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Depuration dynamics of viruses in shellfish

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Abstract

The consumption of shellfish has been associated with viral infections even in cases where shellfish complied with the current regulation, which is based on bacterial analysis. In this study, depuration rates of potential indicators and human viruses have been analysed in order to study the use of complementary parameters for evaluating the microbiological quality of depurated shellfish. Depuration of naturally highly polluted mussels has been evaluated and analyses for *Escherichia coli*, *Clostridium perfringens*, somatic coliphages, F-RNA phages and bacteriophages infecting *Bacteroides fragilis* RYC2056 and HSP40, human adenovirus, enterovirus have been done. Seawater of the depuration tank was disinfected by UV irradiation, ozone and passed through a skimmer and a biological filter. The correct functioning of the depuration tank was monitored by the quantification of total organic carbon (TOC), NH_4^+ and total aerobic bacteria in the seawater. To study the relation between the bacteriophages and the human viruses analysed, a logistic regression model was applied. F-RNA phages are significantly related to human adenoviruses and enteroviruses. Thus, they can be used as a complementary parameter for evaluating the efficiency of the depuration treatment. Somatic coliphages are also significantly associated with enteroviruses. Bacteriophages infecting *B. fragilis* HSP40 were analysed by the double-agar-layer (DAL) method, which quantifies infectious viruses, and by nested PCR, which detects the presence of the genome of these phages. The highest sensitivity of the molecular techniques was demonstrated and the results obtained are an indicator of a close relation between positive results by PCR and the presence of infectious viral particles in shellfish. All shellfish samples were negative for human viruses by PCR after 5 days of depuration treatment and the results obtained applying a regression model also showed negative results or nearly for F-RNA phages and bacteriophages infecting *B. fragilis* RYC2056. Thus, in this specific depuration treatment, 5 days may be necessary to assess the sanitary quality of shellfish.

Keywords: Shellfish; Mussels; Bacteriophages; Viruses; Human adenovirus; Enterovirus; Depuration; UV; Ozone

1. Introduction

Filter-feeding molluscan shellfish accumulate microorganisms, such as bacteria and human viruses, when grown in sewage-polluted waters and can present a significant health risk when consumed raw or lightly cooked (Fleet, 1978; West et al., 1985; Sobsey and Jaykus, 1991). Current regulations of shellfish and their growing waters are based on bacterial standards (faecal coliforms and *Escherichia coli*) and have prevented bacterial gastrointestinal infections. However, they are believed to have limited predictive value for viral

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pathogens such as enteroviruses (Gerba, 1979; Vaughn et al., 1979; Ellender et al., 1980; Jofre, 1992), Norwalk-like virus (NLV) and hepatitis A virus (HAV) (Wanke and Guerrant, 1990; Desenclos et al., 1991).

Several studies have revealed the differential rates of reduction of bacteria and viruses in depurating shellfish (Canzonier, 1971; Metcalf et al., 1979; Scotti et al., 1983; Power and Collins, 1989; De Mesquita et al., 1991; Dore and Lees, 1991, 1995). Thus, there is an urgent need for indicators of human-specific viral faecal pollution in order to improve the biological safety of shellfish. Somatic coliphages (Vaughn and Metcalf, 1974), bacteriophages infecting *Bacteroides fragilis* (Jofre et al., 1986; IAWPRC Study group on Health Related Water Microbiology, 1991; Lucena et al., 1994) and F-specific bacteriophages of RNA (F-RNA) (Havelaar and Hogeboom, 1984; Havelaar et al., 1986; IAWPRC Study group on Health Related Water Microbiology, 1991) have been proposed as potential indicators of infectious viruses. In previous studies (Pina et al., 1998; Muniain-Mujika et al., 2000), human adenoviruses have been proposed as molecular index of human viral contamination and these viruses were detected when enterovirus and/or HAV were also found to be positive.

To evaluate the period of depuration that ensures the microbiological safety of shellfish, we analysed the depuration rates of various parameters in naturally polluted shellfish using highly efficient depuration equipment. We also tested the efficiency of the proposed indicators of human viruses. Three different batches of mussels subjected to a depuration treatment were analysed for several days and treated by a common procedure for the comparative analysis of all microbiological parameters. For this purpose, shellfish samples were analysed for *Clostridium perfringens*, *E. coli*, somatic coliphages, F-RNA bacteriophages, phages infecting *B. fragilis* RYC2056 and HSP40, human adenoviruses, enteroviruses and hepatitis A virus.

2. Material and methods

2.1. Samples

Three batches of 20 kg each of naturally polluted mussels (*Mytilus galloprovincialis*) were collected from faecally polluted areas. The samples were washed in running water and transported to the depuration tank in less than 2 h after collection.

2.2. Depuration treatment

The mussels were placed in a tank of 200 kg of capacity equipped with particle filters. Seawater (1100–1200 l) at 17–18 °C (37‰ salinity and pH 8.00–8.04) was recirculated through the tank 6–8 times per hour, passed through a biological filter (for elimination of NH_4^+) and a skimmer (for elimination of colloids), and disinfected by UV irradiation and ozone.

Shellfish were depurated in plastic mesh baskets placed throughout the length of the tank (3 m). Every 24 h, three samples, from separate locations in the tank, were collected and all analyses began within 4 h of collection. Seawater was also collected every 48 h to quantify NH_4^+, total organic carbon (TOC) and mesophilic aerobic bacteria.

2.3. Treatment of samples

The mussels were washed, scrubbed thoroughly in running water and opened aseptically. The shellfish contents were then processed for microbial analyses. Shellfish meat (80 g/sample) was homogenised in a blender for 3 min.

2.4. Bacterial analysis

Bacterial elution was performed by adding 60 ml of peptone saline to 30 g of shellfish meat. Total coliforms and *E. coli* were assayed with the most probable number procedure, based on the multiple-tube fermentation technique, five tubes with three dilutions (APHA, AWWA, WPCF, 1995). Briefly, the first stage is a resuscitation step that requires inoculation using mineral modified glutamate broth (MMGB, Oxoid, Basingstoke, Hampshire, England) with diluted shellfish homogenates and incubation at 37 °C for 24 h. The presence of *E. coli* is subsequently confirmed by subculturing acid producing tubes onto agar containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronide and detecting β-glucuronidase activity (Donovan et al., 1998). *C. perfringens* spores were assayed by thermal shock at 75 °C for 15 min, inoculated into Sulfit Polymixin Sulfadiazin (SPS) agar (Scharlau-Microbiology, Barcelona, Spain) and incubated for 24 h at

44 °C following the method described by Handford (1974). Mesophilic aerobic bacteria in seawater were analysed by inoculation in tryptic soy agar (TSA, Scharlau-Microbiology) and incubation for 24 h at 37 °C.

2.5. Viral elution

Phages and human viruses were eluted as described by Pina et al. (1998), with some modifications (Muniain-Mujika et al., 2000). Briefly, viruses were eluted by adding 200 ml of glycine buffer 0.25 N at pH 10−50 g of shellfish meat (1:5, w/v). The mixture was homogenised by magnetic stirring for 15 min at room temperature and the pH was adjusted to 7 ± 0.2. This homogenate was then clarified by centrifugation at $2170 \times g$ for 15 min at 4 °C. Phages present in 25 ml of the supernatant (corresponding to 5 g of shellfish meat) were quantified. The rest of the supernatant was centrifuged at $39{,}800 \times g$ for 45 min at 4 °C and the new supernatant was ultracentrifuged, as described below, to concentrate viral particles.

2.6. Analyses of bacteriophages

E. coli WG5 grown on modified Scholten's broth (MSB: 10 g of peptone; 3 g of yeast extract; 12 g of meat extract; 3 g of NaCl; 5 ml of Na_2CO_3 solution (150 g/l); 0.3 ml of Mg solution (100 g $MgCl_2{\cdot}6H_2O$ in 50 ml water) and 1000 ml of distilled water) was used as host strain for the quantification of somatic coliphages. *Salmonella typhimurium* WG49 (Havelaar et al., 1993) grown on tryptone-yeast extract-glucose broth (TYGB: 10 g of trypticase peptone; 1 g of yeast extract; 8 g of NaCl and 1000 ml of distilled water) was used as host strain for F-specific bacteriophages of RNA. *B. fragilis* RYC2056 and HSP40, grown on *Bacteroides* phage recovery medium broth (BPRMB: 10 g of meat peptone; 10 g of casein peptone; 2 g of yeast extract; 5 g of NaCl; 0.5 g of monohydrated L-cysteine; 1.8 g of glucose; 0.12 g of $MgSO_4{\cdot}7H_2O$; 1 ml of $CaCl_2$ solution (0.05g/ml) and 1000 ml of distilled water), were used as host strains for the quantification of *B. fragilis* bacteriophages. All procedures are described in the corresponding standardised protocol (Anonymous, 1996, 1999a,b). All the phages were quantified by the double-agar-layer method. Briefly, 1 ml of appropriately diluted centrifuged mussel homogenate and 1 ml of exponentially growing host strain were added to 2.5 ml of molten agar (Scholten's modified semi-solid agar (ssMSA: MSB with 7.5 g agar) for somatic coliphages, tryptone-yeast extract-glucose semi-solid agar (ssTYGA: TYGB with 7.5 g agar) for F-RNA phages and semi-solid *Bacteroides* phage recovery medium agar (ssBPRMA: BPRMB with 7.5 g of agar) for phages of *B. fragilis*) at 45 °C. This mixture was stirred and poured onto previously prepared agar bases MSA (MSB with 15 g agar); TYGA (TYGB with 15 g of agar) or BPRMA (BPRMB with 15 g of agar) in 90-mm-diameter Petri dishes. The overlays were inverted and incubated overnight at 37 °C.

2.7. Concentration of viral particles and nucleic acid extraction

Fifty milliliters of the supernatants obtained by the elution method was ultracentrifuged ($229{,}600 \times g$ for 1 h at 4 °C) to pellet all the viral particles together with any suspended material. The pellet was resuspended in 50 μl of $1 \times$ PBS (phosphate-buffered saline) and stored at -18 ± 2 °C for nucleic acid extraction.

Nucleic acids were extracted following Boom et al. (1990), with minor modifications (Puig et al., 1994). Briefly, 50 μl of viral suspension was added to a mixture of 50 μl of the silica particle suspension and to 900 μl of lysis buffer. The silica particles (silicon dioxide, SiO_2; Sigma, St. Louis, MO, USA) were suspended in demineralized water and adjusted to pH 2 as described by Boom et al. (1990) and the lysis buffer was prepared by adding 120 g of GuSCN (Guanidine Thiocyanate Salt, Sigma-Aldrich, Steinheim, Germany), 100 ml of 0.1 M Tris−HCl pH 6.4, 22 ml of 0.2 M EDTA adjusted to pH 8 with NaOH and 2.6 g of Triton X-100 (Merck, 64271 Darmstadt, Germany). The mixture was left for 10 min at room temperature and washed twice in 1 ml of washing buffer (120 g of GuSCN in 100 ml of 0.1 M Tris−HCl, pH 6.4), twice more with ethanol 70% and once with acetone. The pellet obtained after the complete evaporation of acetone was resuspended with 50 μl of elution buffer (49.4 μl of DTT (dithiothreitol, Sigma-Aldrich), 1 mM in Tris−EDTA (10 mM Tris (Merck), 0.1 mM Ethylenediaminotetraacetic acid (EDTA, Merck)) and 0.6 μl of RNasin (an RNase inhibitor;

Perkin-Elmer, Applied Biosystems by Roche Molecular Systems, Branchburg, NJ, USA) (5 U/μl final concentration) to allow nucleic acid elution from silica particles. The resulting supernatant was used in cDNA synthesis for enteroviruses and hepatitis A viruses and in PCR amplification for human adenoviruses and phages infecting *B. fragilis* HSP40.

2.8. Oligonucleotide primers

The sequences (Table 1), sensitivity and specificity of the primers used have been defined in previous studies (Pina et al., 1998; Puig et al., 1994, 2000).

2.9. PCR amplification

Five microliters of extracted nucleic acids, corresponding to 1 g of shellfish meat, was used for the detection of enterovirus and HAV by RT-PCR and nested PCR. Ten microliters of extracted nucleic acids, corresponding to 2 g of shellfish meat, was used for the detection of human adenovirus and phages infecting *B. fragilis* HSP40 by nested PCR. The procedures have been described in previous studies (Pina et al., 1998; Muniain-Mujika et al., 2000; Puig et al., 2000). The first step of PCR was performed in a 30-cycle reaction; 1 μl of the amplified DNA was added to a new batch of reaction mixture for nested PCR, which was performed for 30 more cycles.

To reduce cross-contamination by amplified DNA molecules, standard precautions were taken in all manipulations. Separate areas were used for reagents, treatment of samples and manipulation of amplified samples. Undiluted samples and 1/10 dilutions of the nucleic acid extracts were analysed twice by independent experiments and a negative control was added for all samples. As in previous studies (Muniain-Mujika et al., 2000), the estimated sensitivity of the technique was of 10 viral particles/g of animal tissue.

2.10. Statistical analysis

The variables *C. perfringens*, *E. coli*, somatic coliphages, F-RNA bacteriophages and phages infecting *B. fragilis* RYC2056 were transformed by the

Table 1
Oligonucleotide primers used for PCR amplification of human adenovirus, enterovirus, HAV and phages infecting *B. fragilis* HSP40

Virus type (region)	Position	Amplification reaction	Primers	Product size (bp)	Sequence
Ad2[a] (hexon)					
Ad40 (hexon)	18858–18883[b]	First	hexAA1885	301	5′ -GCCGCAGTGGTCTTACATGCACATC-3′
Ad41 (hexon)	19136–19158[b]	First	hexAA1913		5′ -CAGCACGCCGCGGATGTCCAAAGT-3′
Ad2 (hexon)	18937–18960[b]	Nested	nehexAA1893	143	5′ -GCCACCGAGACGTACTTCAGCCTG-3′
Ad2 (hexon)	19051–19079[b]	Nested	nehexAA1905		5′ -TGTACGAGTACGCGGTATCCTCGCGGTC-3′
Polio 1 (5′ NTR)					
CV B4 (5′ NTR)	64–83[c]	First	Ent 1[d]	540	5′ -CGGTACCTTTGTACGCTGT-3′
Polio 1 (5′ NTR)	578–597[c]	First	Ent 2		5′ -ATTGTCACCATAAGCAGCCA-3′
Polio 1 (5′ NTR)	430–450[c]	Nested	neEnt 1	123	5′ -TCCGGCCCTGAATGCGGCTA-3′
CV B4 (5′ NTR)	547–567[c]	Nested	neEnt 2		5′ -GAAACACGGACACCCAAAGTA-3′
HAV (5′ NTR)	332–352	First	HAV1	368	5′ -TTGGAACGTCACCTTGCAGTG-3′
HAV (5′ NTR)	680–700	First	HAV2		5′ -CTGAGTACCTCAGAGGCAAAC-3′
HAV (5′ NTR)	371–391	Nested	neHAV1	290	5′ -ATCTCTTTGATCTTCCACAAG-3′
HAV (5′ NTR)	641–661	Nested	neHAV2		5′ -GAACAGTCCAGCTGTCAATGG-3′
PBF–HSP40 (H–S)[e]	60–79	First	pH5-1	442	5′ -GGGAAAGCACACAAGCG-3′
PBF–HSP40 (H–S)[e]	501–482	First	pH5-3		5′ -CAGAACATTAGTTTTACGG-3′
PBF–HSP40 (H–S)[e]	130–149	Nested	npH5-4	328	5′ -GTGGCACGTGAACTTCCTTC-3′
PBF–HSP40 (H–S)[e]	457–439	Nested	npH5-7		5′ -CGTTTTGCATGGCATCCG-3′

[a] Ad, adenovirus; CV, coxsackievirus.
[b] The sequence positions are referred to the Ad2 hexon region.
[c] The sequence positions are referred to the coxsackievirus B4 5′ NTR.
[d] Modified from Gow et al. (1991).
[e] S–H is *Hin*dIII–*Sal*I subfragment of H5 fragment.

$\log_{10}(x+1)$ function, previously to the statistical analysis. The regression model and the logistic regression models were applied.

The statistical tests were computed using the statistical package SPSS 10.0.7 (SPSS Inc. Headquarters, 233 S. Wacker Drive, 11th floor, Chicago, IL 60606, USA) on a Pentium III computer using MS-W2K Professional. The equality of the regression lines was tested with MS Excel XP.

3. Results

3.1. Microbiological analysis

The mussels collected in faecally polluted areas were three batches of 20 kg each with high levels of contamination. Three samples of each batch placed through the depuration tank were analysed every 24 h for *E. coli*, *C. perfringens*, somatic coliphages, F-RNA phages, phages infecting *B. fragilis* RYC2056 (PBF RYC) and HSP40 (PBF HSP40), human adenoviruses, enteroviruses and hepatitis A virus for 18 days for the first batch, 13 for the second and 6 days for the third. To make sure that the depuration tank worked properly, TOC, NH_4^+ and total aerobic bacteria in water were periodically analysed, obtaining the expected results. TOC results were always <4.3 ppm except 1 day, probably because the sample was placed before the removal of the water sample; NH_4^+ was <1 ppm 3 days after placing shellfish in the tank and total aerobic bacteria were in all the samples <10 CFU/ml confirming the correct functioning of the tank.

The microbiological parameters are shown in Tables 2, 3 and 4. HAV results are not shown because all the samples were negative. Two of the batches were collected in an area classified as type B by the European Union regulation, Directive 91/492/CEE,

Table 2
Microbiological parameters obtained in the depuration treatment of batch 1. Results are the media of the three samples analysed from different locations in the tank each day (except the day 0 when the original sample was analysed), expressed in CFU/g[a] for bacteria, PFU/g[b] for bacteriophages

Day	*E. coli*	*C. perfringens*	Somatic coliphages	F-RNA[c] phages	PBF[d] of RY2056	PBF[d] of HSP40	Human Adenovirus	Enterovirus	PBF[d] of HSP40
0	35	7500	6020	75	21	9	+	+	+
1	1	25	2230	52	5	1	+/+/+	+/+/+	+/+/−
2	0.3	16.7	2152.7	12.3	1.3	<0.2	+/−/−	+/+/+	−/−/−/−
3	1.4	5	1028.3	1	<0.2	<0.2	+/+/+	−/+/+	−/−/−/−
4	0.1	8.3	583.3	0.7	<0.2	<0.2	+/+/+	−/+/−	−/−/−/−
5	<0.2	12.7	740.3	<0.2	<0.2	<0.2	−/−/−/−	−/−/−/−	−/−/−/−
6	<0.2	6.3	378	<0.2	<0.2	<0.2	−/−/−/−	−/−/−/−	−/−/−/−
7	<0.2	7.7	329	<0.2	<0.2	<0.2	−/−/−/−	−/−/−/−	−/−/−/−
8	<0.2	6	364.7	<0.2	<0.2	<0.2	−/−/−/−	−/−/−/−	−/−/−/−
9	<0.2	4.7	328	<0.2	<0.2	<0.2	−/−/−/−	−/−/−/−	−/−/−/−
10	<0.2	3.7	307	<0.2	<0.2	<0.2	−/−/−/−	−/−/−/−	−/−/−/−
11	<0.2	2	352.7	<0.2	<0.2	<0.2	−/−/−/−	−/−/−/−	−/−/−/−
12	<0.2	3	253	<0.2	<0.2	<0.2	−/−/−/−	−/−/−/−	−/−/−/−
13	<0.2	3.3	155.3	<0.2	<0.2	<0.2	−/−/−/−	−/−/−/−	−/−/−/−
14	<0.2	3.3	108.7	<0.2	<0.2	<0.2	−/−/−/−	−/−/−/−	−/−/−/−
15	<0.2	2.3	87.7	<0.2	<0.2	<0.2	−/−/−/−	−/−/−/−	−/−/−/−
16	<0.2	1.7	108	<0.2	<0.2	<0.2	−/−/−/−	−/−/−/−	−/−/−/−
17	<0.2	2.3	86	<0.2	<0.2	<0.2	−/−/−/−	−/−/−/−	−/−/−/−
18	<0.2	1.7	63.7	<0.2	<0.2	<0.2	−/−/−/−	−/−/−/−	−/−/−/−

Results for human viruses and phages infecting *B. fragilis* HSP40 analysed by PCR in 1 g of shellfish are expressed in + or − for each sample.

[a] CFU/g: colony-forming unit per gram.

[b] PFU/g: plaque-forming unit per gram.

[c] F-RNA: F-specific phages of RNA.

[d] PBF: Phages of *B. fragilis*.

Table 3
Microbiological parameters obtained in the depuration treatment of batch 2. Results are the media of the three samples analysed from different locations in the tank each day (except the day 0 when the original sample was analysed), expressed in CFU/g[a] for bacteria, PFU/g[b] for bacteriophages

Day	*E. coli*	*C. perfringens*	Somatic coliphages	F-RNA[c] phages	PBF[d] of RY2056	PBF[d] of HSP40	Human Adenovirus	Enterovirus	PBF[d] of HSP40
0	160	8000	4265	264	145	22	+	+	+
1	1.3	17.7	4276.7	27.3	47.7	13.7	+/+/+	+/−/+	+/+/+
2	0.3	12.3	1301	27.3	42	7.3	+/+/+	+/+/+	+/+/+
3	<0.2	12.3	335.7	2	3.7	<0.2	+/+/−	+/+/+	+/+/−
4	<0.2	8	236	<0.2	<0.2	<0.2	+/+/−	−/−/−	−/−/−
5	<0.2	12.3	316.7	<0.2	<0.2	<0.2	−/−/−	−/−/−	−/−/−
6	<0.2	8	251	<0.2	<0.2	<0.2	−/−/−	−/−/−	−/−/−
7	<0.2	7.7	197.3	<0.2	<0.2	<0.2	−/−/−	−/−/−	−/−/−
8	<0.2	5.3	188	<0.2	<0.2	<0.2	−/−/−	−/−/−	−/−/−
9	<0.2	6.7	153.3	<0.2	<0.2	<0.2	−/−/−	−/−/−	−/−/−
10	<0.2	4	137.7	<0.2	<0.2	<0.2	−/−/−	−/−/−	−/−/−
11	<0.2	4.3	92	<0.2	<0.2	<0.2	−/−/−	−/−/−	−/−/−
12	<0.2	3	86.7	<0.2	<0.2	<0.2	−/−/−	−/−/−	−/−/−
13	<0.2	2.3	75.3	<0.2	<0.2	<0.2	−/−/−	−/−/−	−/−/−

Results for human viruses and phages infecting *B. fragilis* HSP40 analysed by PCR in 1 g of shellfish are expressed in + or − for each sample.
[a] CFU/g: colony-forming unit per gram.
[b] PFU/g: plaque-forming unit per gram.
[c] F-RNA: F-specific phages of RNA.
[d] PBF: Phages of *B. fragilis*.

which specified that less than 6000 faecal coliforms/100 g of mussel meat or less than 4600 *E. coli*/100 g in 90% of samples corresponds to a type B area. One of the batches was collected for depuration from an area considered as C by the European Union regulation, Directive 91/492/CEE, which specified that between 6000 and 60,000 faecal coliforms/100 g of mussel meat or more than 4600 *E. coli*/100 g corresponds to a C area. After 24 h of depuration, the levels of *E. coli* strongly decreased in all the cases and these batches of mussels came to belong to a type A area according to the European Union regulation (less than

Table 4
Microbiological parameters obtained in the depuration treatment of batch 3. Results are the media of the three samples analysed from different locations in the tank each day (except the day 0 when the original sample was analysed), expressed in CFU/g[a] for bacteria, PFU/g[b] for bacteriophages

Day	*E. coli*	*C. perfringens*	Somatic coliphages	F-RNA[c] phages	PBF[d] of RY2056	PBF[d] of HSP40	Human Adenovirus	Enterovirus	PBF[d] of HSP40
0	6	580	1412	73	30	6	+	−	+
1	0.6	8	364.3	13.3	11	4.7	+/+/+	−/−/−	+/+/+
2	0.3	6	323.7	5.7	1.7	<0.2	+/−/+	−/−/−	+/+/+
3	0.2	5	226	3.7	<0.2	<0.2	+/+/−	−/−/−	−/−/−
4	<0.2	4.7	194	2	<0.2	<0.2	−/−/−	−/−/−	−/−/−
5	<0.2	5	153.7	<0.2	<0.2	<0.2	−/−/−	−/−/−	−/−/−
6	<0.2	2.7	113.7	<0.2	<0.2	<0.2	−/−/−	−/−/−	−/−/−

Results for human viruses and phages infecting *B. fragilis* HSP40 analysed by PCR in 1 g of shellfish are expressed in + or − for each sample.
[a] CFU/g: colony-forming unit per gram.
[b] PFU/g: plaque-forming unit per gram.
[c] F-RNA: F-specific phages of RNA.
[d] PBF: Phages of *B. fragilis*.

300 CFU of faecal coliforms or less than 230 CFU of *E. coli* per 100 g of shellfish meat), being suitable for direct human consumption. However, within only 24 h of depuration, human viruses can be detected, indicating that *E. coli* is not a good parameter to assess viral contamination in shellfish (Jofre, 1992; Dore and Lees, 1995).

Phages infecting *B. fragilis* HSP40 were analysed in all the experiments by enumeration of plaques following the double-agar overlaying method and by nested PCR to compare the effectiveness of these methods and the stability of viral genomes versus infectious particles.

3.2. Statistical analysis

The statistical significance of the linear regression was tested over time for *C. perfringens*, *E. coli*, somatic coliphages, F-RNA bacteriophages and phages infecting *B. fragilis* RYC2056, as was the significance between regression lines at different batches (Rawlings, 1988). The prediction for the fifth day and its upper limit confidence interval were also computed (Table 5).

The binary variables human adenovirus and enterovirus were analysed following three logistic regression models (Agresti, 1990). We aimed to measure the predictive capacity of time, the batch and the variables associated with phages on the binary variables human adenoviruses and enteroviruses. The three models included the batch as classificatory variable and time as covariate, but they differed in the latest covariate, which was, respectively, somatic coliphages, F-RNA bacteriophages and phages infecting *B. fragilis* RYC2056. The purpose of separately testing a model for each covariate associated with phages was to reduce any possible masking effect due to multicollinearity.

Table 5
Predicted values obtained in the regression model for F-specific phages of RNA and bacteriophages infecting *B. fragilis* RYC2056 in the fifth day of depuration treatment. Results are expressed in PFU/g[a]

	Batch 1	Batch 2	Batch 3
F-RNA[b] phages	− 0.36	− 0.45	0.13
PBF[c] of RYC2056	− 0.37	− 0.36	− 0.45

[a] pfu/g: plaque forming unit per gram.
[b] F-RNA: F-specific phages of RNA.
[c] PBF: Phages of *B. fragilis*.

4. Discussion

In accordance with current regulations, shellfish are commercialised and consumed when *E. coli* levels are less than 230 CFU per 100 g of shellfish meat. This value is detected in shellfish grown in A and B areas often after 24 h of depuration. However, the results obtained demonstrate that 24 h are not sufficient to achieve microbiological safety of this product. This is also confirmed by the frequent outbreaks of diarrhoea and hepatitis associated with the consumption of shellfish with accepted levels of *E. coli* (Heller et al., 1986; Chalmers and Mcmillan, 1995; Perret and Kudesia, 1995; Ang, 1998).

Shellfish passively bioaccumulate different solid particles and microorganisms like bacteria and viruses, mainly in the digestive gland. When a depuration treatment is applied in seawater with low levels of nutrients, shellfish feed on the material accumulated in this gland. Accordingly, the weight of these digestive diverticula during the depuration treatment diminished with time and even disappeared in some cases (data not shown).

Bacteriophages infecting *B. fragilis* HSP40 were analysed by the double-agar-layer (DAL) method, which quantifies infectious viruses, and by nested PCR, which is used to detect the presence of viral particles. PCR gave positive results even when the results obtained by DAL were negative. The difference between positive results for PCR and DAL could be explained for the highest sensitivity of the molecular techniques as it has been described (Puig et al., 2000). These results also strongly indicate a close relation between positive results by PCR in shellfish and the presence of infectious viral particles.

The elimination of the microbiological parameters was evaluated by applying the logistic regression model. The significant relation observed between F-RNA phages and human adenoviruses ($P = 0.08$) and enteroviruses ($P < 0.001$) presents an overall correct classification of the model of 83.3% for human adenoviruses and 87.5% for enteroviruses and indicates that F-RNA phages could be used as a comple-

mentary parameter for evaluating the efficiency of the depuration treatment. Somatic coliphages have also a significant relation with enteroviruses ($P=0.06$), with an overall correct classification of 93.8%. The behaviour of these phages in the depuration tanks, with positive results even after 18 days, suggests that their depuration dynamics differs from that of other viruses. However, they could be used as a complementary parameter to study a depuration process due to their high levels in the environment and their easy quantification. The model predicting the presence of human viruses does not include phages infecting *B. fragilis* RYC2056 ($P=0.15$ for human adenoviruses and $P=0.27$ for enteroviruses). In previous studies, a significant relation has been detected between F-RNA phages (Chung et al., 1998) or phages infecting *B. fragilis* RYC2056 (Muniain-Mujika et al., 2000) with human viruses, suggesting that these phages are potential indicators of faecal viral contamination in shellfish. The lack of a significant relation between phages infecting *B. fragilis* RYC2056 and the viruses studied in these depuration experiments may be due to the low number of these bacteriophages detected in the analysed samples.

One of the objectives of this study was to evaluate the duration of the depuration treatment that is required to produce shellfish without viral genomes detectable by PCR. The regression model allows an alternative approach to point estimates of the specific number of days. It is possible to make accurate predictions considering the dynamic of the entire depuration process. The values of F-RNA phages and bacteriophages infecting *B. fragilis* RYC2056 observed on the fifth day of depuration are always <5 PFP/g, even in the sample collected in an area classified as C and not allowed for consumption (batch 2). The predictions done with the regression model also show negative values or nearly zero for these phages and the results obtained for human viruses were also negative the fifth day of depuration in all the experiments. Realistically, the characteristics of the market do not permit shellfish to be kept in depuration tanks for long periods of time. Moreover, further experiments are required to confirm the presented results but there are data enough to suggest that shellfish should be depurated in tanks with the technical characteristics described here (UV irradiation, ozone, skimmer and biological filter) for about 5 days.

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