Inadequacy of bacterial indicators for assessing elimination rates of viruses from molluscan shellfish

Insuffisance des indicateurs bactériens dans l'évaluation des taux d'élimination des virus dans les coquillages

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Abstract

The reported incidence of shellfish-borne illness in the United States increased dramatically during the last decade. Most of the outbreaks are attributed to diseases of viral aetiology. These human health problems result primarily from the ingestion of raw shellfish which have accumulated enteric pathogens from environmental waters or from wet storage facilities. Our studies have demonstrated that hard-shelled clams concentrate different indicator micro-organisms at variable and unpredictable rates during the year. In temperate waters, this has been observed at two abbreviated periods in mid-spring and again in late fall when accumulation rates increased dramatically. Moreover, these rates were not generally coincident for viruses and bacteria. In addition, our studies have shown that, using conventional depuration technologies, the elimination rates for viruses and bacteria are profoundly different. Relative to vegetative bacterial indicators (fecal coliforms, *E. coli*), the male-specific coliphage group may take as long as ten times that of the conventional indicator for a similar degree of elimination. Our results, coupled with those of other investigators as well as with certain epidemiological reports, demonstrate that viral behaviour within molluscan shellfish is not indexed by bacterial indicator organisms.

Keywords: Bacterial indicators, depuration, elimination rates, shellfish, viruses.

Résumé

Les épisodes de maladies dues aux coquillages décrits aux États-Unis se sont multipliés de façon spectaculaire au cours de la dernière décennie. La plupart de ces épisodes sont attribués à des maladies à étiologie virale. Ces problèmes de santé humaine résultent principalement de la consommation de coquillages crus ayant accumulé des pathogènes entériques provenant des eaux du milieu naturel ou des installations de conservation en eau. Nos études démontrent que les clams *Mercenaria mercenaria* concentrent différents microorganismes indicateurs à des taux variables et imprévisibles dans le courant de l'année. Dans les eaux tempérées, ce phénomène a été observé pendant deux brèves périodes au milieu du printemps et à la fin de l'automne, avec une augmentation spectaculaire des taux d'accumulation. En outre, ces taux ne coïncident généralement pas pour les virus et les bactéries. Par ailleurs, nos études ont montré que les taux d'élimination des virus et des bactéries diffèrent fortement lorsqu'on utilise des techniques de purification traditionnelles. Par rapport aux indicateurs bactériens viables (coliformes fécaux, *E. coli*), le groupe des coliphages spécifiques-mâle peut nécessiter jusqu'à dix fois plus longtemps qu'un indicateur traditionnel pour atteindre un niveau similaire d'élimination. Nos résultats, associés à ceux d'autres chercheurs ainsi qu'à certains rapports épidémiologiques, démontrent que le comportement viral dans les coquillages n'est pas indexé par les indicateurs bactériens.

Mots-clés : Indicateurs bactériens, purification, taux d'élimination, coquillages, virus.

INTRODUCTION

Raw and partially cooked molluscan shellfish (including clams, oysters, and mussels) have been directly implicated in many outbreaks of infectious disease of viral aetiology. Public health problems that are associated with the consumption of these shellfish range from mild gastro-enteritis to hepatitis A. These illnesses appear to have a seasonal occurrence (Rippey S.R., 1991). To reduce their incidence, certain public health safeguards have been implemented over the past several decades. Among these safeguards are National Shellfish Sanitation Program (NSSP) procedures (U.S. Food and Drug Admin., 1992) for purifying contaminated shellfish by relay or depuration. Both of these purification practices are premised upon the ability of contaminated shellfish to purge themselves of microbial pathogens in a short period.

Fecal coliform bacteria are the traditional sanitary indicators of water and foods, including shellfish (American Public Health Association, 1970). However, this bacterial group is criticised as a reliable indicator of sanitary quality for several reasons: (a) fecal coliforms often do not index the presence of viral pathogens in marine environments (Gerba C.P., 1979), presumably because of differential rates of inactivation due to the effects of such factors as salinity, sunlight, and temperature (Borrego J.J., F. Arrabal, A. deVicente, L.F. Gomez, P. Romero., 1983; Kapuscinski R.B., R. Mitchell, 1980; Shuval H.I., A. Thompson, B. Fattal., S. Cymbalista., Y. Wiener, 1971); (b) chlorine disinfection of treated waste waters will inactivate fecal coliforms more efficiently than certain of the enteric viral pathogens which have been implicated in shellfish-associated illnesses (Grabow W.O.K., V. Gauss-Muller, O.W. Prozesky, F. Deinhardt, 1983; Havelaar A.H., T.J. Nieuwstad, 1985; Keswick B.H., T.K. Satterwhite, P.C. Johnson, H.D. Dupont, S.L. Secor, J. Bitsura, G.W. Gary, J.C. Hoff, 1985); (c) fecal coliform bacteria are accumulated by shellfish to significantly lower levels then either *C. perfringens* (an alternative sanitary indicator) or male-specific bacteriophage, simulants of enteric viruses (Burkhardt III W., W.D. Watkins, S.R. Rippey, 1992); moreover, the densities of fecal coliforms do not reliably index the level of accumulation of these enteric viral simulants; and, (d) fecal coliform bacteria appear to be eliminated more rapidly from shellfish undergoing depuration than are certain enteric viral pathogens (Grohmann G.S., A.M. Murphy, P.J. Christopher, G. Auty, H.B. Greenberg, 1981; Grun R.A., H.T. Janowski, S. Lieb, E.C. Prather, H.B. Greenberg, 1982; Timoney J.F., A. Abston, 1984). As such, fecal coliforms may not reliably index the presence of certain human enteric pathogens, particularly viruses and, thus, the sanitary quality of shellfish growing waters or shellfish meats themselves cannot be confidently assessed. Other micro-organisms have been, and continue to be, examined as potentially more reliable indices of viral contamination. The objectives of this study were to determine the relative rates of elimination (depuration) of certain of these microbial indicator organisms in relation to the currently accepted fecal coliform group. Trials were conducted in two depuration systems under a variety of conditions using both the northern quahog (*Mercenaria mercenaria*) and the eastern oyster (*Crassostrea virginica*).

Materials and methods

Shellfish

Hard-shelled clams and eastern oysters were harvested from Narragansett Bay, Rhode Island and relocated to a marine laboratory at Allen Harbor, RI. Clams and oysters were stored (and contaminated for trials) separately in circular, fiberglass resin tanks (90 cm diameter; capacity 127 l). Each tank received a continuous flow of ambient sea water (4-5 l/min for storage) from Narragansett Bay.

Shellfish Contamination

Raw waste water was collected in polypropylene carboys (20 l) from a municipal treatment facility (E. Greenwich, RI) receiving primarily domestic waste. Collected sewage was held under refrigeration (3-5°C) and continuously delivered to a contamination tank using a proportioning pump (Technicon Corp., Tarrytown, NY) for a 72 h contamination period. A submersible pump (Model 1, Little Giant, Oklahoma City, OK) was used to thoroughly mix waste water with sea water in the tank. For each depuration trial, 150 animals (either species) were placed in a tank receiving a constant flow of sea water adulterated with a continuous input of waste water. Flow rates of sea water and waste water were varied (1-3 l/min and 0.6-3.9 ml/min, respectively) to attain preselected densities of microbial indicator organisms in both the overlying tank water and the shellfish. Temperatures and salinities in contamination tanks ranged from 10-20°C and 28-31 ppt, respectively. Dissolved oxygen concentrations ranged from 6.4-8.4 ppm, and turbidity was consistently less than 2.0 nephelometry turbidity units.

Depuration Systems

Two types of continuous-flow sea water systems were employed for depuration experiments.

A. Flow-Through

Trials using a flow-through design of fresh sea water were conducted in rectangular, plywood-reinforced fiberglass resin tanks measuring 350 x 30 x 25 cm, with capacities of 210 l. Prior to depuration, contaminated shellfish were rinsed and placed upon galvanised hardware cloth (65 mm mesh) in a monolayer suspended 10 cm above the bottom. Sea water was disinfected by a 4-bulb Kelly-Purdy (Kelly C.B., 1961) UV irradiation unit (15 volt lamps, General Electric germicidal lamp, Cleveland, OH) prior to entering the tanks. The effectiveness of disinfection was monitored daily by assaying UV disinfected sea water for the presence of the indicators of interest. Salinity, temperature, dissolved oxygen and turbidity also were determined daily.

B. Recirculating

The recirculating system consisted of two rectangular, polypropylene tanks (45 x 32 x 24 cm, Nalgene Laboratories, Rochester, NY), connected by latex

tubing (20 mm diameter). In one tank, shellfish (approximately 100 animals) were stacked in a stainless steel basket (mesh size 30 mm) to a maximum height of 7.5 cm (about 4 animals deep) to allow free flow of water about them. In the other tank, a submersible pump circulated sea water through the UV disinfection unit at 6 l/min, and then back to the shellfish tank. This set-up was devised to prevent the resuspension of settled material. Again, microbial, chemical, and physical parameters were monitored daily.

Sea water

Trials were conducted using either natural sea water (28-31 ppt) from Narragansett Bay, RI, or artificial sea water. Artificial sea-salts (Aquarium Systems, East lake, OH) were reconstituted in deionized water to a final salinity of 28-31 ppt. Salinities and temperatures of sea water were determined using a electrode-less induction salinometer (Model RS 5-3, Beckman, Cedar Grove, NJ). Dissolved oxygen was measured using a YSI Model 57 oxygen meter (Yellow Springs Instruments, Yellow Springs, OH). Turbidities were determined with a nephelometer (Model 21PE, Monitek Inc., Hayward, CA).

Microbiological Analyses

Sea water. Samples were collected in sterile polypropylene sample bottles and held on ice until examined. Microbial analyses were performed within 4 h of collection. Appropriate volumes of samples were filtered through 0.7µm membranes (HC filters, Millipore Corp., Bedford, NH), and these then were placed onto various agar enumeration media and incubated. Fecal coliforms and *E. coli* densities were determined using the mTEC procedure (Dufour A.P., E.R. Strickland, V.J. Cabelli, 1981), enterococci levels were determined using the modified mE procedure (Dufour A.P., 1989; Levin M.A., J.R. Fischer, V.J. Cabelli, 1975), and *C. perfringens* densities were determined using the mCP procedure (Bisson J.W., V.J. Cabelli, 1979). Male-specific bacteriophage were enumerated using a modified double-agar overlay procedure and *E. coli* HS(pFamp)R as the host (DeBartolomeis J., V.J. Cabelli, 1991).

Shellfish. Samples were collected and processed according to recommended procedures (American Public Health Assoc., 1970). Between 12-20 animals were randomly collected from experimental populations at selected times during each depuration trial. Homogenised shellfish meats and liquors were held on ice (up to 60 minutes) until assayed. Fecal coliforms and *E. coli* levels in homogenates were determined using the APHA most probable number procedure (American Public Health Assoc., 1970) as either a 20-tube, single dilution MPN (samples consisted of 20 animals) or a 5-tube, multiple dilution MPN (3-4 sub-samples consisting of 4 animals each). Enterococci were determined using azide dextrose broth (Difco) as a presumptive, selective, enrichment medium in one of the two multiple tube MPN procedures described above. Tubes positive for growth after 24 and 48 h at 35°C were confirmed by streaking onto membrane filters which had been placed on modified mE agar. The modified mE plates were incubated for 24 h at 41°C, and tubes were scored positive if blue growth was found along streaks. Levels of *C. perfringens* in shellfish were determined using the iron milk MPN procedure (Abeyta C.,

1983), again using one of the two MPN procedures described above. Male-specific bacteriophage densities were determined using a modified double-agar-overlay procedure (Cabelli V.J., 1988). In essence, the procedure consists of extraction of these bacteriophage from shellfish by high speed homogenisation for 2 min. followed by low speed centrifugation (9,000 x g), then analyses of supernatants using host strain *E. coli* HS(pFamp)R in a double-agar-overlay technique. Densities are determined by measuring the supernatant weights relative to the total homogenate weights and making the appropriate calculations.

Results

Hard-shelled Clams

The elimination rates of fecal coliforms, *E. coli* (data not shown) and enterococci were consistently more rapid than those for either *C. perfringens* or the male-specific bacteriophage in hard-shelled clam depuration trials using a flow-through natural sea water system (table I). Data for *E. coli* are not shown because they are very similar to the fecal coliform results. In three replicate trials, each of the vegetative bacterial species required less than 2 days for a 99% reduction in the shellfish. This resulted in fecal coliform, *E. coli,* and enterococci MPNs of less than 20 per 100 g of homogenate. A comparable reduction (99%) in shell stock levels of *C. perfringens* required over 7 days. Male-specific bacteriophage persisted far longer than any of the other indicators, and required more than 4 times as long as fecal coliforms for comparable reductions.

Table I: Elimination of microbial indicators from *Mercernaria mercenaria* in a flow-through natural sea water system [a]

Microbial Indicator	Range of Initial Levels /100 g[b]	Mean Elimination Times in hours [c]		Mean Ratios to Fecal coliforms[d]	
		T_{90}[e]	T_{99}[f]	T_{99}[e]	T_{90}[f]
Fecal coliforms	2.7-4.2	19.1	47.6	—	—
Enterococci	2.7-3.9	19.6	45.8	1.0	1.0
Clostridium perfringens	3.1-3.3	50.6	172.2	2.6	3.6
Male-specific bacteriophage	1.5-5.0	77.6	204.8	4.1	4.3

(a). Temperature maintained at 19-23°C; salinities from 28-31 ppt.
(b). Range of initial densities for 3 trials expressed as $\log_{10}$ values.
(c). Mean of 3 replicate depuration trials.
(d). Ratios of mean elimination times for a given indicator to those for fecal coliforms.
(e). Time of a 1 $\log_{10}$ (90%) reduction from initial levels.
(f). Time of a 2 $\log_{10}$ (99%) reduction from initial levels.

Table II: Depuration of microbial indicators from *Mercernaria mercenaria* in recirculating artificial sea water[a]

Microbial Indicators	Trial	Log_{10} Initial Levels /100 g	Elimination Times in hour		Ratios to Fecal coliforms[b]	
			T_{90}[c]	T_{99}[d]	T_{90}[c]	T_{99}[d]
Fecal coliforms	1 2	3.6 2.8	71.9 89.8	125.6 173.7	—	—
Enterococci	1 2	4.1 3.9	86.8 87.7	174.1 167.3	1.1	1.2
Clostridium perfringens	1 2	3.2 2.8	154.1 160.1	317.2 384.1	2.0	2.0
Male-specific bacteriophage	1 2	3.6 4.1	353.5 309.3	611.2 615.6	4.2	4.2

(a). Temperatures maintained at 10-12°C; salinities of 28-31 ppt.
(b). Ratios of mean elimination times for a given indicator to those for fecal coliforms.
(c). Time of a 1 log_{10} (90%) reduction from initial levels.
(d). Time of a 2 log_{10} (99%) reduction from initial levels.

In trials using stacked shellfish in artificial sea water (10-12°C), fecal coliforms, *E. coli* (data not shown), and enterococci again were eliminated from shellfish at similar rates (table II). However, 99% reductions in the initial densities of fecal coliforms, *E. coli*, and enterococci required up to 174 h, a period that is appreciably longer than that found for the flow-through system. Elimination rates of *C. perfringens* were considerably slower in comparison to fecal coliforms, requiring up to 16 days under these conditions for similar reductions. Male-specific bacteriophage were the most refractory to elimination from the hard-shelled clams, requiring as long as 615 h (26 days) for 99% reductions in initial densities.

Eastern oysters

Reductions in the levels of fecal coliforms, *E. coli* (data not shown), and enterococci were similar in depuration trials conducted with *C. virginica* in a flow-through natural sea water system (table III). Two orders of magnitude reductions in each of these vegetative indicators generally occurred within 48 h. The times required for comparable reductions of *C. perfringens* and male-specific bacteriophage were consistently and substantially longer than those found for the vegetative bacterial indicators. A 99% reduction in the levels of *C. perfringens* in oysters required from 96 to 235 h, and comparable decreases in densities of the bacteriophage required 162 to 237 h (6.8-9.9 days).

In the recirculating artificial sea water system, oyster depuration rates for all indicators were consistently more prolonged relative to those found with the flow through system (table III). Times required to eliminate 99% of the fecal coliforms, *E. coli*, and enterococci from oysters were similar, and required up to 3 days. Equivalent reductions of *C. perfringens* and male-specific bacteriophage required 8 and 25 days, respectively, as much as 10 times longer.

Table III: Depuration of microbial indicators from *Crassostrea virginica* in two types of seawater system[a]

Microbial Indicators	Trial	Log_{10} Initial Levels /100 g	Elimination Times in hour		Ratios to Fecal coliforms[(b)]	
			T_{90}[(c)]	T_{99}[(d)]	T_{90}[(c)]	T_{99}[(d)]
Fecal coliforms	1-F	4.1	9.9	32.5	—	—
	2-F	5.0	8.6	28.0		
	3-F	4.8	14.5	49.0		
	4-F	4.5	12.9	33.4		
	5-R	3.6	15.3	37.0		
	6-R	3.4	25.1	59.4		
Enterococci coliforms	1-F	3.2	13.4	32.6	1.4	1.1
	2-F	3.0	10.6	21.2		
	3-F	3.6	13.8	30.2		
	4-F	2.5	28.4	55.6		
	5-R	3.2	12.4	24.7	1.1	1.0
	6-R	2.6	34.0	70.1		
Clostridium perfringens	1-F	3.5	54.2	119.3	6.3	4.5
	2-F	2.9	91.7	234.6		
	3-F	4.8	71.2	166.5		
	4-F	3.3	48.0	96.0		
	5-R	3.6	50.1	196.8	2.2	4.0
	6-R	3.7	37.0	190.0		
Male-specific bacteriophage	1-F	3.9	79.8	162.1	9.2	6.0
	2-F	5.4	100.9	198.9		
	3-F	5.0	108.3	235.9		
	4-F	4.0	123.4	237.3		
	5-R	2.7	229.2	455.1	13.2	10.9
	6-R	2.5	303.1	599.6		

(a) Seawater temperatures (°C) in the flow-through natural (F) system were: Trial 1, 19.5-22.5; Trial 2, 18.0-20.0; Trial 3, 19.0-20.0; Trial 4, 22.0-25.5. Temperatures in the recirculating artificial (R) seawater system were maintained at 10-13°C, and salinities were 28-31 ppt.

(b) Ratios of mean elimination times for a given indicator to those for fecal coliforms.

(c) Time of a 1 log_{10} (90%) reduction from initial levels.

(d) Time of a 2 log_{10} (99%) reduction from initial levels.

Discussion

This study compared the elimination rates of fecal coliforms, the currently accepted sanitary indicator group, to those determined for other microbial indicators of fecal pollution from two commercially important molluscan bivalve species (*M. mercenaria* and *C. virginica*). Commercial depuration facilities can process shellfish using a variety of seawater systems. Accordingly, several key parameters were manipulated to reflect conditions commonly encountered at

commercial operations. These parameters included: (a) the sea water itself (natural or artificially prepared); (b) system hydraulics (flow-through or recirculating); (c) temperature; and, (d) shellfish stacking.

Ideally, the effects of each parameter should be investigated individually during separate trials. However, this was not possible due to practical considerations and logistical constraints. Therefore, the elimination rates for five microbial indicator groups were determined under the 'optimum' (flow-through natural sea water, elevated temperatures, shell stock in monolayers) and 'minimal' (recirculating artificial sea water, low temperatures, stacked shell stock) conditions found commercially. Each depuration system and parameter (water flow, temperature, dissolved oxygen, turbidity, shellfish density and stacking) used met the U.S. NSSP minimum guidelines for depuration of molluscan shellfish (U.S. Food and Drug Admin., 1992).

Both the oyster and the clam depuration trials demonstrated that these shellfish species were more efficient in eliminating each of the indicators in a flow-through natural sea water system, and were considerably less efficient in the recirculating artificial sea water system. In the former, fecal coliforms, *E. coli*, and enterococci densities were generally reduced by more than 2 orders of magnitude within 2 days, whereas similar reductions in the latter system required up to 7 days. *C. perfringens* and the male-specific bacteriophage persisted longest in shellfish under all depuration conditions examined. Elimination of these indicators required 2-4 times longer than that needed for fecal coliforms. Male-specific bacteriophage were found to be the indicators most refractory to depuration. The mean times necessary to eliminate 99% of these bacterial viruses from oysters was 208 h (> 8 days) in the flow-through system and 528 h (22 days) in the recirculating system.

These results are supported, in part, by the findings of other investigators. While *poliovirus* is expelled from shellfish at a rate similar to that for coliforms (Liu O.C., H.R. Seraichekas, B.L. Murphy, 1967; Sobsey M.D., A.L. Davis, V.A. Rullman, 1987), other depuration studies have demonstrated the prolonged persistence of bacteriophage S-13 in hard-shelled clams (Canzonier W.J., 1971) and hepatitis A virus in oysters (Sobsey M.D., A.L. Davis, V.A. Rullman, 1987). Male-specific bacteriophage, or at least a large portion of this group, appear to depurate similarly to these latter two viral groups in both clams and oysters.

Depuration rates of the *Norwalk* virus are not known because there are no quantitative methods available for its enumeration. Nevertheless, the bacteriophage data from this study are disturbing because they continue to support the findings (in lieu of the poliovirus work) on the persistent nature of viruses in molluscan shellfish. Since the *Norwalk* virus is presumably the principal agent of gastro-enteritis among raw shellfish consumers (Rippey S.R., 1991), and since it may not be eliminated in a commercially feasible time period (48 h) from molluscan shellfish (Gunn R.A., H.T. Janowski, S. Lieb, E.C. Prather, H.B. Greenberg, 1982), depuration may not provide an effective barrier for public health protection. When quantitative methods for assaying the Norwalk agent become available, further investigations will be needed to address these

questions. Should it be found that Norwalk viruses are very refractory to depuration using conventional practices, as determined here for the male-specific bacteriophage, a parallel line of investigation should be undertaken to examine factors which promote shellfish metabolic activity with the intention of enhancing elimination rates of microbial contaminants, especially viruses (Rheault R.B., 1984).

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