The behaviour of F specific bacteriophage in depurating shellfish with reference to their use as pollution indicator organisms

Comportement du bactériophage spécifique F dans la décontamination des coquillages en référence avec leur utilisation comme indicateurs de pollution

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Abstract

The behaviour of sex-pilli specific (F+) bacteriophage during the depuration process was investigated using Pacific oysters *(Crassostrea gigas)* and edible mussels *(Mytilus edulis)* naturally contaminated with untreated sewage. F+ bacteriophage was eliminated considerably slower than *E. coli*. This effect was most pronounced in oysters which showed average T90 bacteriophage values of 62.5 hours compared with 11 hours for *E. coli*. F+ bacteriophage in mussels was largely confined to the digestive tract. The majority of *E. coli* were similarly located but, in contrast, counts were also distributed throughout the other internal tissues. Investigation of ultra-violet (UV) dosage showed that the difference in depuration rate was unlikely to be due to the higher resistance of F+ bacteriophage to UV irradiation. These results demonstrate the potential usefulness of F+ bacteriophage as an alternative indicator organism for depuration of shellfish.

Keywords: F+ bacteriophage, shellfish, depuration, indicators

Résumé

Le comportement du bactériophage spécifique F+ au cours du processus de purification a été étudié chez des huîtres creuses (*Crassostrea gigas*) et des moules (*Mytilus edulis*) contaminées dans le milieu naturel par des rejets d'égout bruts. Le bactériophage F+ s'élimine beaucoup plus lentement que *E. coli*. Cet effet est le plus prononcé pour les huîtres, avec des valeurs moyennes de T90 de 62,5 heures pour les bactériophages par rapport à 11 heures pour *E. coli*. La présence du bactériophage F+ dans la moule est principalement limitée au tractus digestif. *E. coli* est localisé principalement au même endroit mais par contre il se retrouve aussi réparti dans l'ensemble des autres tissus internes. L'étude avec irradiation UV indique que la différence entre les vitesses de décontamination n'est vraisemblablement pas due à une plus grande résistance du bactériophage F+ à l'irradiation aux UV. Ces résultats démontrent l'utilité potentielle du bactériophage F+ comme indicateur possible dans la purification des coquillages.

Mots-clés : bactériophage F+, coquillages, décontamination, indicateurs

INTRODUCTION

Viruses causing gastro-enteritis and hepatitis A are the main aetiological agents associated with human infection following consumption of sewage contaminated bivalve molluscan shellfish (Richards, 1985). The direct detection of these agents in shellfish is not, however, currently feasible. Health controls, therefore, rely on the presence of traditional bacterial pollution indicator species such as *E. coli* and the faecal coliforms (Bukman, 1991). However, several well documented disease outbreaks have occurred following consumption of depurated shellfish judged to be clean by bacteriological criteria (Gill *et al.*, 1983; Grohmann *et al.*, 1981). These epidemiological considerations, and laboratory studies showing that elimination of viruses tends to be considerably slower than that of bacteria (Richards, 1988; Power and Collins, 1989), challenge the traditional use of *E. coli* and the faecal coliforms as indicators during the depuration process. Bacterial indicators are currently used as index organisms to assess risk in processed shellfish and as model organisms to evaluate depuration during process development. There is a need for indicators more representative of virus behaviour for both applications. Such indicators should be ubiquitous in sewage and sewage-contaminated waters, easy and cheap to assay, and be similar in character to the viruses causing gastro-enteritis and hepatitis. Suitable alternative indicators would also display demonstrably similar behaviour to human enteric viruses during the depuration process. This study investigated the behaviour of one such potential indicator, sex-pilli specific (F+) bacteriophage (Havelaar *et al.*, 1984; Havelaar, 1987). Investigations included F+ bacteriophage depuration kinetics in mussels and oysters, site of tissue localisation, and the effect of one depuration variable, namely UV dosage, on depuration kinetics.

Material and methods

Historical monitoring

Mussels *(Mytilus edulis)* and oysters *(Crassostrea gigas)* were contaminated with *E. coli* and F+ bacteriophage to high levels by exposure to untreated sewage from a point discharge for 1-3 weeks in a natural estuarine environment. The outfall served a small village and discharged approximately 400 m^3 dry weather flow per day.

Following contamination, shellfish were held in a model depuration tank measuring 1,350 x 380 mm with a working volume of 150 litres. Sea-water was recirculated lengthways at one cycle per hour passing through a cascade and a 15W UV sterilising tube (type 15/3P, UVAQ Ltd, Sudbury, UK). Alternative UV tubes investigated included a 30W tube (type 30/3P, UVAQ Ltd) and a 15W narrow bore tube (Model 15, Tropical Marine Centre Ltd, Rickmansworth,UK). The narrow-bore tube had a void space distance of 12 mm as opposed to 25 mm for UVAQ Ltd tubes. Flow rate was constant (1 cycle per hour) for all tube configurations. Temperature was maintained at 15°C, dissolved oxygen at, or above. 60% saturation and salinity at 30-33‰. Shellfish were thoroughly washed externally before depuration,

using potable water, and loaded into plastic mesh trays as a single overlapping layer for oysters or to a depth of 8 cm for mussels. Trays were raised from the tank floor by 22 mm to prevent recontamination by faeces. All depuration experiments were conducted over a 48-hour period and commenced within 4 hours of shellfish collection. At each time point, meat and liquors from duplicate samples of 12 mussels or 6 oysters were pooled, homogenised as described by West and Coleman (1986), and assayed for *E. coli* and F+ bacteriophage.

For tissue location experiments, mussels were collected and thoroughly washed ; gaping animals were discarded. Haemolymph was extracted from the posterior adductor muscle sinuses using a 23-gauge needle and syringe. Fluid from 5-10 individuals was pooled for assay. Subsequently, mussels were dissected and samples of mantle, gill, foot, adductor muscle, labial palps and whole digestive gland, respectively, were pooled, diluted 1:10 in 0.1% peptone water and homogenised for 1 min using a waring blender. In addition, the intestinal tract was dissected from 20-30 animals and the contents separated from the intestinal wall by extrusion using blunt forceps. Intestinal contents were diluted 1:10 in 0.1% peptone water and homogenised using sterile sand and a pestle and mortar.

E. coli was assayed by a most probable number (MPN) procedure as described by West and Coleman (1986). F+ bacteriophage was assayed using the engineered *Salmonella Wg 49* host described by Havelaar *et al.* (1984). Duplicate 1 ml homogenates were mixed with 2.5 ml molten 1% tryptone-yeast extract-glucose agar (TYGA) and held at 48°C. 1 ml of host culture, prepared as described by Havelaar (1987), was added and the mix layered onto a prepoured base plate of 2% TYGA. Plaques were counted after overnight incubation at 37°C and mean values determined.

Depuration rates were expressed in the form of T90 values, which is the time required for a 90% reduction in micro-organism numbers in shellfish flesh (Mesquita *et al.*, 1991). Values were taken from Figures 1-3 or calculated by linear regression analysis following logarithmic transformation of data where they exceeded 48 h.

Results

The comparative kinetics of elimination for *E. coli* and F+ bacteriophage are plotted for mussels (figure 1) and oysters (figure 2). Two experiments were conducted for each species of mollusc. Both species were initially contaminated to high levels with both *E. coli* and F+ bacteriophage. Although little variation was observed in uptake levels for either organism, major differences were observed in elimination rates. T90 values are listed in table I. In both species of shellfish, *E. coli* was eliminated considerably faster than F+ bacteriophage. In addition, depuration of *E. coli* and F+ bacteriophage was faster in mussels than in oysters. It was noted that in mussels, microbial indicator titres converged towards the end of the depuration period (figure 1). This trend was not apparent in oysters (figure 2).

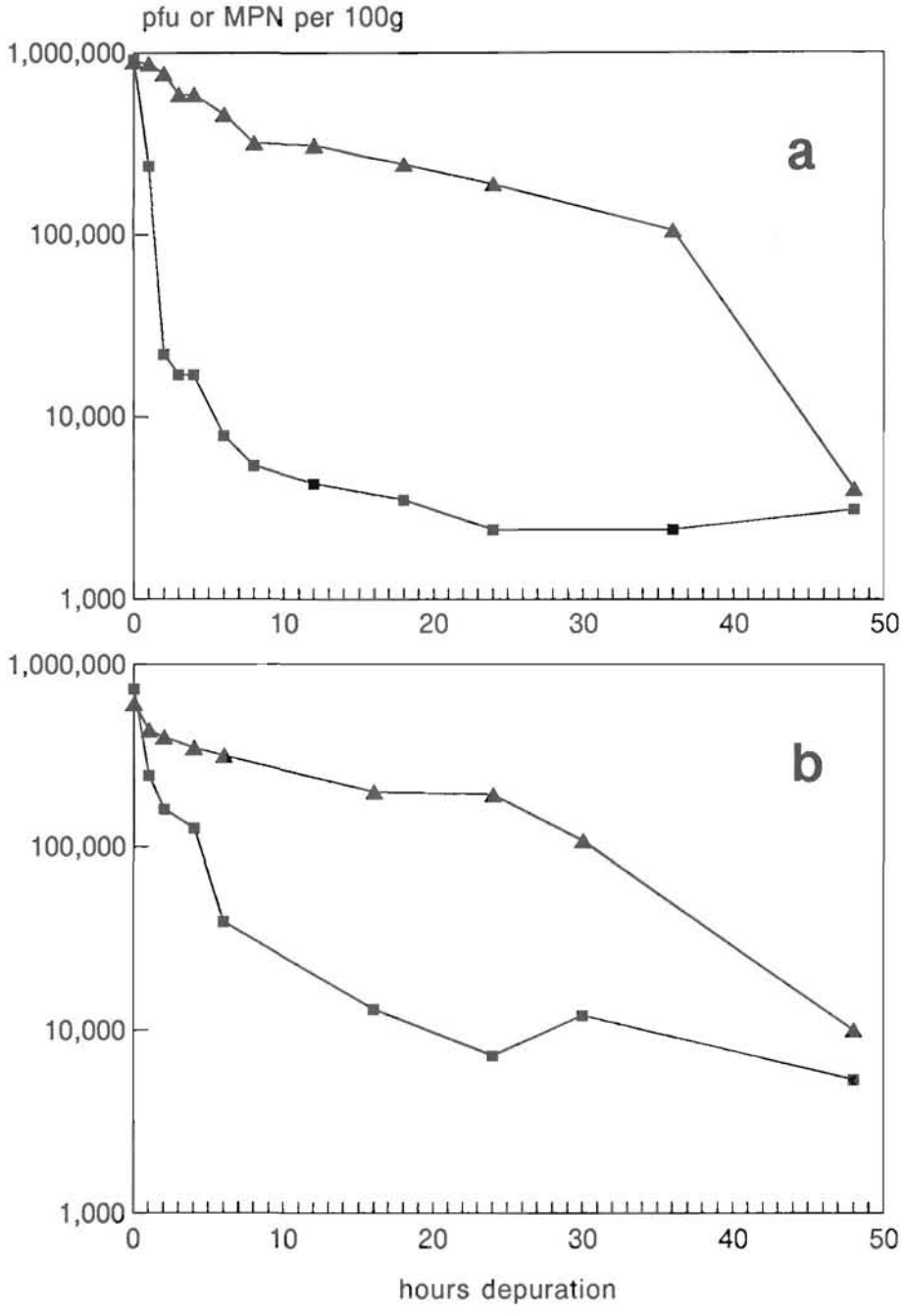

Figure 1: Elimination of F+ bacteriophage (PFU per 100 gram) (▲) and *E. coli* (MPN per 100 gram) (■) in mussels during depuration.
a: experiment conducted 6.11.90 ; b: experiment conducted 27.11.90

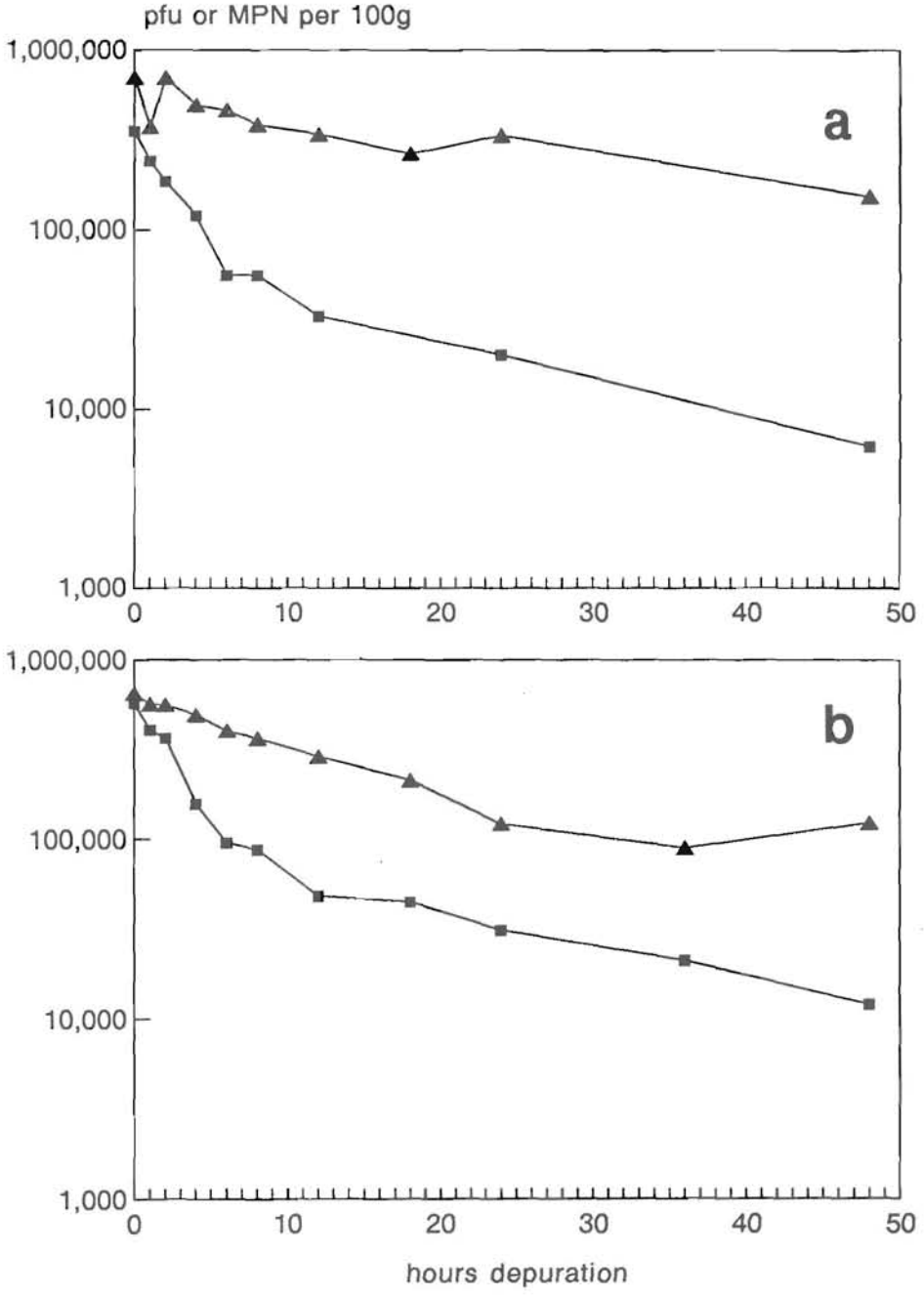

Figure 2: Elimination of F+ bacteriophage (PFU per 100 gram) (▲) and *E. coli* (MPN per 100 gram) (■) in oysters during depuration.
a: experiment conducted 17.12.90 ; b: experiment conducted 14.01.91

Table I: T90 values (in hours) for microbial indicators during depuration of mussels and oysters

	Mussels (*M. edulis*)		Oysters (*C. gigas*)	
	E. coli	F+ phage	*E. coli*	F+ phage
Experiment A	1.5	36.5	11.5	74.5
Experiment B	5.0	34.5	10.5	50.5
Mean	3.2	35.5	11.0	62.5
Standard deviation	2.5	1.4	0.7	17.0

The depuration kinetics of oysters following alteration of one process variable, UV tube configuration, are plotted in figure 3. T90 values are listed in table II. The values for both microbial indicators remained unaffected by either doubling the UV tube wattage (30W UV tube) or reducing void space distance from 25 mm to 12 mm (15W narrow bore UV tube) and thus increasing UV dosage.

The distribution of *E. coli* and F+ bacteriophage in mussel tissues following contamination and dissection is shown in table III. Three separate experiments were conducted. The majority of both microbial indicators were consistently detected in the intestine and digestive gland. *E. coli* was also detected in all other tissues examined. By contrast, F+ bacteriophage was exclusively confined to the organs concerned with filtration or digestion of food; the digestive tract, gills and labial palps.

Table II: T90 values (in hours) for microbial indicators during depuration of oysters *(C. gigas)* using different UV tube configurations

	E. coli	F+ phage
30 W UV tube	12.0	53.5
15 W narrow bore UV tube	12.5	66.5

Table III: Distribution of *E. coli* and F+ bacteriophage in mussel tissues following contamination

	Exp. of 25.09.91		Exp. of 19.11.91		Exp. of 9.12.91	
	*E. coli**	F+	*E. coli**	F+	*E. coli**	F+
Whole mussel	2 400	100	2 400	2 750	5 400	4 800
Mantle	49	<10	<2	<10	49	<10
Gill	1 600	nd	540	120	540	220
Foot	nd	nd	79	<10	49	<10
Adductor muscle	240	<10	33	<10	54	<10
Haemolymph	540	<10	49	<10	240	<10
Labial palps	240	180	130	220	540	<10
Intestine contents	92 000	80 000	240 000	75 000	49 000	95 000
Digestive gland	nd	nd	2 400	2 050	nd	2 750

* Counts expressed as MPN or PFU per gram ; nd = not done

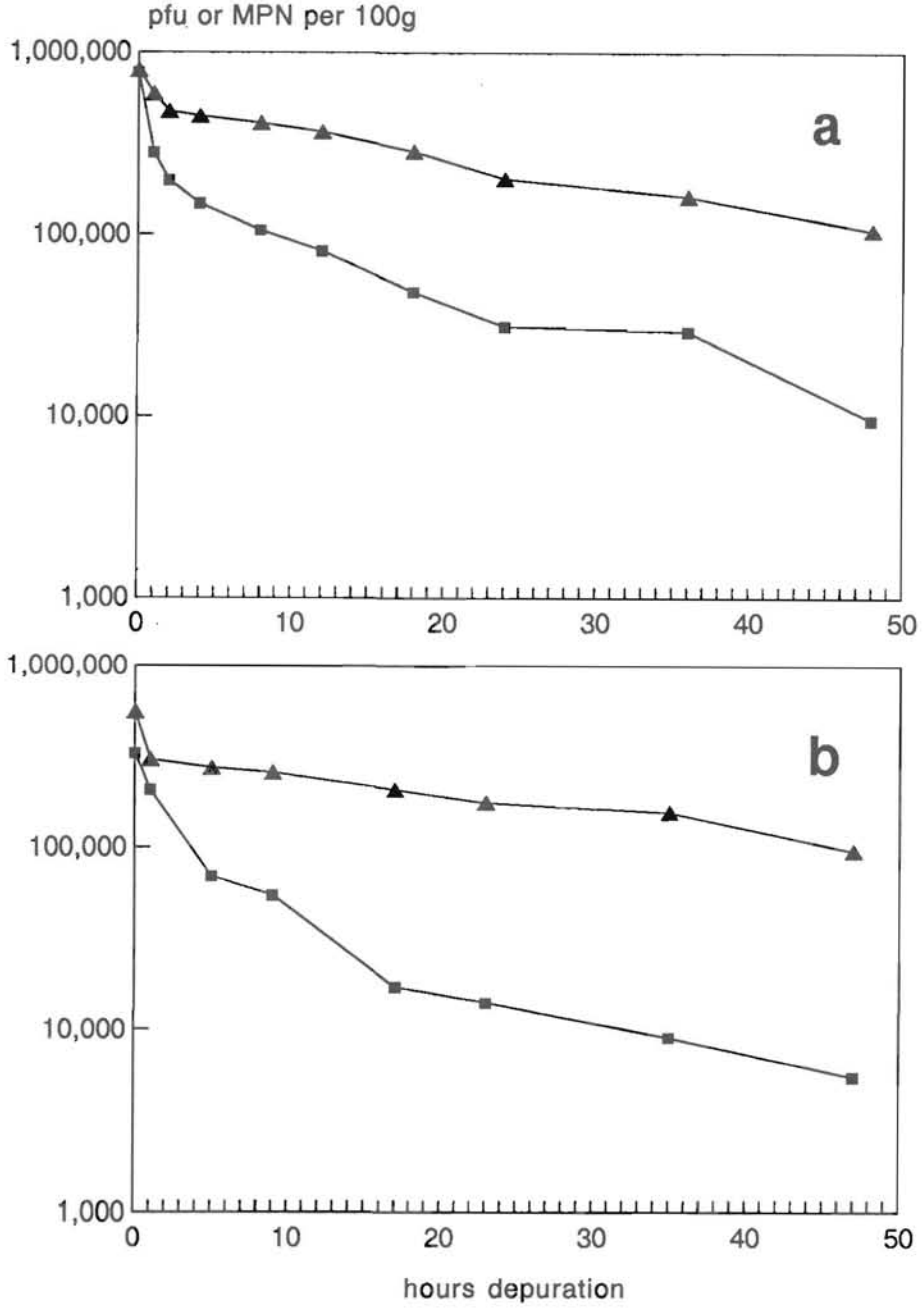

Figure 3: Elimination of F+ bacteriophage (PFU per 100 gram) (▲) and *E. coli* (MPN per 100 gram) (■) in oysters using different UV tube configurations during depuration.
a: 30W UV tube (UVAQ Ltd) ; b: 15W narrow bore UV tube (Tropical Marine Ltd)

Discussion

E. coli and the faecal coliforms are traditional indicators of water and shellfish quality and have been incorporated into shellfish standards (Bukman, 1991). However, their use as an end product standard for depurated shellfish has acknowledged limitations (Richards, 1988). Other faecal indicator bacteria (*Streptococci, Clostridium spores*) have been investigated but depuration rates were not found to be significantly different to those of *E. coli* (Mesquita *et al.*, 1991). Human enteroviruses (Schild *et al.*, 1987) potentially offer advantages of specificity for human faecal pollution and have similar virion characteristics to the pathogens causing hepatitis A and viral gastro-enteritis. Enteroviruses have been used extensively in artificial contamination experiments (Richards, 1988). Currently available methods for enumeration are, however, complex and expensive and enterovirus levels in sewage are low. The reliability of enteroviruses as indicators in naturally-contaminated shellfish remains unknown (Richards, 1988). Bacteriophages are ubiquitous in sewage, cheap and easy to detect, and are gaining acceptance as potential "virus indicators" in the water cycle (IAWPRC Study Group, 1991). Of this diverse group, male-specific (F+) bacteriophage are of particular interest as their physical characteristics closely resemble the viruses of concern (Havelaar, 1987; IAWPRC Study Group, 1991). Investigation of their behaviour during the depuration process facilitates consideration of their potential role as indicator organisms for viral contamination of shellfish.

Mesquita *et al.*, (1991) recently demonstrated that F+ bacteriophage depuration rates in naturally contaminated mussels were significantly slower than those of *E. coli*. Our results confirm these findings and those of other investigators using shellfish artificially contaminated with bacteriophage (Power and Collins, 1989) and enteroviruses (Richards, 1988). We also demonstrate for the first time that naturally-contaminated oysters display differential depuration kinetics and that elimination of F+ bacteriophage is considerably slower in oysters than in mussels. Average T90 values of 62.5 hours are in excess of the commercial depuration period in the UK (42-48 h) and are of particular significance for oysters which are traditionally consumed raw and frequently implicated in shellfish-transmitted disease (Richards, 1985).

The differential elimination patterns of bacteria and viruses have led to suggestions that unsuccessful virus elimination may be due to sequestration in internal tissues or cells (Girolamo *et al.*, 1975; Pain, 1986). Laboratory studies using bacteriophage (Power and Collins, 1990) or enterovirus (Liu *et al.*, 1966; Richards, 1988) have invariably demonstrated that, although the digestive tract harbours the large majority of contaminants, virus can also be detected in other internal tissues. However, Metcalf *et al.* (1979) showed that naturally acquired contaminants depurated more slowly than laboratory-induced contaminants which indicates caution. Our results confirm the digestive tract as the site of contamination in naturally-contaminated mussels and further show that F+ phage, unlike *E. coli*, is exclusively confined to the organs concerned with filtration or digestion of food. This intriguing result is not consistent with internal sequestration of virus as an explanation for differential elimination patterns.

UV irradiation of depuration water (Wood, 1961) has gained wide acceptance in the UK and the United States. It is feasible that differential elimination of bacteriophage (and enterovirus) during depuration is influenced by their higher resistance to UV irradiation (Havelaar, 1987). Our findings suggest that this is unlikely to be a major factor responsible for failure to clear virus during depuration and moreover demonstrates the potential of the F+ bacteriophage system for investigation of process variables.

In conclusion F+ bacteriophage, like human enteroviruses (Richards, 1988), are eliminated only slowly during the depuration process. They may therefore be suitable alternatives to *E. coli* as pollution indicator organisms for depurated shellfish. They may also be useful model viruses for further exploration of the differential depuration phenomena and for investigation of depuration process parameters. Further necessary work includes an evaluation of F+ bacteriophage levels in commercially-depurated shellfish and investigation of the clearance of F+ bacteriophage following contamination at threshold levels (4,600 *E. coli*/100 g) indicated by recent legislation (Bukman, 1991).

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