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Bacteriophage as models for virus removal from Pacific oysters (*Crassostrea gigas*) during re-laying

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SUMMARY

A study was undertaken to examine the feasibility of using naturally-occurring bacteriophages to assess the impact of re-laying on levels of viral contamination in *Crassostrea gigas*, the Pacific oyster. Two phages were chosen. One, male-specific (F+), was enumerated using *Salmonella typhimurium*. The other, a somatic phage, was detected using an, as yet, uncharacterized *Escherichia coli*. Investigations, using a variety of re-laying sites, demonstrated that numbers of F+ phage in oyster tissue declined more rapidly than those of somatic phage. For example, in oysters placed in commercially-used sea water ponds, F+ phage reached undetectable levels within 2–3 weeks, whereas somatic phage could still be detected 5 weeks after re-laying. The studies suggest that F+ phage may not be a suitable indicator for virus removal and that somatic phage may be better suited to this role.

INTRODUCTION

Bivalve molluscan shellfish grown in sewage-polluted waters can become contaminated with a variety of micro-organisms potentially pathogenic to man. Principal among these are small round structured viruses (SRSV), and shellfish are recognized internationally as important vehicles for human infection [1]. It is common practice in the European Community for bivalve molluscan shellfish such as oysters, and mussels, to be cleansed with disinfected sea water in specially designed units [2], except for those grown in the cleanest waters. This process, known as depuration, has been successful in reducing both the levels of bacterial contamination in shellfish and associated human illness [3]. However, depuration is much less effective in removing enteric viruses [4], and outbreaks of infection with Norwalk agent, a SRSV, have been transmitted by oysters in which no indictor bacteria, such as *Escherichia coli*, could be demonstrated [3]. Contaminated shellfish have also been implicated in outbreaks of hepatitis A (HAV) infections [5].

The relative inefficiency of depuration as a means of reducing the viral load in shellfish has focused attention on other procedures. The most important would appear to be re-laying in water less polluted than that in growing areas [6–9]. Re-laying can bring about a rapid reduction in the levels of bacterial contamination [3] but less is known about its impact on the number of viruses.

Bacteriophages can be found in high numbers in sewage and polluted waters. They have been used to measure the efficacy of sewage treatment [10] and as indicators of contamination in the water cycle [11]. They may also have an application as indicators of potential hazards in shellfish, and as models for virus removal during depuration [12, 13] and re-laying.

The study reported in this paper was undertaken to assess the feasibility of using phage to monitor re-laying efficacy. Previous investigations on water system hygiene [10, 11] concentrated on pili-specific (F+) phage. The results of a small initial survey by this laboratory [14] indicated that F+ phage could not be detected in some oyster samples, even though other phages were often present in high numbers. This observation, coupled with the fact that F+ phages have been shown to be largely absent from human faeces [15] caused the study to be widened to include somatic phages.

In an investigation lasting over 2 years, F+ phages of Bradley Group E [16], and some somatic phages, mainly belonging to Bradley Group D [16], were used to study the impact of re-laying on phage levels in naturally contaminated *Crassostrea gigas*, the Pacific oyster. The F+ phages were detected using *Salmonella typhimurium* (strain WG-49) and the somatic phages with an, as yet, uncharacterized strain of *E. coli*.

MATERIALS AND METHODS

Shellfish and re-laying

Samples of *C. gigas* were obtained from growing areas in the West Country, UK. They were used either without further experimental treatment or exposed to untreated sewage from a small riverside town for up to 25 weeks. The shellfish, in lots of 100 in plastic mesh bags used for commercial production, were placed at 5 potential re-laying sites for up to 20 months. In general, bags of oysters were laid on metal frames and exposed at low tide. In separate studies the impact of re-laying in sea-water ponds was also examined.

Sewage samples

At intervals during the investigations, samples of sewage effluent were also collected for phage estimation.

Microbiological examination

Depending upon season, re-laying site, and the length of the study, sampling frequency varied between 3 days and 4–6 weeks. Approximately ten oysters were collected each time.

Shellfish were examined either individually or in batches of five either on the day of collection, or after overnight storage at +4 °C.

Little experimental work has been undertaken on the estimation of phages in the Pacific oyster and it was necessary to carry out some developmental investigations. These indicated that estimated phage numbers could be maximized if oyster samples were homogenized in a blender for 45 sec followed by centrifugation of homogenates at 1000 g for 15 min. These preparatory techniques were used with all samples. Plating techniques were those of Havelaar and

Hogeboom [17] for F+ phage and a simple pour plate, using 12 ml of agar, incubated at 30 °C, for somatic phage [14].

For all estimations, oyster homogenates were tested within 2 h of preparation. Water and sewage samples were examined for phages using the same plating techniques as for shellfish. Numbers of *E. coli* were estimated using the technique of West and Coleman [18].

RESULTS

Phage types in oysters either from commercial growing areas or following exposure to sewage

On the basis of differing plaque morphologies, oysters were found to contain a range of phages which were capable of attacking either *S. typhimurium* or *E. coli* following exposure to sewage. For example, some batches of oysters contained up to six different phages which formed plaques on cultures of *E. coli*.

The uptake of either phages or *E. coli* by oysters exposed to sewage showed essentially the same kinetics irrespective of season or length of exposure to sewage effluent. Uptake was rapid and levels of both the bacterium and the phages in oyster tissues reached a maximum approximately 1–2 days after placing at the outfall (Fig. 1). The data shown in Figure 1 were derived from an experiment in July 1991 when the heavily contaminated water into which the oysters were placed was shown to contain, on average, $3{\cdot}8 \times 10^3$ ($\pm 2 \times 10^3$) p.f.u./ml somatic phage during the 7-day exposure period (range $7{\cdot}6 \times 10^2$–$1{\cdot}8 \times 10^4$ p.f.u./ml) and $4{\cdot}9 \times 10^3$ ($\pm 1{\cdot}7 \times 10^3$) p.f.u./ml F+ phage (range $1{\cdot}5 \times 10^3$–$1{\cdot}3 \times 10^4$ p.f.u./ml). Very similar results were obtained in an experiment carried out in January 1992, when the mean levels of somatic and F+ phage, during the 4-week exposure period, were $4{\cdot}4 \times 10^2$ ($\pm 1 \times 10^2$) p.f.u./ml (range $3{\cdot}6 \times 10^1$–9×10^2 p.f.u./ml) and $1{\cdot}4 \times 10^3$ ($\pm 3{\cdot}9 \times 10^2$) p.f.u./ml (range $4{\cdot}4 \times 10^2$–$3{\cdot}2 \times 10^3$ p.f.u./ml) respectively. Data from most of the uptake experiments are shown in Table 1 and indicate that contamination levels in oysters exposed to sewage for 1 day were little different from those in oysters exposed for 25 weeks.

In general, shellfish from commercial growing areas appeared to contain only one type of F+ phage and one type of somatic phage on the basis of plaque morphology. The latter was given the code KM and was used, in conjunction with the F+ phage, in the studies on re-laying reported in this paper.

Removal of phages and Escherichia coli *during re-laying*

Oysters were exposed to untreated sewage effluent for up to 15 weeks and then removed to areas in estuaries adjacent to commercially used re-laying sites. Examination of batches of oysters collected at intervals revealed significantly different rates with which phages, and *E. coli*, were removed during re-laying. Eleven experiments were carried out at different times of the year at five sites in four estuaries. All experiments at all re-laying sites gave essentially the same results although there were minor experiment-to-experiment differences. There was also some seasonal variation with rates of removal being more rapid in summer (Fig. 2). In general, however, numbers of F+ phage were reduced by 50% within 4–6 days and by 90% within 10–14 days of re-laying. The comparative figures for the KM somatic phage were 10–14 days and 6–8 weeks respectively. In

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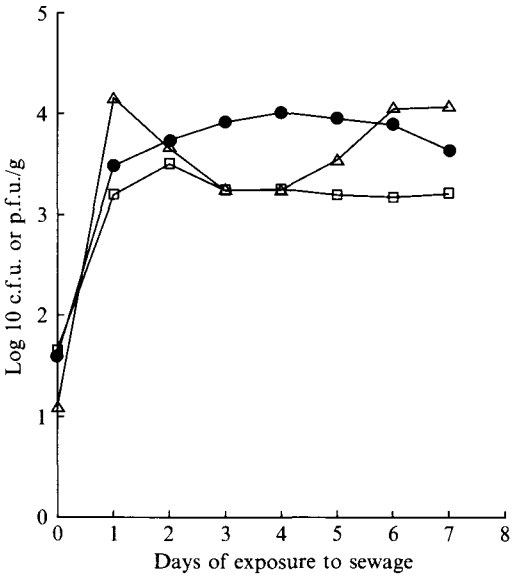

Fig. 1. Uptake of phages and *Escherichia coli* by oysters exposed to untreated sewage effluent. □, Somatic phage; ●, F+ phage; △, *E. coli*.

Table 1. *Length of exposure to untreated sewage effluent and phage and* E. coli *levels in* Crassostrea gigas

Date	Length of exposure to sewage* (weeks)	c.f.u. or p.f.u./g		
		Somatic phage	F+ phage	*E. coli*
Jan 1991	2	56	1222	900
Mar 1991	10	424	3560	800
May 1991	2	461	2903	> 900
May 1991	15	740	4415	> 900
July 1991	1	1650	4250	12000
July 1991	25	1450	1650	18000
Sept 1991	2	27200	2920	4600
Sept 1991	0·14†	1200	399	850
Feb 1992	4	632	4610	850

* Mixed domestic and agricultural from a small riverside town.
† Oysters exposed for one day.

all experiments, *E. coli* levels fell more rapidly than those of either phage, although in some trials rates of removal were close to those of F+ phage. Data from typical experiments are presented in Fig. 2. Other phages found in oysters following exposure to sewage were removed rapidly during re-laying and generally reached very low levels within 1–2 weeks.

Seasonal variations in contamination levels in oysters

As the data in Figure 2 illustrate, most oysters re-laid in the estuaries had detectable levels of the F+ and KM phages many weeks after placing. At some time after re-laying, dependent upon site and season, numbers of phage and *E. coli*

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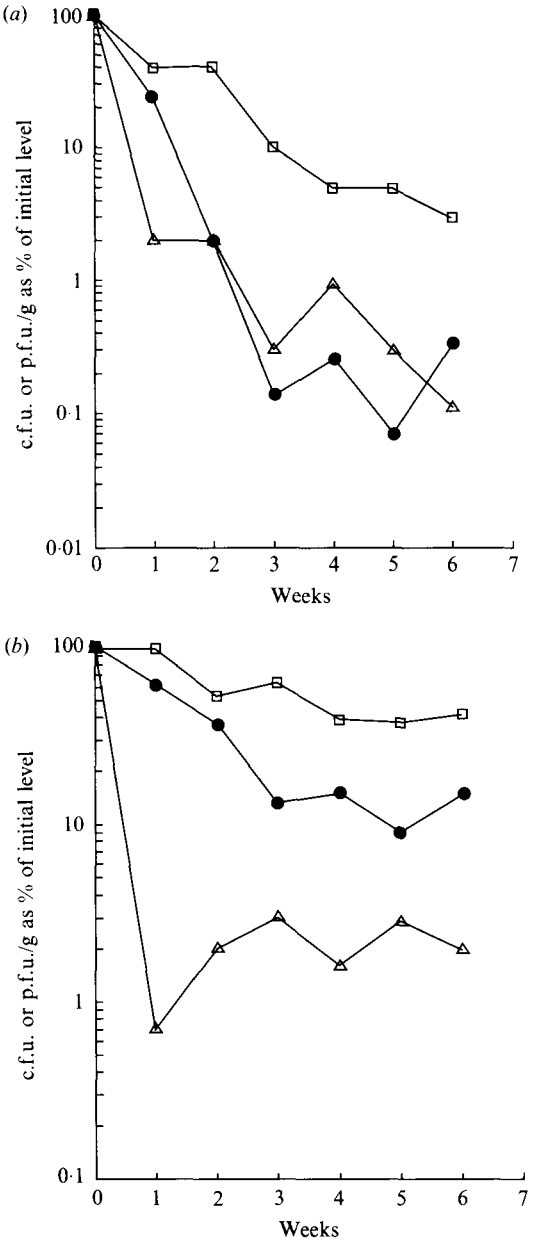

Fig. 2. Removal of phages and *Escherichia coli* from oysters during re-laying in estuaries. (*a*) Oysters relaid in May/June; (*b*) oysters relaid in Feb/Mar. □, Somatic phage; ●, F+ phage; △, *E. coli*.

reached a plateau after which there was fluctuation around a mean value (Fig. 2). At this point, levels were very similar to those in commercially produced oysters from areas at or around the experimental sites.

There were often marked variations in the background numbers of phage and *E. coli*. This can best be illustrated by an experiment where 200 oysters were taken directly from a growing area and placed at a potential re-laying site in May 1990.

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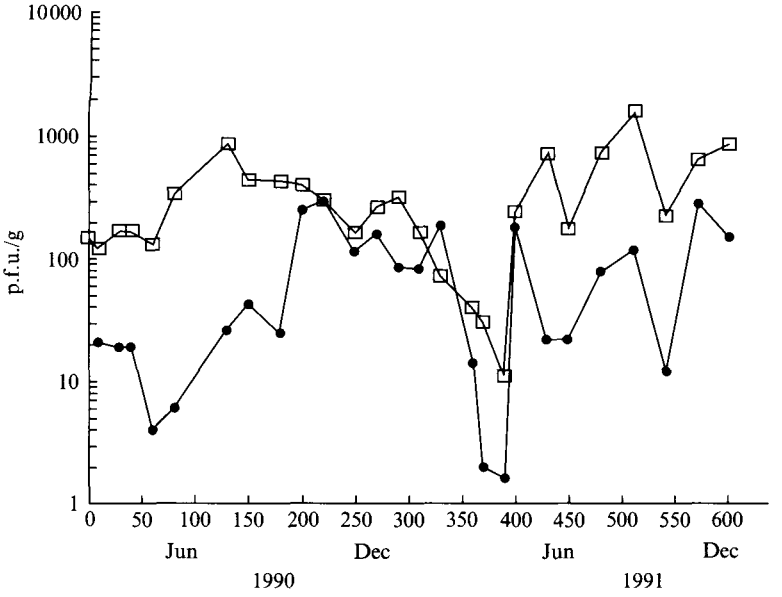

Fig. 3. Seasonal variations in phage concentrations levels in oysters. □, P.f.u./g somatic phage; ●, p.f.u./g F+ phage.

Samples of oysters were collected for examination every 2–6 weeks until January 1992.

The results, presented in Fig. 3, show that in addition to week-to-week variation in phage levels, somatic and F+ phages appear to have different seasonal patterns. Similar fluctuations in *E. coli* levels were also observed although these results are not presented.

The effects of re-laying in sea water ponds

Oysters re-laid at the estuary sites were generally exposed to air at low tide. Other systems, where oysters are placed in non-tidal waters and thus not exposed to air, are also used. Such a system, which used sea-water ponds, was investigated. The results of these trials are in general agreement with data from oysters at estuary sites and confirm that there are significant differences in the rate with which F+ and somatic phages are removed during re-laying (Fig. 4). In oysters re-laid in April/May 1992 F+ phage reached undetectable levels (< 1 p.f.u./5 g tissue) within 3 weeks, whereas somatic phage was still detectable, albeit in small numbers, 5 weeks after placing (Fig. 4). The same pattern was observed in experiments carried out in January/February 1993.

Effect of length of exposure to sewage and initial levels of contamination on rates of phage removal during re-laying

The rate with which phages were removed from *C. gigas* was unaffected by either length of exposure to untreated sewage effluent before re-laying or initial levels of contamination. Thus the kinetics of phage removal were identical whether oysters had been exposed to sewage for either 24 h or 15 weeks or where initial phage levels were either high or low. Data presented in Figure 5 are from

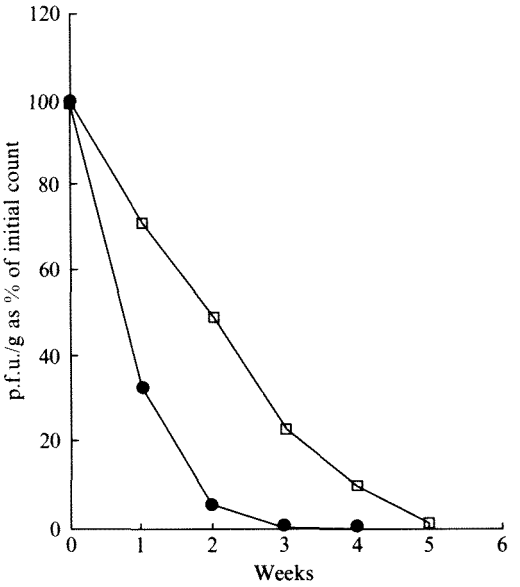

Fig. 4. Removal of phages from oysters by re-laying in sea water ponds. ●, F+ phage; □, somatic phage.

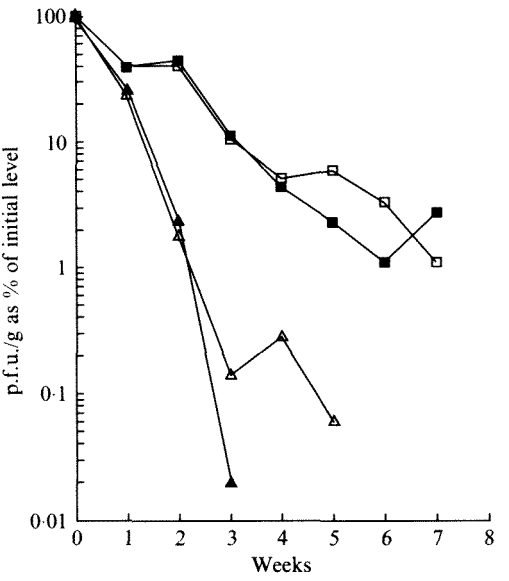

Fig. 5. Influence of length of exposure to sewage effluent on the subsequent removal of phages during relaying. □, Somatic phage in oysters exposed to sewage for 2 weeks; ■, somatic phage in oysters exposed for 14 weeks; △, F+ phage in oysters exposed to sewage for 2 weeks; ▲, F+ phage in oysters exposed for 14 weeks.

an experiment carried out in May 1991. Oysters were exposed to sewage effluent for either 2 or 15 weeks. In the shellfish exposed for 2 weeks, levels of F+ and somatic phages were $2{\cdot}9 \times 10^3$ and $4{\cdot}6 \times 10^2$ p.f.u./g respectively. Those exposed for the longer period contained $4{\cdot}4 \times 10^3$ F+ phage/g and $7{\cdot}4 \times 10^2$ somatic phage.

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Table 2. *Phage levels in sewage*

Effluent type	No. of outfalls*	No. of samples	p.f.u./ml ± S.E. (Range) F+ phage	p.f.u./ml ± S.E. (Range) Somatic phage
Crude	7	45	8023 ± 1557 (52–54000)	4604 ± 973 (9–31000)
Treated	5	35	191 ± 128 (1–4500)	169 ± 49 (1–232)

* Outfalls varied from ones serving small local, rural communities to large treatment works serving urban populations.

Phage levels in sewage effluent

Large numbers of phages were present in most samples of crude sewage tested. There were marked site-to-site and day-to-day variations but, in general, numbers of F+ phage exceeded those of somatic phage (Table 2). Samples of treated effluent from five treatment works were also examined. Phage numbers were substantially reduced although high levels were found in occasional samples (Table 2).

In a separate study, samples, taken before and after treatment, were collected from a treatment works serving a population of approximately 150000. Sewage was subjected to primary screening and settlement, biological oxidation and final settlement. Numbers of F+ and somatic phages in the incoming raw sewage were $1{\cdot}1 \times 10^4$ ($\pm 1{\cdot}4 \times 10^3$) p.f.u./ml and $1{\cdot}10^4$ ($\pm 1{\cdot}3 \times 10^3$) p.f.u./ml respectively. In the final effluent, there were $1{\cdot}4 \times 10^2$ ($\pm 4 \times 10^1$) p.f.u./ml of F+ phage (a reduction of 99%) and $3{\cdot}5 \times 10^2$ ($\pm 1{\cdot}2 \times 10^2$) p.f.u./ml of somatic phage (a reduction of 96%).

Oyster-to-oyster variations in phage levels

Examination of individual oysters, not exposed to sewage and taken from the same bag, revealed that there could be marked variation in phage levels. For example, in one batch of five oysters with a mean F+ phage count of 86 ± 25 p.f.u./g the number of phages in individual oysters ranged from 1 to 151 p.f.u./g. With somatic phage, the mean count was 759 ± 189 p.f.u./g and the range 21–1025 p.f.u./g.

DISCUSSION

It is not yet possible to culture Norwalk agent or other SRSV. Thus detection largely relies upon the use of electron microscopy. The insensitivity of this technique precludes its use with shellfish. Estimation of *E. coli* may have a potentially useful role in assessing contamination levels in shellfish cultivation areas or as an indication that depuration has been carried out. However, *E. coli*, in common with other bacterial indicators, is unreliable as a measure of potential hazard from enteric viruses [3]. Other viruses, such as the vaccine strains of poliovirus, have been suggested as potential indicators of SRSV, but their detection can be expensive and time-consuming [19]. Virus extraction from shellfish tissue would also appear to be unreliable and no one method has been shown to be entirely satisfactory. It must also be recognized that the absence of one enterovirus, such as poliovirus, does not necessarily guarantee absence of

other enteric viruses. In contrast to the extraction and detection difficulties with enteroviruses, results presented in this paper illustrate that both somatic and F+ phages can be detected in oysters and enumerated using simple, inexpensive, standard microbiological techniques.

The purpose of our investigations was to examine the feasibility of using naturally occurring phages as models for the removal of potentially pathogenic viruses during re-laying of *C. gigas*, the Pacific oyster. Other studies, using a variety of molluscan shellfish, have examined phage removal during re-laying [3]. In general, these used shellfish contaminated artificially with only one type of phage and have not involved *C. gigas*. This present study is the first to use naturally contaminated Pacific oysters and to compare the behaviour of two different phages.

The intestinal tract of animals, including humans, contains many different types of phage [15]. It was decided to concentrate upon the phages described in this paper because they are spherical and approximately the same size as the Norwalk agent and other SRSV. Other phages are present in oysters, particularly following exposure to sewage, but they are removed so rapidly after transfer to cleaner waters that they would appear to have little use as indicators. They are also only rarely present in significant numbers in commercially produced oysters.

It is of interest that, although phage and *E. coli* levels in the sewage polluted waters into which oysters were placed were often high, the shellfish did not appear to continue to accumulate the organisms after the first 1–2 days of exposure (Fig. 1). The often marked fluctuations in contamination levels in oysters exposed to sewage are probably a reflection of phage levels in sewage, which can vary markedly (Table 2) [20]. Levels may also be influenced by possible different feeding patterns in individual oysters.

Despite the almost identical rate at which the two phages are accumulated by oysters there are marked differences in the rate with which they are removed during re-laying (Figs. 2, 4, 5). The decline in phage numbers was unaffected by either levels of contamination or length of exposure to sewage (Fig. 5). This is in contrast to earlier reports on phage removal from the edible mussel, *Mytilus edulis* [21]. There is the possibility that the kinetics of virus removal from shellfish differ from species to species.

Advances have been made in the processing of certain species of shellfish and alternative procedures of heat treatment of molluscs such as cockles have proved very effective in preventing viral illness [5]. There is still an active market, however, for shellfish which are consumed uncooked. With these, a potentially important part of any programme of control of shellfish-associated viral illness is the identification of procedures which permit the removal of viruses from the live shellfish. Re-laying away from growing areas may have an important role in future control programmes. It is not yet possible to define optimum re-laying procedures. Whatever are adopted, and the European Community has suggested 8 weeks [2], it will be important to monitor their effectiveness. Phage may have a role in this respect and it may be possible, in the future, to establish microbiological guidelines for shellfish which include phage levels. Data presented in this paper could be taken to suggest that should phage be used, somatic phage may be a better choice as an indicator than F+ phage. Somatic phage are easier to estimate,

requiring only a simple pour plate, and would appear to be less affected by laboratory procedures than F+ phage. Their slower removal from oysters may also have advantages. No data are available, at present, on the effects of re-laying on numbers of SRSV in oysters. It would not be unreasonable to assume, however, that procedures which can be demonstrated to result in the removal of somatic phage may also have an impact on the numbers of potential human pathogens. The speed with which *E. coli* and F+ phage are removed may make them less suitable as indicators of re-laying efficacy.

SRSV and HAV are both infectious in low doses. The aim of shellfish treatment procedures, such as re-laying, must be to eliminate viruses and not just reduce levels. The results from this study suggest that oysters should be re-laid for at least 6 weeks in the warmer months of the year and possibly longer in the winter when lower water temperatures will facilitate virus survival [14] and reduce the physiological activity of the shellfish.

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