LEVELS OF F-SPECIFIC RNA BACTERIOPHAGE IN BIVALVE MOLLUSCAN SHELLFISH FROM COMMERCIAL HARVESTING AREAS

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Introduction

Current measures for controlling public health problems associated with the consumption of sewage contaminated bivalve molluscan shellfish rely on the use of faecal bacteria indicator, such as *E. coli*, to indicate the sanitary quality of shellfish and their suitability for consumption. In the UK and other EU countries shellfish harvesting areas are categorised depending on the *E.coli* and/or faecal coliform content of shellfish within the harvesting area. Four categories exist ranging from class A (<230 *E. coli*/100g) requiring no treatment prior to consumption, class B (<4600 *E. coli*/100g in 90% of samples) with shellfish requiring depuration, class C (<60,000 faecal coliforms/100g) with shellfish requiring relaying for 2 months, through to prohibited for consumption (>60000 faecal coliforms/100g). Despite these control measures outbreaks of viral illness, particularly gastro-enteritis caused by Norwalk-like virus and hepatitis A caused by Hepatitis A virus, continue to occur. Shellfish associated with such outbreaks are frequently shown to be free of *E.coli* and therefore compliant with the relevant regulatory requirements. There is therefore a widely recognised need to develop alternative means for assessing the virological quality of bivalve molluscan shellfish.

F-Specific RNA (FRNA) bacteriophages are a group of single-stranded RNA viruses with a simple cubic capsid measuring 24-27 nm. These genomic and physical properties are similar to the viral pathogens of concern and their behaviour in shellfish during contamination and elimination is similar. This allied to their abundance in sewage and their ease of enumeration makes them a candidate alternative indicator of viral contamination in shellfish (Doré *et al*., 2000). However relatively little is known about the distribution of FRNA bacteriophage in commercial shellfish harvesting areas. This study investigated levels of *E.coli* and FRNA bacteriophage in oysters and mussels from a variety of commercial harvesting areas in the UK to determine the potential implications of the use of FRNA bacteriophage as an indicator for shellfish.

Materials and Methods

Shellfish sampling: Oysters (*Crassostrea gigas* and *Ostrea edulis*) and mussels (*Mytilus edulis*) were collected by local food authorities from 49 commercial harvesting sites throughout England and Wales in parallel with sampling carried out for classification of harvesting areas under European Union Directive 91/492. Sampling was carried out at approximately monthly intervals over an 18 month period. Samples were sent to the laboratory at ambient temperature and received within 24 hours of sampling.

***E.coli* and FRNA bacteriophage analysis:** For each sample a minimum of 10 oysters or 15 mussels were analysed for both *E.coli* and FRNA bacteriophage content on the day of receipt in the laboratory by previously described methods (Doré *et al*., 2000).

Results

During this study a total of 608 samples from 49 separate sampling locations were tested for *E.coli* and FRNA bacteriophage. To assess the pollution status of each sampling location *E.coli* results from each site were used to classify the harvesting area into categories in accordance with the European Directive 91/492. Table 1 shows the study data classified according to this *E.coli* criteria and by species. The majority of samples (389) were obtained from locations classifiable as category B which reflects the current status of classifications according to Directive 91/492 in England and Wales. No chosen monitoring locations conformed with the criteria for category A classification. 140 samples were from sites conforming to category C classification and 79 samples from sites conforming to a prohibited classification.

Table 1. Study data set classified according to E.coli *status of monitoring location*

Classification	*C. gigas*	*O. edulis*	*M.edulis*	All Species
Category B	108	127	154	389
Category C	56	24	60	140
Prohibited			79	79
All categories	164	151	293	608

Initial analysis compared paired *E.coli* and FRNA bacteriophage results for all sites and all species. The results are shown in figure 1 as a scatter plot with a fitted linear regression line. Although a correlation can be observed between *E.coli* and FRNA bacteriophage titre there is wide variability in the data with an R^2 value for the fit of the regression line of only 0.29. It is however apparent from the fitted regression line for this data set that, on average, FRNA bacteriophage titres in shellfish were higher than *E.coli* titres.

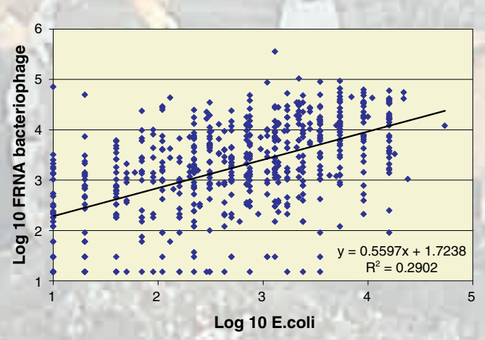


Figure 1. Scatter plot of E.coli *against FRNA bacteriophage*

This is confirmed in figure 2 which shows a box plot of the data set for *E.coli* and FRNA bacteriophage. Geometric mean values for the entire data set were 1800 and 538 counts per 100g shellfish for FRNA bacteriophage and *E.coli* respectively. Application of the students T test showed these values to be highly significantly different (p = >0.0001).

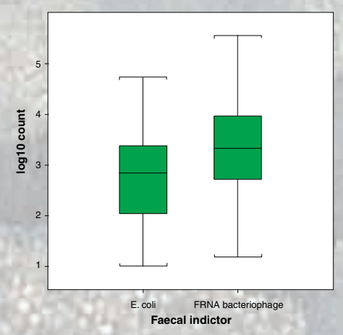


Figure 2. Box plot of the E.coli *and FRNA bacteriophage data set*

Table 2 shows an analysis of geometric mean values for *E.coli* and FRNA bacteriophage grouped according to the classification of the monitoring location. It is clear that geometric mean values for both *E.coli* and FRNA bacteriophage reflected the assessment of pollution level according to the EC Directive criteria and that FRNA bacteriophage levels were, on average, 3-4 fold higher than *E.coli* levels for all classifications.

Table 2. Geometric mean E.coli and FRNA bacteriophage counts grouped according to the classification of the monitoring location.

Classification	No. samples	Geometric mean	
		E. coli	FRNA bacteriophage
Category B	389	244	835
Category C	140	1387	5028
Prohibited	79	4956	12,736
All categories	608	538	1800

Further inspection of the data set showed a significant effect of season on FRNA bacteriophage counts. This is shown in figure 3 where geometric mean values for *E.coli* and FRNA bacteriophage for the entire data set are grouped by month of shellfish collection. It is clear that FRNA bacteriophage levels in the winter months are significantly higher than in the summer months whereas *E.coli* values were more stable. Geometric mean values for all samples taken during the winter months (October to March inclusive) were 786 and 4503 counts per 100g for *E.coli* and FRNA bacteriophage respectively (259 observations). Whereas geometric mean values for samples taken during the summer months (April to September inclusive) where 405 and 910 counts per 100g for *E.coli* and FRNA bacteriophage respectively (349 observations).

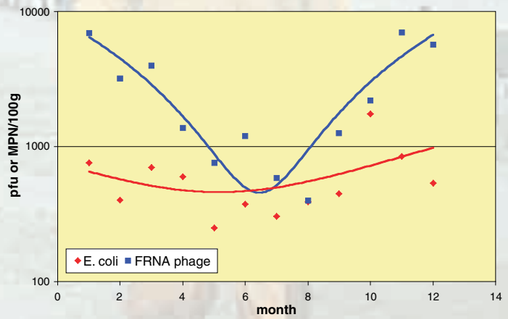
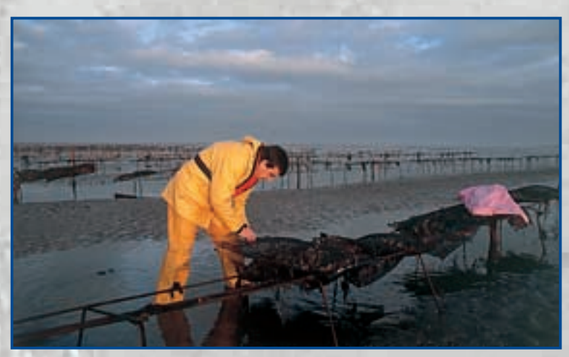


Figure 3. Geometric mean values of FRNA bacteriophage and E.coli *in shellfish by month of sample collection.*

It is clear from this study that monitoring sites classified as category B may contain significant levels of FRNA bacteriophage. European Directive 91/492 requires shellfish harvested from category B sites to be purified (depurated) prior to sale for human consumption. Previous work by this laboratory evaluated the potential of FRNA bacteriophage as a 'viral' indicator in bivalve shellfish sold for human consumption and showed that removal of FRNA bacteriophage by commercial shellfish depuration was more problematical than removal of *E.coli*, particularly during the winter months (Doré *et al*., 2000). In this study we show that levels of FRNA bacteriophage in shellfish harvesting areas are elevated during winter months which may contribute towards difficulty in eliminating them during commercial shellfish depuration. It is clear from previous work that temperature, time, and contamination level are important factors influencing clearance of contaminants during shellfish depuration.



Commercial oyster harvesting

Table 3 groups samples from category B monitoring locations according to FRNA bacteriophage level and season and explores the implications of this for shellfish depuration at various temperatures. European Directive 91/492 requires removal of *E.coli* during depuration to the end product level of <230 per 100g shellfish flesh. Equivalent standards have not been set for FRNA bacteriophage. However for illustration in this study removal of FRNA bacteriophage to an arbitrary level of <100 pfu per 100g shellfish was taken as the depuration criteria. Table 3 shows that 29% of category B samples already complied with this threshold level during the summer months compared with only 6 percent during the winter months. Table 3 shows the depuration times required to reduce higher levels of FRNA bacteriophage contamination to <100 pfu per 100g and the influence on this of depuration temperature. Table 3 shows that during the winter months more than 50% of shellfish samples tested would have required depuration for extended periods to remove FRNA bacteriophage even at elevated temperatures. It should be noted that shellfish depuration protocols currently used in most countries (based on compliance with *E.coli* standards) commonly require depuration for 36 to 42 hours at minimum temperatures of 5-8°C. It is clear from table 3 that such depuration protocols would be unlikely to clear FRNA bacteriophage from the majority of shellfish harvested from category B areas in this study.

Table 3. Implications of FRNA bacteriophage levels in category B shellfish harvesting areas for removal during shellfish depuration.

FRNA bacteriophage count (pfu per 100g)	% category B samples complying in study data set during			Depuration time (in days) to reduce FRNA bacteriophage to <100 pfu per 100g at		
	Summer (n = 168)	Winter (n = 221)	All (n = 389)	8°C	14°C	20°C
≤ 100	29.2	6.3	16.2	-	-	-
≤ 500	48.8	22.6	33.9	5*	3.5	1.9
≤ 1000	62.5	34.3	46.5	7.1	5.0	2.8
≤ 2000	79.8	53.8	65.0	9.3	6.5	3.6
≤ 5000	91.1	74.2	81.5	12.2	8.5	4.7

*depuration times are established from unpublished data

Discussion

Recent studies have investigated the use of FRNA bacteriophage as a potential indicator for the presence of pathogenic viruses in bivalve molluscan shellfish (Doré *et al*., 2000; Chung *et al*., 1998). However relatively little is known about the distribution of FRNA bacteriophage in commercial shellfish harvesting areas. This study investigated levels of *E.coli* and FRNA bacteriophage in oysters and mussels from a variety of commercial harvesting areas in the UK to determine the potential implications of the use of FRNA bacteriophage as an indicator for shellfish.

FRNA bacteriophage were found to be widely distributed in the studied shellfish harvesting areas present, on average, at levels of about 3 fold greater than *E.coli*. A correlation could be observed between levels of *E.coli* and FRNA bacteriophage however there was wide variability in individual samples. Interestingly a strong seasonal effect was noted with levels of FRNA bacteriophage elevated during the winter months. This effect was not seen for *E.coli*. It is interesting to note that elevated levels of FRNA bacteriophage during the winter months concurs with the period of higher risk for shellfish associated viral gastroenteritis (Doré *et al*., 2000). It is possible that shellfish related biological factors could similarly contribute both to the observed higher levels of FRNA bacteriophage contaminant concentrations, and retention of viral pathogens, during the winter months.

Significantly FRNA bacteriophage in shellfish from category B sites were regularly detected at levels which would not be easily removed by conventional depuration, the usual treatment recommended for such shellfish.

Acknowledgement

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References

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