants and reduce the likelihood of human pathogen transmission to consumers. Nevertheless, for

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decades the study of food-borne diseases linked to contaminated molluscs has mainly focused on bacterial pathogens. Consequently, controlling these pathogens has been the main objective of the shellfish sanitary controls and depuration systems and practices. The bacterial indicators are indeed the unique parameter used in current standards and depuration controls (Anon, 2004).

The compliance with the end product bacterial standards (≤230 *Escherichia coli*/100 g shellfish flesh in EU) is frequently seen as evidence of satisfactory design and operation of purification plants. However, virus removal is known to be less effective than bacterial removal and the compliance with standards cannot guarantee the viral absence (Loisy, Atmar, LeSaux, et al., 2005; Richards, McLeod, & Le Guyader, 2010; Romalde et al., 2002; Schwab, Neill, Estes, Metcalf, & Atmar, 1998; Ueki et al., 2007), a fact evidenced by the periodic outbreaks of Hepatitis A and gastroenteritis following the consumption of depurated shellfish (Chalmers & McMillan, 1995; Heller et al., 1986; Le Guyader et al., 2003, 2006).

From a virological point of view, shellfish safety continues to be a sanitary challenge and the severe impact of the viral enteric diseases on human populations has brought awareness by European authorities. Recently, it has been developed a standard method based on real time RT-PCR (RT-qPCR) for NoV and HAV quantification in foodstuffs, to be incorporated into EU legislation as a reference method (ISO/TS 15216).

This study focuses on the evaluation of the effectiveness and depuration kinetic of HAV, NoV (genogroups I and II) and F+RNA bacteriophage (a potential indicator for enteric viruses) from clam and mussels. The objective was to compare the behaviour of these viruses under commercial depuration in an authorized plant, extending the 48 h purification period applied commercially for a longer period of 7 days.

2. Material and methods

2.1. Samples

A total of 13 and 9 depuration trials were carried out with pullet carpet shell clams (*Venerupis pullastra*) and Mediterranean mussels (*Mytilus galloprovincialis*) respectively. Molluscs were naturally contaminated during 15 days in an intertidal zone near of a sewage discharge zone. After this period, the initial level of contamination (t0) for F+RNA, HAV and NoV was determined and then molluscs were collected and transferred to the depuration plant to undergo purification during 7 days. Each trial was carried out with 200 kg of mollusc. Bivalves were randomly sampled before (t0) and during the purification process (t1−t7) every 24 h. All samples were kept at 4 °C during shipment, washed and scrubbed thoroughly in water, shucked aseptically and analysed within 24 h.

2.2. Depuration system

Depuration process was carried out in an authorized commercial depuration plant in a separate area to avoid interferences with the normal operations in the plant. The depuration system consisted on a vertical system with two or three bins per column (100 m^3 each), with open water flow circuit and water treatment by chlorination (Fig. 1). Initial concentration of chlorine in the disinfection tank (Fig. 1: DT) was around 2 ppm. Due to the toxic nature of chlorine, the water is dechlorinated through a cascade system to provide vigorous aeration before contact with the shellfish (Fig. 1: AC and AT). This avoids the presence of chlorine in the depuration bins. Physico-chemical parameters of the water (conductivity, salinity, dissolved oxygen, pH, Temperature, and chlorine) were exhaustively controlled over the complete depuration period.

2.3. F+RNA bacteriophages detection and quantification

For the F+RNA bacteriophages analysis, shellfish flesh was homogenised in a blender for 2 min with peptone water (0.1% pH = 7.4; 1:2 w:v). Diluted homogenates were then centrifuged at 1000× *g* for 5 min at room temperature. The supernatant was decanted and a 1:10 and 1:100 dilutions with peptone water were prepared. Ten millilitres of the undiluted supernatant and ten-fold dilutions were then assayed by using 1-ml portions in 90 mm-petri dishes.

Quantification was performed according to the standard double agar overlay method ISO 10705-1 with *Salmonella typhimurium* WG49 as bacterial host. Briefly, replicate 1-ml portions of undiluted, 1:10 and 1:100 shellfish homogenates and 1-ml portions of a WG49 host culture were added to 2.5-ml portions of molten 1% tryptone−yeast extract agar at 45 °C. The melted agar and sample

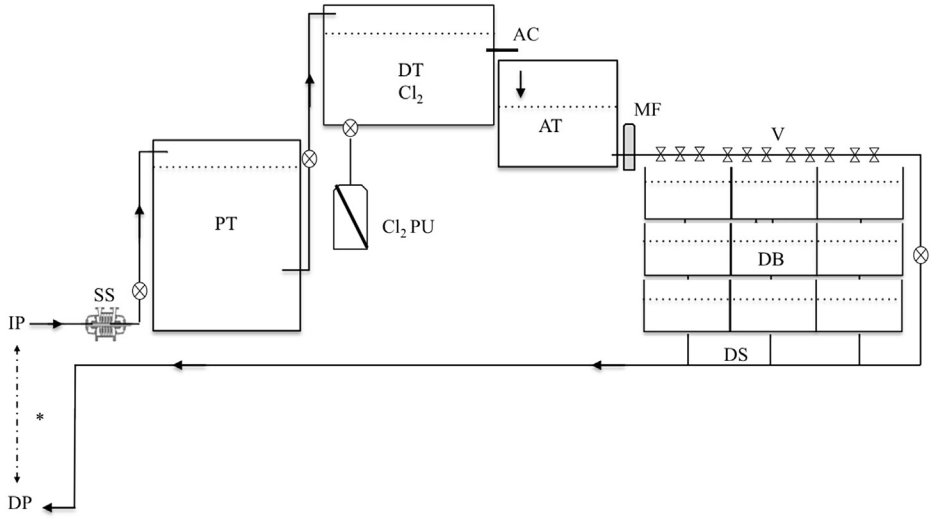

Fig. 1. Commercial depuration system. IP: Intake point; SS: Seawater system supply; PT: Primary tank for filtration and settlement; DT Cl_2: Disinfection tank with chlorine (Cl_2); Cl_2 PU: Production unit of Cl_2; AC: Aerator column; AT: Aerator tank; MF: Micro filter; V: Venturi Device; × : Water flow regulator; DB: Depuration bins; DS: Drain system; DP: Dischsrge point; *: Intake and discharge points are separated in time and space to avoid cross contamination.