THE USE OF MALE-SPECIFIC RNA BACTERIOPHAGE TO MODEL AND PREDICT VIRUS REMOVAL BY PACIFIC OYSTERS DURING DEPURATION

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Introduction

Sewage contaminated bivalve molluscan shellfish present a considerable public health risk when consumed raw or lightly cooked. Treatment of such shellfish by self-purification in tanks of clean seawater (depuration) has been employed extensively to reduce this risk (Richards, 1988). Despite this, outbreaks of viral illness following the consumption of depurated oysters continue (Chalmers and Mcmillan1995; Kohn *et al*., 1995) and viruses have been shown to persist in depurated oysters in the absence of *E. coli* the traditional indicator of the sanitary quality of shellfish. The principle illness associated with the consumption of depurated oysters in the UK is gastro-enteritis caused by Norwalk Like Viruses (NLVs). Difficulty in detecting these viruses has hindered research into viral elimination during depuration.

Male-specific RNA (FRNA) bacteriophages have similar physical and genomic characteristics as the NLVs and are commonly found in sewage. Because of this and their ease of analysis they have been proposed both as an indicator of the behaviour of viruses in shellfish (Doré and Lees, 1995) and as an index organism for the viral risk associated with shellfish (Doré *et al*., 2000). Investigations by this laboratory have demonstrated that depurated oysters associated with outbreaks of gastro-enteritis and containing NLVs also contained levels of FRNA bacteriophages even in the absence of *E. coli*. Conversely to date, depurated oysters which have been shown to be free of FRNA bacteriophages have also been shown to be free from NLVs. It is considered that if depuration procedures were developed which produced oysters free from FRNA bacteriophage such procedures would also be likely to produce oysters free from NLVs and other human viruses. In this study we investigated the effect of temperature on the elimination of viruses from oysters (*crassostrea gigas*) during depuration using FRNA bacteriophage as an indicator organism.

Materials and Methods

Contamination of Oysters

Pacific oysters (*Crassostrea gigas*), obtained from a commercial harvesting area, were contaminated by relaying approximately 800m from a sewage outfall for a minimum of two weeks. The sewage outfall discharged approximately 3.6x10 M^3 dry weather flow of secondary treated effluent per day. During relaying oysters were held in mesh bags on trestles.

Depuration

Experimental systems had tanks with dimensions of 1,050 mm (length) by 500mm (width) by 450mm (high) with a working volume of 150 litres. Seawater (30-33â salinity) was recirculated lengthways through the tank at a rate of 1200 litre/hour and sterilised by irradiation in a 15W UV steriliser (type 15/3p UVAQ Ltd., Sudbury, UK.). Temperature was maintained by the use of aquarium heaters (elevated temperatures) or by placing the whole tank in controlled temperature room (low temperatures). Dissolved oxygen was maintained above 80% saturation by the use of a spray bar for recirculated water. Shellfish were depurated in plastic mesh baskets (no. 41042; Sommer Alibert [UK] Ltd) and raised of the base of the tank to avoid recontamination by voided faecal material. Filtered seawater was circulated through the depuration system and irradiated for at least 24 hours prior to each experiment. Contaminated oysters were thoroughly washed using a pressure washer and damaged or gaping shellfish discarded. Oysters were loaded into baskets in a single overlapping layer. For all experiments depuration was started within 18 hours of shellfish collection. Depuration was carried out under various conditions to investigate specific parameters.

Sampling and analysis

A minimum of duplicate samples of 6-10 oysters were taken from all experiments on each day of depuration over a minimum period of depuration of 5 days. Samples were assayed for FRNA bacteriophage within 24 hours of collection. Samples were assayed for FRNA bacteriophage using methods described previously (Doré *et al*, 2000)

Results

Temperature was found to have a significant effect on the rate of elimination of FRNA bacteriophage with elimination rates increasing with depuration temperature. Elimination curves at 8, 14 and 20°C from one experiment are shown in figure 1 and are typical of all experiments conducted. FRNA bacteriophage levels in oysters depurated at 8°C for 5 days were reduced to levels ranging between 6.6 and 42.5% (n=6) of initial contaminant levels whereas levels in oysters depurated at 20°C (n=14) were reduced to levels ranging between 0.2 and 3.8% of initial levels in the same time.

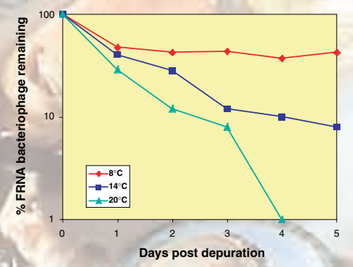


Figure 1. Elimination of FRNA bacteriophage from C. gigas during depuration at 8, 14 and 20°C.

Significantly, the increased rate of FRNA bacteriophage elimination associated with elevated temperature was found to be consistent and independent of season or harvesting area temperature. Given that the rate of FRNA bacteriophage elimination was constant at any given temperature regardless of season it was possible to determine the rate of elimination of F+bacteriophage from oysters at the three temperatures investigated during this study. Log_{10} values of FRNA bacteriophage were plotted against time and a best fit linear regression curve applied using the Excel computer programme. From this best fit curve it was possible to apply a straight-line equation;

Y = mX+C

Where **Y** = Dependent variable (Log_{10} phage levels pfu/100g)

X = Independent variable (days post depuration)

C = Intercept (Log_{10} phage levels pfu/100g)

m = Rate of elimination (Log_{10}) of FRNA bacteriophage/ day

Given that for each experiment X, Y and C are known it is possible to calculate m, the rate of elimination. The values obtained for m from each experiment performed for 8, 14 and 20°C are shown in table 1. along with the coefficient of determination (r^2) as a measure of the fit of the curve. Average rates of elimination were determined as 0.14, 0.2 and 0.36 for 8, 14 and 20°C respectively.

Table 1. Calculated rates of FRNA bacteriophage elimination expressed as Log_{10} reduction of pfu/100g per day during depuration at 8, 14 and 20°C.

	8°C		14°C		20°C	
Date	Rate	r^2	Rate	r^2	Rate	r^2
7/1/98	0.17	0.96			0.4	0.99
15/1/98	0.06	0.56			0.25	0.88
27/1/98			0.2	0.87	0.28	0.96
17/3/98	0.10	0.87			0.34	0.86
18/5/98	0.27	0.57			0.32	0.91
19/6/98	0.09	0.61			0.34	0.96
20/7/98	0.13	0.91			0.45	0.93
4/11/98					0.43	0.99
17/11/98					0.3	0.93
1/12/98					0.56	0.98
9/12/98					0.42	0.99
8/1/99					0.36	0.99
25/1/99					0.52	0.94
16/8/99					0.14	0.85
31/8/99	0.15	0.83				
4/10/99			0.15	0.97		
15/11/99			0.18	0.97		
7/12/99					0.37	0.85
24/1/00			0.29	0.98		
6/3/00			0.21	0.96		
Average	0.14		0.20		0.36	

Levels of FRNA bacteriophage in oysters from the experimental site prior to depuration varied with season (Figure 2). A clear seasonal trend was observed with geometric mean values for FRNA bacteriophage levels in the summer months (April to September) almost three times lower than during the winter months (October to March). Significantly complete elimination of FRNA bacteriophage was only achieved after five days depuration at 20°C on two occasions with both occasions occurring in the summer (18th May and 20th July) when FRNA bacteriophage levels were at their lowest (14,006 and 3,450 pfu/100g respectively). This suggests that initial contaminant loading, as well as depuration temperature is a critical factor in determining whether complete elimination of FRNA bacteriophage can be achieved during depuration.

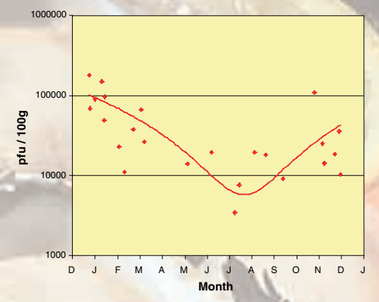


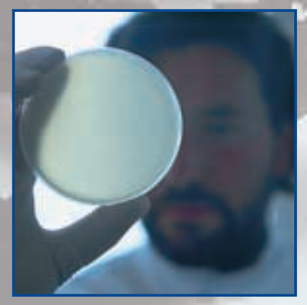
Figure 2. Seasonal distribution of FRNA bacteriophage in C. gigas at experimental harvest area.

Using the elimination rates determined above it was possible to predict the level of FRNA bacteriophage remaining at any time during depuration at each temperature for any starting level. Predicted final FRNA bacteriophage levels for each experiment are compared with the actual levels obtained by assay in in table 2. The predicted values correlated well with the actual levels obtained with correlation factors of 0.91, 0.74 and 0.84 calculated for temperatures of 8,14 and 20°C respectively.

Table 2. Comparison of predicted levels of FRNA bacteriophage with actual levels (determined by assay) remaining in oysters following depuration at 8, 14 and 20°C.

		FRNA bacteriophage levels (pfu/100g)					
		8°C		14°C		20°C	
Date	Initial	Predicted	Actual	Predicted	Actual	Predicted	Actual
17/1/98	178,000	35,515	27,390			2,821	1,380
15/1/98	90,000	17,957	38,265			1,426	2,490
27/1/98	49,500			4,950	5,220		
23/2/98	11,025					400[1]	950[1]
17/3/98	67,000	13,368	13,658			1,061	571
18/5/98	14,006	2,794	1,740			221	<30
19/6/98	19,500	3,890	12,900			309	390
20/7/98	3,450	688	810			54	<30
23/9/98	9,100					144	44
4/11/98	108,300					1716	995
17/11/98	4,331					68	125
1/12/98	8,569					135	60
9/12/98	10,331					163	75
8/1/99	69,000					1,093	1,120
25/1/99	96,750					1,533	255
26/7/98	7,260	797[2]	150[2]			23[2]	<30[2]
15/11/99	25,200			2,520	3,300		
24/1/00	149,813			14,981	4,800		
16/2/00	23,000			2,300	2,200		
6/3/00	37,950					601	400
21/3/00	26,650					442	1,000

[1]Values are for 4 days depuration
[2]Values are for 7 days depuration



Detection of F+ bacteriophage in shellfish

Table 3 shows the predicted level of FRNA bacteriophage remaining each day up to 7 days of depuration for a range of theoretical initial contamination levels from 100 to 10,000 pfu/100g. From this table it can be predicted that FRNA bacteriophage levels as low as 1000 pfu/ 100g would not be eliminated within 7 days at 8°C which is the currently accepted minimum temperature for commercial depuration of Pacific oysters in the UK. By comparison the same level of FRNA bacteriophage would be eliminated after 4.2 days at 20°C whilst at 14°C the predicted time for elimination is 7.6 days.

Table 3. Predicted levels of FRNA bacteriophage remaining in oysters following depuration periods 8, 14 and 20°C for selected initial contaminant loading.

	Days post depuration							
Depuration temperature	0	1	2	3	4	5	6	7
8°C	100	72	52	38	**28**	**20**	**14**	**10**
	1000	724	524	380	275	200	145	105
	5000	3620	2620	1900	1375	1000	725	525
14°C	100	63	40	**25**	**16**	**10**	**6**	**4**
	1000	630	400	250	160	100	60	40
	5000	3150	2000	1250	800	500	300	200
20°C	100	44	**19**	**8**	**4**	**2**	**0**	**0**
	1000	437	191	83	36	**16**	**7**	**3**
	5000	2185	955	415	180	80	35	**15**

Predicted levels of FRNA bacteriophage pfu/100g
Values in **bold** are predicted levels below the limit of sensitivity of the assay (30pfu/100g)

Depuration System

Discussion

From this study it appears that the use of elevated temperature during depuration would significantly increase viral elimination during depuration as judged by FRNA bacteriophage removal. The fact that the effect of increased temperature on viral elimination is consistent regardless of season or harvest area temperature may be of major significance. As a consequence elevated temperature could be applied in the winter months when incidence of viral illness associated with depurated oysters occurs most frequently. It is possible therefore that the application of elevated depuration temperatures during this period could significantly reduce the viral risk associated with the consumption of such shellfish. However before depuration at elevated temperatures could be recommended further work is required to establish the relationship between FRNA bacteriophage elimination and NLV elimination. Similarly further work is required to establish any effect on the shelf-life or quality of the product.

FRNA bacteriophages have been proposed as a potential index organism for the viral risk associated with shellfish consumption and clearly the potential for setting an end product standard exists. In this study, the calculation of FRNA bacteriophage elimination rates during depuration for Pacific oysters allows an upper level of FRNA bacteriophage which can be eliminated by a given depuration regime (time and temperature) to be determined. This study therefore provides a basis for assessing and setting depuration parameters for Pacific oysters which could be used to successfully reach a target FRNA bacteriophage level in end-product shellfish.

References

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