APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1995, p. 2830–2834 Vol. 61, No. 8
0099-2240/95/$04.00+0

Behavior of *Escherichia coli* and Male-Specific Bacteriophage in Environmentally Contaminated Bivalve Molluscs before and after Depuration

WILLIAM J. DORÉ AND DAVID N. LEES*

Fish Diseases Laboratory, Directorate of Fisheries Research, Ministry of Agriculture, Fisheries and Food, Weymouth, Dorset DT4 8UB, England

Received 17 January 1995/Accepted 17 May 1995

We monitored the differential reduction rates and elimination patterns of *Escherichia coli* and male-specific (F^+) bacteriophage during UV depuration for 48 h in oysters (*Crassostrea gigas*) and mussels (*Mytilus edulis*) contaminated by short-term (1 to 3 weeks) and long-term (more than 6 months) exposure to sewage in the marine environment. The time taken to reduce levels of *E. coli* by 90% was 6.5 h or less in all cases. In contrast, the amounts of time needed to reduce levels of F^+ bacteriophage by 90% were considerably longer: 47.3 and 41.3 h (after short- and long-term exposures, respectively) in mussels and 54.6 and 60.8 h (after short- and long-term exposures, respectively) in oysters. No differences in the rates of reduction of indicators of viral pollution following exposure of the shellfish to either short- or long-term sewage contamination were observed. Further experiments were conducted with mussels to determine the relative distributions of *E. coli* and F^+ bacteriophage in tissue before and during depuration. Prior to depuration the majority of *E. coli* organisms (90.1%) and F^+ bacteriophage (87.3%) were detected in the digestive tract (i.e., the digestive gland and intestine). *E. coli* and F^+ bacteriophage were reduced in all tissues except the digestive gland to undetectable levels following depuration for 48 h. Within the digestive gland, levels of F^+ bacteriophage were reduced to 30% of initial levels, whereas *E. coli* was reduced to undetectable levels. These results confirm previous laboratory studies showing the differential reductions of levels of *E. coli* and F^+ bacteriophage during depuration. They also demonstrate that these differential elimination patterns are not affected by the duration of sewage contamination and that F^+ bacteriophage are retained only in the digestive gland and are not sequestered into other internal tissues.

Filter-feeding molluscan shellfish accumulate microorganisms, including human pathogenic bacteria and viruses, when grown in sewage-polluted waters and can present a significant health risk when consumed raw or lightly cooked (8, 24, 27). Controlled self-purification (depuration) of shellfish is used extensively throughout the world (8, 22, 25) and has been successful in reducing to low levels bacterial illness associated with shellfish consumption in the United Kingdom (24). However, there remains a significant risk of viral infection following the consumption of depurated shellfish (9, 10, 21). Viral diseases predominantly associated with the consumption of sewage-contaminated molluscan shellfish are gastroenteritis, caused by small round structured viruses, and infectious hepatitis, caused by the hepatitis A virus (23).

Several studies have demonstrated differential rates of reduction of bacteria and viruses in depurating shellfish (3, 5, 7, 18, 19, 23). Generally, bacteria are rapidly reduced to nondetectable levels, usually within 48 h, while viruses are more slowly reduced and may persist for several days (22, 25). Some workers have suggested that differential elimination rates are caused by virus sequestration from the digestive tract into other (unspecified) internal tissues (3, 4, 6). It is proposed that viruses sequestered into such tissue depots are refractory to elimination by the digestive process. However, there is little evidence to support this hypothesis, and, indeed, most available studies (13, 16, 20) show that viruses and virus indicators are almost exclusively confined to the digestive tract and are not found in significant numbers in other tissues after depuration. Conditions in such studies may not, however, be representative of environmental conditions, as the researchers examined shellfish artificially contaminated in the laboratory over a relatively short period. Virus sequestration into shellfish tissue may be a function of time and may thus be more apparent in shellfish contaminated in the marine environment over longer periods of time. It is also possible that contamination with laboratory strains of a virus and virus indicators may not be fully representative of contamination in the marine environment.

Male-specific (F^+) bacteriophage have been suggested to be indicators of viral pollution in the marine environment (11) and have been used previously by this laboratory in shellfish depuration studies (7). This group of single-stranded RNA bacteriophage, with a simple cubic capsid measuring 24 to 27 nm, is present in large numbers in sewage. The genomic and physical structures of F^+ bacteriophage are similar to those of the human enteroviruses and the viruses causing human gastroenteritis and hepatitis following shellfish consumption. F^+ bacteriophage thus provide an attractive model system for studying the behavior of viruses during depuration of environmentally contaminated molluscan shellfish.

In this study we contaminated Pacific oysters (*Crassostrea gigas*) and mussels (*Mytilus edulis*) for short and protracted periods by exposing them to sewage in the marine environment and then monitored the depuration kinetics of their distribution of *Escherichia coli* and F^+ bacteriophage. We make particular reference to the location of these indicators in mussel tissue before and after depuration to establish if sequestration of virus can occur, whether this sequestration is linked to the degree of contamination exposure, and whether this seques-

* Corresponding author.

tration may be responsible for residual levels of virus in shellfish after depuration.

MATERIALS AND METHODS

Shellfish and environmental contamination. Commercially purified oysters (*C. gigas*) and mussels (*M. edulis*) were contaminated by relaying them in bags on trestles approximately 800 m from a sewage outfall for 1 to 3 weeks (short-term exposure) or for more than 6 months (long-term exposure). The outfall discharged approximately 3.6×10 m^3 (dry-weight flow) of secondary treated effluent per day.

Environmental bioaccumulation. Approximately 50 mussels were placed in individual plastic mesh bags. The bags were placed on trestles at the contamination site at a low water level. One bag was removed at 0, 2, 6, 9, 23, and 92 h after initial immersion in seawater. Duplicate samples of 12 mussels each were taken from each bag and assayed for *E. coli* and F^+ bacteriophage within 24 h.

Laboratory depuration tank design. The depuration tank had dimensions of 1,050 mm (length) by 500 mm (width) by 450 mm (height), with a working volume of 150 liters. Seawater (30 to 33‰ salinity) was recirculated lengthways through the tank at a rate of 300 liters/h and sterilized by irradiation in a 15-W UV sterilizer (type 15/3p; UVAQ Ltd., Sudbury, United Kingdom). The temperature was maintained at 12°C for all experiments by placing the entire apparatus in a controlled-temperature room. Dissolved oxygen was maintained above 60% saturation by the use of a spray bar for recirculating seawater. Shellfish were depurated in plastic mesh baskets (no. 41042; Sommer Alibert [UK] Ltd., Droitwich, United Kingdom) and raised off the base of the tank by 22 mm to avoid recontamination by voided fecal material.

Depuration experiments. Filtered seawater (Hi-Rate Sandfilter [diameter, 24 in. (60.96 cm)]; Lacron Ltd., Sittingbourne, United Kingdom) was circulated through the depuration tank and UV irradiated for at least 24 h prior to each experiment. Contaminated shellfish were thoroughly washed, and damaged or gaping animals were discarded. Oysters and mussels were loaded into baskets to form a single overlapping layer or to a depth of 8 cm, respectively. For all experiments depuration was commenced within 4 h of shellfish collection. Duplicate samples of 12 mussels or 6 oysters each were removed for *E. coli* and F^+ bacteriophage analysis on a minimum of nine occasions throughout the 48-h depuration period. Shellfish were opened, and meats and liquors were pooled, diluted in 0.1% (wt/vol) peptone water (L37, pH 7.2; Oxoid Ltd., Basingstoke, United Kingdom), and homogenized in a blender (Waring Products Division, New Hartford, Conn.) as described by West and Coleman (26).

Tissue location experiments. Contaminated mussels (after 1 to 3 weeks of exposure) were thoroughly washed, and damaged or gaping animals were discarded. All analyses were commenced within 4 h of collection. For whole-animal analysis triplicate samples of 10 mussels each were opened, pooled, and homogenized as described by West and Coleman (26). Further triplicate samples of 10 mussels each were dissected for phage location experiments. Initially, hemolymph was extracted from the posterior adductor muscle sinuses of each animal with a 23-gauge needle and was pooled. The adductor muscle was then severed, and tissues were carefully dissected from each animal in the following order: foot, labial palps, gills, intestine, mantle lobes, digestive gland, and posterior adductor muscle. Tissues from each triplicate sample were pooled, diluted 1:5 in 0.1% (wt/vol) peptone water, and homogenized as described above. These analyses were performed twice with contaminated animals and with animals following 24- and 48-h depuration.

In addition to being subjected to the above-described procedures, samples from contaminated mussels were further analyzed by separation of the intestinal contents from the intestinal wall. The contents were extruded with a seeker, and the intestinal wall was thoroughly washed with 0.1% (wt/vol) peptone water. Each component was assayed for *E. coli* and F^+ bacteriophage separately.

***E. coli* analysis.** Shellfish and shellfish tissues were assayed in duplicate experiments for *E. coli* with a most-probable-number procedure as described by West and Coleman (26).

F^+ bacteriophage analysis. Shellfish and shellfish tissues were assayed for F^+ bacteriophage with the host of *Salmonella typhimurium* WG 49 as described by Havelaar and Hogeboom (12). Briefly, to 2.5 ml of molten 1% tryptone–yeast extract–glucose agar, held at 45°C, were added duplicate 1-ml volumes of appropriately diluted shellfish homogenates and 1 ml of WG 49 host culture. This mixture was stirred and poured onto a previously prepared 2% tryptone–yeast extract–glucose agar base in a 90-mm-diameter petri dish. The overlays were inverted and incubated overnight at 37°C.

RESULTS

E. coli and F^+ bacteriophage were both bioaccumulated rapidly in mussels, with levels reaching an equilibrium after 24 h (data not shown). Initial contamination levels observed in oysters ranged from 180,000 to 400,000 *E. coli* organisms and 70,000 to 80,000 F^+ bacteriophage per 100 g of shellfish. Contamination levels in mussels were similar, ranging from 80,000 to 220,000 *E. coli* organisms and 55,000 to 120,000 F^+ bacteriophage.

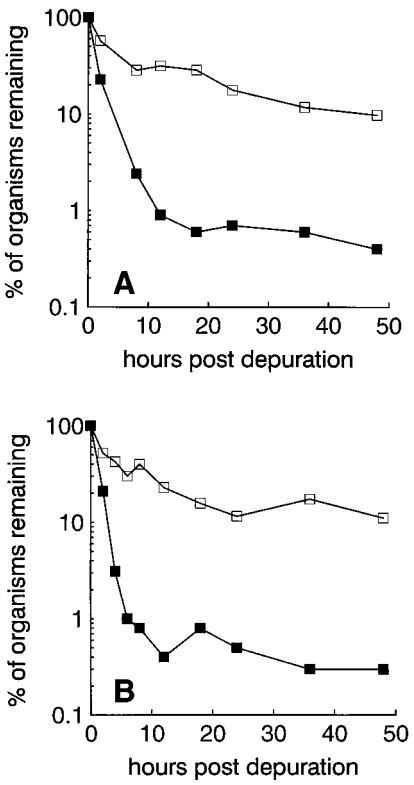

FIG. 1. Percentages of *E. coli* (■) and F^+ bacteriophage (□) remaining after depuration in mussels contaminated by short-term exposure (A) and long-term exposure (B) to sewage. Datum points are average values from two experiments.

The rates of reduction of *E. coli* and F^+ bacteriophage titers during depuration of contaminated mussels and oysters following either short- or long-term exposure to sewage are plotted in Fig. 1 and 2. *E. coli* levels were reduced rapidly in all cases, with mussels achieving >99% reduction within 12 h (Fig. 1). The rates of reduction of *E. coli* in oysters were marginally lower, however, with reductions of >98% achieved within the 48-h depuration period (Fig. 2). Although levels of *E. coli* appeared to increase after 24 h from 0.5 to 1.7% in oysters exposed over a long period (Fig. 2), this was not considered significant, falling within the expected test variation. The rates of reduction of *E. coli* did not appear to be influenced by the duration of exposure to contamination in either species. By contrast, F^+ bacteriophage removal during depuration was markedly more inefficient, with final rates of reduction over the 48-h depuration period of about 90% for mussels (Fig. 1) and 80% for oysters (Fig. 2). Again, the degree of F^+ bacteriophage removal did not appear to be influenced by the duration of exposure to contamination. To aid a comparison of the rates of elimination of these indicator organisms during depuration, T90s (time taken, in hours, to reduce 90% of the contaminants) were calculated (Table 1). T90s for both indicators were consistently shorter in mussels than in oysters. Importantly, F^+ bacteriophage appeared to behave significantly differently from *E. coli*, with T90s of, on average, 10-fold longer duration in both mussels and oysters. However, there was no evidence that these extended F^+ bacteriophage removal times were influenced by the duration of exposure to sewage contaminants (Table 1).

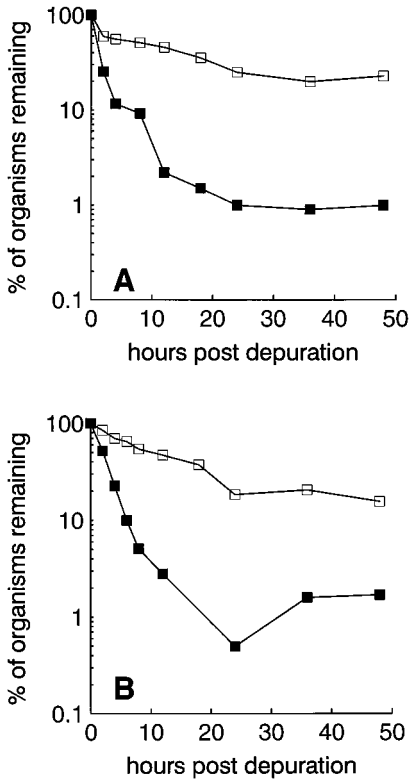

FIG. 2. Percentages of *E. coli* (■) and F^+ bacteriophage (□) remaining after depuration in oysters contaminated by short-term exposure (A) and long-term exposure (B) to sewage. Datum points are average values from two experiments.

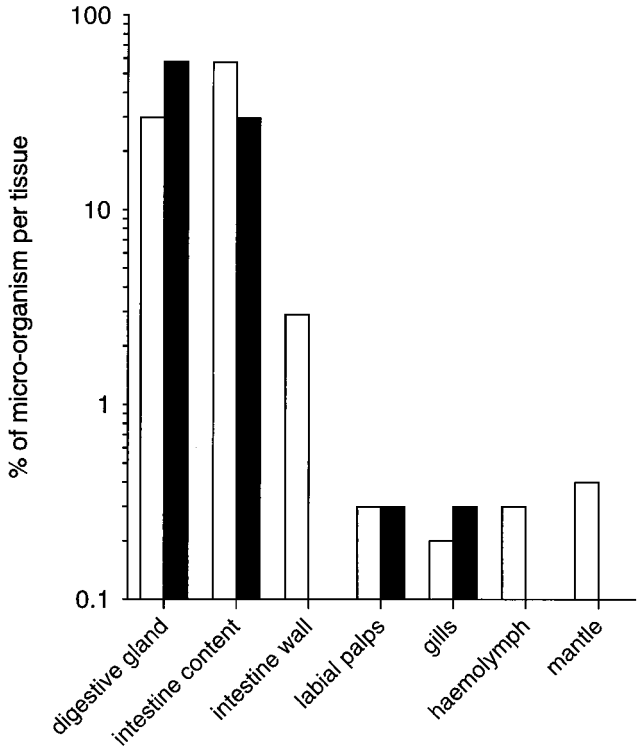

FIG. 3. Distributions of *E. coli* (open bars) and F^+ bacteriophage (solid bars) in the tissues of sewage-contaminated mussels as percentages of levels in whole animals. Data are average values from two experiments.

The significantly different reduction rates of *E. coli* and F^+ bacteriophage prompted further investigation of the location of contaminants within mussel tissues. The relative percent distribution of *E. coli* and F^+ bacteriophage in tissues compared with that in whole animals is shown in Fig. 3. *E. coli* was detected in all tissues except the posterior adductor muscle and the foot. The majority of *E. coli* organisms were confined to the digestive tract, with 90.1% found in the digestive gland and intestine (wall and content). All other tissues accounted for just 1.2% of *E. coli* organisms found in the whole animal. F^+ bacteriophage was detected only in four tissues, the digestive gland, intestine (intestinal contents), labial palps, and gills, with 87.3% confined to the digestive gland and intestinal contents. Other tissues accounted for just 0.6% of the F^+ bacteriophage levels found in the whole animals. It was noted after dissection that the total levels of *E. coli* and F^+ bacteriophage found in the tissues accounted only for 91.3 and 87.9%, respectively, of levels found in whole animals. This discrepancy was probably due to incomplete dissection of heavily contaminated intestinal tissues.

TABLE 1. T90s[a] for *E. coli* and F^+ bacteriophage after exposure to sewage and depuration by shellfish

Exposure	T90s (h) for:			
	Mussels		Oysters	
	E. coli	F^+ phage	*E. coli*	F^+ phage[b]
Short-term	4.5	47.3[b]	6.5	54.6
Long-term	3	41.3	6	60.8

[a] T90s are derived from Fig. 1 and 2.
[b] Where 90% reductions were not achieved, predicted T90s were determined by using the statistical computer package MINITAB.

The percent reductions of *E. coli* and F^+ bacteriophage levels from whole animals, digestive glands, and intestines during depuration are plotted in Fig. 4. *E. coli* and F^+ bacteriophage in all other tissues were reduced to undetectable levels within 24 h. *E. coli* and F^+ bacteriophage in the intestines were reduced to undetectable levels within 48 h. While *E. coli* was reduced to undetectable levels in the digestive glands within 48 h, significant levels of F^+ bacteriophage, 29.7%, remained in the digestive glands after depuration.

DISCUSSION

This study was undertaken to investigate the hypothesis that residual levels of viruses found in shellfish after depuration are due to sequestration into tissues not associated with the digestive process. This is the first published report of an investigation of this hypothesis using shellfish contaminated by sewage while in the marine environment.

The accumulation of microbial contaminants by mussels was similar to patterns reported previously for bivalve shellfish (14). Both *E. coli* and F^+ bacteriophage were accumulated rapidly over 24 h, thereafter reaching an apparent equilibrium, suggesting that similar mechanisms are responsible for both viral and bacterial accumulation in bivalve shellfish.

The rates and patterns of *E. coli* and F^+ bacteriophage reduction observed during the depuration of oysters and mussels after short-term sewage contamination confirm previous findings (7). Shellfish showed no significant differences in T90s or in reductions or in residual levels of both viral and bacterial indicators after short- or long-term exposure to sewage. These

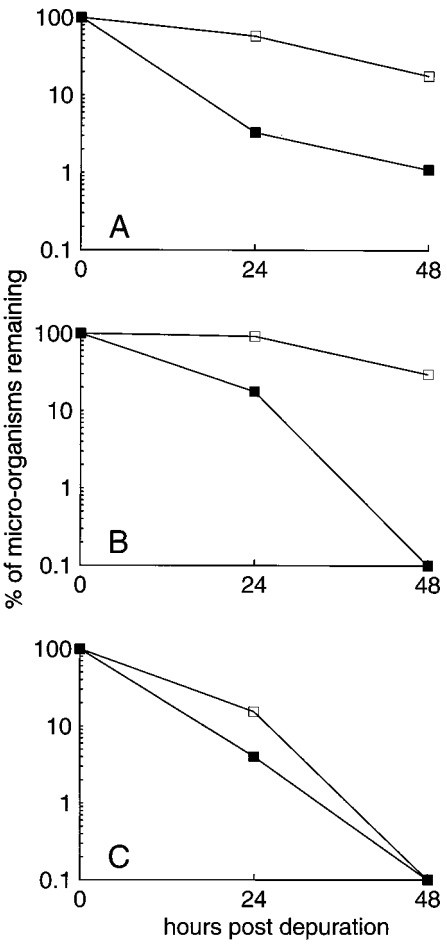

FIG. 4. Percentages of *E. coli* (■) and F^+ bacteriophage (□) remaining after depuration in whole mussels (A) and digestive glands (B) and intestines (C) from mussels contaminated with sewage. Datum points are average values from two experiments.

results do not concur with the suggestion that the degree of viral sequestration is linked to duration of exposure (3).

Significantly, dissection of mussel tissues demonstrated that F^+ bacteriophage were exclusively confined to the organs associated with digestion, namely, the digestive gland, intestine, labial palps, and gills, with the vast majority detected within the digestive gland and the intestine. By contrast, *E. coli* organisms were also detected in low numbers in tissues not associated with the digestive tract, namely, the mantle lobes and notably the hemolymph. These results show that sequestration of F^+ bacteriophage in contaminated mussels is not demonstrable and is therefore unlikely to be responsible for the residual levels following depuration reported in this and previous studies (5, 7). *E. coli* and F^+ bacteriophage were reduced to undetectable levels within 24 h in all tissues except the intestine and digestive gland. By 48 h, levels in the intestine were also undetectable, confirming the visual observation that intestinal contents were evacuated completely during depuration. The only tissue showing a significant, and differential, level of retention of F^+ bacteriophage above that of *E. coli* was the digestive gland. It appears, then, that this is the main site of F^+ bacteriophage and, by implication, viral retention in depurated mussels.

Contaminant reduction in the digestive gland is primarily a function of defecation or digestion or both. Digestion can be a function of absorption into the cells of the digestive diverticulum or by direct ingestion by hemocytes (1). It is well documented that bacteria are utilized as a food source by molluscan shellfish (17), and the widespread distribution of *E. coli* in hemolymph and other tissues in this study tends to confirm this. Extensive and rapid extracellular degradation of *E. coli* by lysosomal activity in the digestive gland has also been documented (1, 2). These mechanisms may account for the rapid reduction of *E. coli* levels in the digestive gland observed in this study and suggest that this may be due largely to digestion as opposed to elimination through defecation. Hay and Scotti (13) used radiolabelled cricket paralysis virus to show that while the majority of viruses in oysters were eliminated during depuration, some were absorbed intracellularly in the digestive diverticulum. The value of this observation in explaining differential depuration rates critically depends on the resistance of viruses to inactivation in the cells of the digestive diverticulum following intracellular absorption. This has not yet been explored.

This, and other studies (15), demonstrates that bacterial reductions may be predominantly influenced by digestive processes. Previous work related to shellfish depuration has, however, assumed that a reduction in levels of contaminants equates to elimination by defecation. This is significant, as the inference from this assumption was that all sewage microorganisms not sequestered into tissues or intracellularly absorbed should be equally rapidly eliminated in feces. However, while gut transit times for molluscs are normally rapid (1), the time required to clear the digestive tract may be considerably longer under starvation conditions such as those routinely found during depuration. It is possible that the rates of reduction observed for F^+ bacteriophage in this study are the actual times required for shellfish to eliminate contaminants from the digestive tract under depuration conditions. Initial rapid reductions of viruses, often observed during depuration, may be attributable to the evacuation of material from the intestine, as demonstrated in this study.

In conclusion, this study demonstrates that duration of sewage exposure does not affect the differential reductions of *E. coli* and F^+ bacteriophage from depurating oysters and mussels. It further demonstrates that the sequestration of F^+ bacteriophage into nondigestive tract tissues does not occur in environmentally contaminated mussels and identifies the digestive gland as the major site of F^+ bacteriophage retention. The mechanism primarily responsible for virus retention has considerable importance, as intracellular absorption would probably preclude the further development of depuration as an effective procedure to remove viruses of public health significance from shellfish. However, if viral retention is due to gut clearance times longer than previously anticipated, extended depuration under favorable conditions may in fact be effective in removing viruses from shellfish.

REFERENCES

1. **Bayne, B. L., R. J. Thompson, and J. Widdows.** 1976. Physiology: 1. Feeding and digestion, p. 122–159. *In* B. L. Bayne (ed.), Marine mussels. Their ecology and physiology. University Printing House, Cambridge.
2. **Birbeck, T. H., and J. G. McHenery.** 1982. Degradation of bacteria by *Mytilus edulis*. Mar. Biol. (Berlin) **72:**7–15.
3. **Canzonier, W. J.** 1971. Accumulation and elimination of coliphage S-13 by the hard clam, *Mercenaria mercenaria*. Appl. Microbiol. **21:**1024–1031.
4. **Canzonier, W. J.** 1982. Depuration of bivalve molluscs—what it can and cannot accomplish and some practical aspects of plant design and operation. *In* Proceedings of the International Seminar on Management of Shellfish Resources. Irish Marine Farmers Association, Tralee, Ireland.
5. **de Mesquita, M. M. F., L. M. Evison, and P. A. West.** 1991. Removal of faecal indicator bacteria and bacteriophages from the common mussel (*Mytilus edulis*) under artificial depuration conditions. J. Appl. Bacteriol. **70:**495–501.

6. **di Girolamo, R., J. Liston, and J. Matches.** 1975. Uptake and elimination of poliovirus by West Coast oysters. Appl. Microbiol. **29:**260–264.
7. **Doré, W. J., and D. N. Lees.** 1991. Differential depuration of *Escherichia coli* and F+ bacteriophage from sewage-contaminated bivalve molluscs, p. 113–119. *In* R. Morris, L. Alexander, P. Wyn-Jones, and J. Sellwood (ed.), Proceedings of the First United Kingdom Symposium of Health-Related Water Microbiology, University of Strathclyde, Glasgow, 3 to 5 September 1991. International Association on Water Pollution Research and Control.
8. **Fleet, G. H.** 1978. Oyster depuration—a review. Food Technol. Aust. **30:** 444–454.
9. **Gill, O. N., W. D. Cubitt, D. A. McSwiggan, B. M. Watney, and C. L. R. Bartlett.** 1983. Epidemic of gastro-enteritis caused by oysters contaminated with small round structured viruses. Br. Med. J. **287:**1532–1534.
10. **Grohmann, G. S., A. M. Murphy, P. J. Christopher, E. Auty, and H. B. Greenberg.** 1981. Norwalk virus gastro-enteritis in volunteers consuming depurated oysters. Aust. J. Exp. Biol. Med. Sci. **59:**219–228.
11. **Havelaar, A. H., K. Furuse, and W. M. Hogeboom.** 1986. Bacteriophages and indicator bacteria in human and animal faeces. J. Appl. Bacteriol. **60:**255–262.
12. **Havelaar, A. H., and W. M. Hogeboom.** 1984. A method for the enumeration of male-specific bacteriophages in sewage. J. Appl. Bacteriol. **56:**439–447.
13. **Hay, B., and P. Scotti.** 1986. Evidence for intracellular absorption of virus by the Pacific oyster, *Crassostrea gigas*. N. Z. J. Mar. Freshwater Res. **20:**655–659.
14. **Humphrey, T. J., and K. Martin.** 1993. Bacteriophage as models for virus removal from Pacific oysters (*Crassostrea gigas*) during re-laying. Epidemiol. Infect. **111:**325–335.
15. **Kueh, C. S. W.** 1987. Uptake, retention and elimination of enteric bacteria in bivalve molluscs. Asian Mar. Biol. **4:**113–128.
16. **Liu, O. C., H. R. Seraichekas, and B. L. Murphy.** 1966. Fate of poliovirus in northern quahaugs. Proc. Soc. Exp. Biol. Med. **121:**601–607.
17. **McHenery, J. G., and T. H. Birbeck.** 1985. Uptake and processing of cultured microorganisms by bivalves. J. Exp. Mar. Biol. Ecol. **90:**145–163.
18. **Metcalf, T. G., B. Mullin, D. Eckerson, E. Moulton, and E. P. Larkin.** 1979. Bioaccumulation and depuration of enteroviruses by the soft-shelled clam, *Mya arenaria*. Appl. Environ. Microbiol. **38:**275–282.
19. **Power, U. F., and J. K. Collins.** 1989. Differential depuration of poliovirus, *Escherichia coli*, and a coliphage by the common mussel, *Mytilus edulis*. Appl. Environ. Microbiol. **55:**1386–1390.
20. **Power, U. F., and J. K. Collins.** 1990. Tissue distribution of a coliphage and *Escherichia coli* in mussels after contamination and depuration. Appl. Environ. Microbiol. **56:**803–807.
21. **Public Health Laboratory Service, London.** 1994. Gastro-enteritis associated with oysters. Commun. Dis. Rep. Weekly **4:**43.
22. **Richards, G. P.** 1988. Microbial purification of shellfish: a review of depuration and relaying. J. Food Prot. **51:**218–251.
23. **Scotti, P. D., G. C. Fletcher, D. H. Buisson, and S. Fredericksen.** 1983. Virus depuration of the Pacific oyster (*Crassostrea gigas*) in New Zealand. N. Z. J. Sci. **26:**9–13.
24. **Sobsey, M. D., and L. A. Jaykus.** 1991. Human enteric viruses and depuration of bivalve molluscs, p. 71–114. *In* W. S. Otwell, G. E. Rodrick, and R. E. Martin (ed.), Molluscan shellfish depravation. CRC Press, Boca Raton, Fla.
25. **Sockett, P. N., P. A. West, and M. Jacob.** 1985. Shellfish and public health. Public Health Lab. Service Microbiol. Digest. **2:**29–35.
26. **West, P. A., and M. R. Coleman.** 1986. A tentative national reference procedure for isolation and enumeration of *Escherichia coli* from bivalve molluscan shellfish by most probable number method. J. Appl. Bacteriol. **61:** 505–516.
27. **West, P. A., P. C. Wood, and M. Jacob.** 1985. Control of food poisoning risks associated with shellfish. J. R. Soc. Health **1:**15–21.