

Strategies for removal of indicator and pathogenic bacteria from commercially harvested shellfish

Stratégies d'élimination des germes indicateurs et pathogènes dans les coquillages d'élevage

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Abstract

The Great Bay/Piscataqua River Estuary in New Hampshire and Maine has an abundant oyster resource in sewage-contaminated water. The only approved area in the Maine portion of the Estuary is Spinney Creek, where fecal indicator bacteria are present at reduced levels and *Vibrio vulnificus* is absent. Spinney Creek Oyster Company (SCOC) of Eliot, Maine, operates relay lagoons and a depuration facility for oysters harvested commercially from restricted areas of the Salmon Falls River in Maine. Oysters naturally contaminated with vibrios and fecal indicator bacteria were used to evaluate depuration and relaying as strategies for eliminating these bacteria from shellfish. Oysters and water were analysed for the presence of total and fecal coliforms, *V. vulnificus*, *V. parahaemolyticus*, and total vibrios. Coliforms were always detected at varying levels in water and oyster samples. *V. vulnificus* was detected consistently during July-October at the harvest site, but never in Spinney Creek water or anywhere else during November-June. Relaying oysters for 7 days to the SCOC relay lagoons consistently decreased levels of fecal coliforms and *V. vulnificus*. Depuration of oysters for 48 hours significantly reduced total and fecal coliforms, but did not decrease vibrio levels. These results demonstrate the effectiveness of depuration and relaying in reducing fecal contamination in oysters, and a unique, additional benefit of relaying for eliminating otherwise recalcitrant *V. vulnificus*.

Keywords : depuration, relaying, pathogenic bacteria, bacterial indicators, vibrios, oysters.

Résumé

L'estuaire du fleuve Piscataqua/Great Bay dans les états du New Hampshire et du Maine représente une source abondante d'huîtres dans des eaux contaminées par les égouts. La seule zone autorisée de la section de l'estuaire située dans le Maine est Spinney Creek où les bactéries témoins de contamination fécale sont présentes à des niveaux limités et dont *Vibrio vulnificus* est absent. La société Spinney Creek Oyster Company (SCOC), basée à Eliot dans le Maine, exploite des bassins de reparcage et une station de purification d'huîtres d'élevage provenant de zones protégées de la rivière Salmon Falls. Des huîtres contaminées naturellement par les *Vibrio* et par les bactéries fécales ont été utilisées pour évaluer la purification et le reparcage en tant que stratégies d'élimination de ces bactéries. La présence de coliformes fécaux et la contamination totale en coliformes, ainsi que la présence de *V. vulnificus*, de *V. parahaemolyticus* et la contamination totale en vibrios

ont été analysées dans les huîtres et dans les eaux. Les coliformes sont détectés en permanence à différents degrés dans les échantillons d'huîtres et d'eau. *V. vulnificus* a été détecté régulièrement sur le site d'élevage entre juillet et octobre, mais jamais dans les eaux de Spinney Creek ni ailleurs entre novembre et juin. Le reparaillage des huîtres pendant 7 jours dans les bassins de la SCOC a toujours entraîné une diminution des concentrations en coliformes fécaux et en *V. vulnificus*. La purification des huîtres pendant 48 heures a permis de réduire considérablement les coliformes totaux et les coliformes fécaux, mais sans diminution des niveaux de vibrions. Ces résultats démontrent l'efficacité de la purification et du reparaillage pour réduire la contamination fécale chez les huîtres, en même temps que l'intérêt supplémentaire présenté par le reparaillage dans l'élimination de *V. vulnificus* qui s'avère récalcitrant à d'autres traitements.

Mots-clés : purification, reparaillage, bactéries pathogènes, indicateurs bactériens, vibrions, huîtres.

INTRODUCTION

Water pollution and increasing pressure from competing interest groups have had significant negative impacts on the shellfishing industry in the U.S. As the area that remains open to shellfishing decreases, problems with illegal harvesting (DuPont, 1986), outbreaks of food poisoning from consumption of tainted shellfish, and negative publicity have further depressed the shellfish industry. Shellfish may be legally harvested from mildly polluted waters only if the shellfish undergo some process of purification before being marketed for direct consumption. Two basic strategies, depuration and relaying, are used to purify shellfish. Both have been shown to be generally effective in reducing the regulatory target organisms, fecal coliforms, and other enteric bacteria to acceptable levels (Son and Fleet, 1980; Power and Collins, 1989; Jones *et al.*, 1991a), thus minimising the public health risk from fecal-borne bacterial pathogens in purified shellfish (Richards, 1988). However, outbreaks of shellfish-borne hepatitis A and viral gastroenteritis continue to occur in the U.S. (Rippey, 1991). In general, depuration and relaying are relatively ineffective in removing viruses from shellfish in reasonable periods of time (Canzonier, 1971 ; Power and Collins, 1989 ; de Mesquita *et al.*, 1991 ; Sobsey and Jaykus, 1991).

The serious diseases and fatalities associated with consumption of bivalves contaminated with pathogenic vibrios are a growing concern in the U.S. (Richards, 1988). *Vibrio* sp. are common, natural inhabitants of coastal waters (Oliver *et al.*, 1983), are constituent of the natural microflora of shellfish (Colwell and Liston, 1960), are involved in larval shellfish diseases (Prieur, 1987), and some are clinically significant in the southern U.S. (Janda *et al.*, 1988). The impact of depuration on indigenous, estuarine *Vibrio* sp. is inconsistent (Greenberg *et al.*, 1982 ; Kelly and Dinuzzo, 1985; Jones *et al.*, 1991b), in contrast to the response of fecal-borne bacteria. However, little information is available on the effect of relaying on vibrios, probably because the intent of regulations and studies has been to document the effectiveness of relaying for removing only fecal-borne microorganisms from bivalves. Son and Fleet (1980) showed some reduction in *V. parahaemolyticus* levels in oysters relaid to a less fecally-contaminated site. The concept of relaying shellfish from areas contaminated with pathogenic vibrios to uncontaminated areas has not been tested, possibly because of a lack of documented uncontaminated areas close to conta-

minated, harvest areas. The conditions that account for the absence of pathogenic vibrios in uncontaminated areas could induce their elimination from contaminated shellfish.

Studies in the Great Bay Estuary have shown the distribution of *V. vulnificus* and *V. parahaemolyticus* to be spatially and temporally heterogeneous (Bartley and Slanetz, 1971 ; O'Neill *et al.*, 1990 ; Jones *et al.*, 1991a ; O'Neill *et al.*, 1991). The distribution of *V. vulnificus* in the Estuary seems to be governed by a combination of salinity, temperature, and as yet unidentified factors (O'Neill, 1991). In a recent study conducted to determine if this heterogeneous distribution could be exploited for shellfish sanitation purposes, the relaying of contaminated oysters to uncontaminated areas in Maine and New Hampshire resulted in significant reductions in *V. vulnificus* levels (Jones *et al.*, 1994). The purpose of this paper is to summarise these past studies and more recent work to give a comparison of the effectiveness of depuration and relaying for commercial production of safe shellfish for direct consumption.

Material and methods

Oysters were harvested from the restricted waters of the Salmon Falls River in Maine during 1989 and 1990 and brought to the Spinney Creek Oyster Company (SCOC) controlled purification/relay facility located on Spinney Creek in Eliot, Maine. The oysters were cleaned and culled before being placed into relay lagoons or depuration tanks. Further details of the design and operation of the SCOC CP process are described by Howell and Howell (1989). Oysters harvested in 1989 were transferred directly to depuration tanks and processed for 48 hours, while oysters harvested in 1990-91 were kept in the relay lagoons for 7 days prior to depuration.

Oysters were harvested by dredging and transported to SCOC at the end of each harvest day. Subsamples were collected before purification and after purification from the same harvest lots. Water samples were collected in sterile plastic 1 liter bottles from the harvest and relay sites on the same days that oysters were sampled. Temperature and salinity were measured onsite using a mercury thermometer and refractometer, respectively. Oysters and water samples were processed at SCOC for fecal coliforms. Samples for fecal coliform and/or vibrio detection were refrigerated and transported to the Jackson Estuarine Laboratory (JEL) located on Great Bay in Durham, New Hampshire. Samples were processed for analysis within two hours of arrival at either laboratory.

Bacteriological analyses were performed on water samples and both freshly harvested and purified oysters. Twelve to sixteen individual oysters were aseptically shucked and the contents homogenised with equal parts of buffered peptone water. The MPN, multiple tube fermentation method (APHA, 1985) used for the detection of total and fecal coliforms in oysters and water was carried through confirmed and completed tests in accordance with recommended procedures, as previously described (Jones *et al.*, 1991b). Vibrio analyses involved decimal dilution of samples in alkaline peptone water as a three-tube MPN assay (O'Neill *et al.*, 1990). Turbid dilution broth tubes were streaked onto

thiosulfate-citrate-bile-sucrose (TCBS) agar and all different resultant colony types transferred to peptone broth containing 0% and 3% NaCl. Isolates that did not grow in 0% NaCl were considered vibrios (total vibrios). Vibrios that formed blue-green colonies on TCBS, and were thus suspected *V. vulnificus* or *V. parahaemolyticus*, were further characterised using growth in 6%, 8%, and 10% NaCl, ornithine and lysine decarboxylase, arginine dehydrogenase, and cellobiose and salicin fermentation tests, the API 20E identification system, and a latex-bound antigen specific for *V. vulnificus*.

Results

Oysters were harvested from beds in mildly polluted, restricted areas of the Salmon Falls River and purified in water from Spinney Creek, which is classified as approved. Temperature ranges at the two sites were similar, while the salinity of the water at Spinney Creek was always higher than of the Salmon Falls River (table I). *V. vulnificus* was consistently detected in oysters from the Salmon Falls River each year only during July-October when the water was also contaminated, and never detected at the harvest site during November-June.

Table I: Environmental and microbial contamination characteristics of water at harvest and relay sites: 1989-1991

| Site | Temperature range (°C) | Salinity range (ppt) | Fecal coliform concentration (per 100 ml) | Incidence of <i>Vibrio vulnificus</i> in water* |
|--------------------|---------------------------|-------------------------|--|---|
| Salmon Falls River | 0-26 | 1-17 | 120±6" | 11/29+ |
| Spinney Creek | 0-26 | 18-29 | 9±4 | 0/24 |

* *V. vulnificus* only detected during July-October; samples collected from June-November.

" Geometric mean ± standard deviation.

+ Number of positive samples per total number of samples.

Table II: Effect of depuration on microbiological quality of oysters

| Microorganisms* | Concentration in fresh oysters (MPN per 100 g) | Concentration in depurated oysters + (MPN per 100 g) |
|----------------------------|---|---|
| Fecal coliforms (n = 14) | 660 | 8" |
| Total coliforms (n = 14) | 7 000 | 130" |
| Pathogenic vibrios (n = 7) | 25,000 | 11,000 |
| Total vibrios (n = 14) | 67,000 | 140,000 |

* Numbers in parentheses are sample dates in which microorganisms were detected (July-December, 1989).

+ Oysters were depurated for 48 hours by Spinney Creek Oyster Company.

" Reduction in concentration significant at P = 0.01.

During 1989, harvested oysters were purified only by depuration. Oysters were held in tanks while UV light-disinfected water was recirculated for 48 hours. This process was very effective in reducing the fecal coliform levels to below the target level of 20 MPN/100 g, resulting in a significant, nearly 2 \log_{10} reduction (table II). Total coliforms levels were also significantly reduced during depuration. *V. vulnificus* and *V. parahaemolyticus* were only detected in some of the samples collected during July-October and not always in paired fresh and depurated shellfish from the same harvest lots. Depuration had little beneficial impact on these bacteria or total vibrios.

Detailed studies of the effect of seven-day relaying on fecal bacteria and *V. vulnificus* were conducted during the summer and autumn of 1990 and 1991. Relaying oysters from a contaminated site to an uncontaminated site for reducing *V. vulnificus* levels was consistently and dramatically effective (figure 1). When detected in fresh oysters, levels of *V. vulnificus* were always reduced after relaying. Generally, *V. vulnificus* levels in water and oysters were highest during August and September when water temperatures were $> 20^{\circ}\text{C}$, decreased during October as temperatures decreased to $< 15^{\circ}\text{C}$, and were undetectable in November when temperatures were $< 10^{\circ}\text{C}$. Fecal coliforms were also always reduced following relaying (figure 2). Reductions were less dramatic in October and November when fecal coliforms in harvest and relay waters were elevated in association with heavy autumn rain storms.

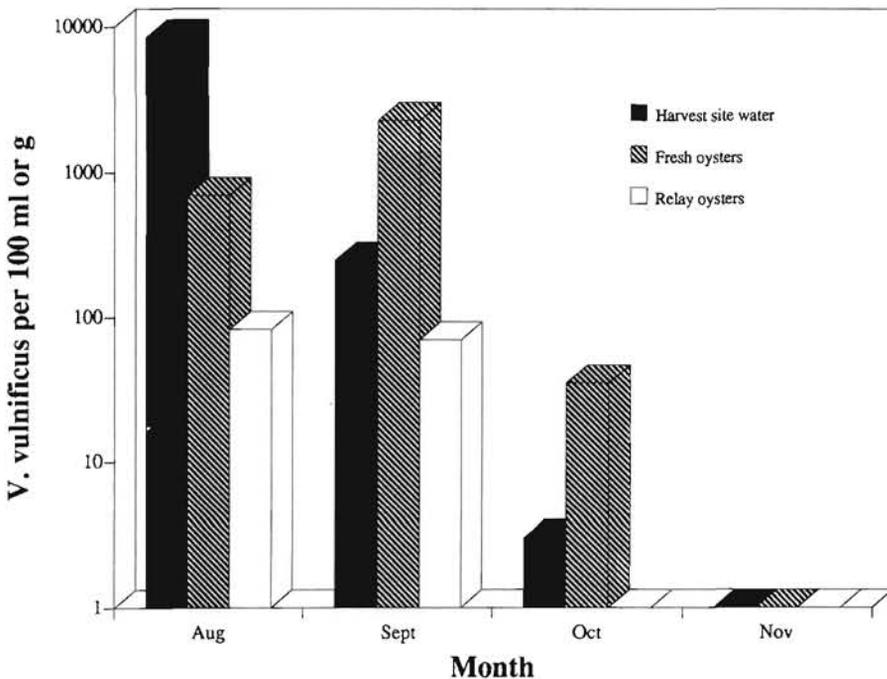


Figure 1: Effect of relaying and water quality on *Vibrio vulnificus* concentrations in oysters during August-November of 1990 and 1991

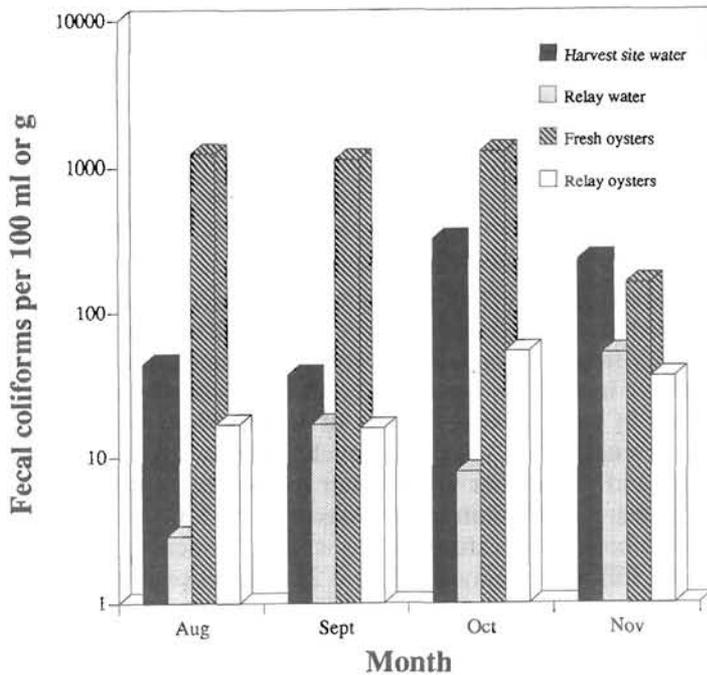


Figure 2: Effect of relaying and water quality on fecal coliform concentrations in oysters during August-November of 1990 and 1991

Discussion

The controlled purification facility at Spinney Creek Oyster Company is unique in that both relaying and depuration are used to purify shellfish. This facility provides a meaningful setting for evaluation of these processes under conditions that are actually employed to produce safe shellfish for human consumption. Oysters were harvested over a wide range of environmental conditions, they were naturally contaminated, and conditions during purification were maintained to minimise stress to the shellfish. The responses of the fecal bacteria and the vibrios are thus a direct indication of their fate in commercial-scale controlled purification of shellfish for direct marketing to consumers.

Depuration of shellfish for 48 hours at SCOC was consistently and dramatically effective in eliminating fecal coliforms. Even in December with water temperatures near 5°C, fecal coliforms were generally reduced to the target level of 20 per 100 g. A significant factor that contributed to this success was careful maintenance of conditions within the facility to minimise stress to the oysters, because stressful conditions can impair the elimination of microorganisms during depuration (Power and Collins, 1989). The fact that coliform levels were reduced confirms that the depuration process was functional. Comparison of the synoptic responses of the vibrios to the coliforms is convin-

cing evidence of the ineffectiveness of depuration for consistently eliminating vibrios from shellfish. The vibrios are natural constituents of the microflora of oysters (Colwell and Liston, 1960) and may have developed mechanisms that allow them to remain associated with oyster tissue and resist depuration. Conversely, fecal-borne bacteria are allochthonous organisms in coastal waters and are thus not adapted for persistence in shellfish tissue.

Relaying oysters from a restricted harvest area to an approved area was virtually as effective in eliminating fecal bacteria from oysters as depuration. Relaying is dependent on the quality of non-disinfected water over 7 days, during which time bacterial levels can vary according to meteorological influences. All oysters at SCOC were depurated following relaying to account for occasional elevated levels of fecal coliforms in Spinney Creek. The combined effects of relaying and depuration thus constitute an effective strategy for eliminating fecal bacteria from shellfish. An additional benefit of relaying prior to depuration at Spinney Creek was that *V. vulnificus* were also eliminated during two years of study. It is unlikely, based on our own observations, that there were significant differences in water characteristics between 1989 and 1990-91. If the water characteristics alone were responsible for the success of relaying to eliminate vibrios, then depuration would probably also have shown some beneficial effect. The major remaining differences in the two processes are the process times (7 days relay vs. 2 days depuration) and the microflora of the water. More detailed studies on the kinetics of disappearance of *V. vulnificus* from relaid oysters are needed to determine the significance of process time.

The removal of *V. vulnificus* in the presence of a natural, indigenous bacterial microflora in relay water compared to no removal in disinfected depuration water suggests that a biological factor may be significant in eliminating *V. vulnificus* from oysters during relaying. Results from nearly three years of analysis have never indicated that *V. vulnificus* is present in Spinney Creek water. This may be related to the consistently high salinity in Spinney Creek compared to other areas in the Great Bay Estuary where *V. vulnificus* is found (O'Neill *et al.*, 1990). The microbial community present in Spinney Creek water is adapted to the environmental conditions there, and probably replace *V. vulnificus* in relaid oysters through competition on tissue surfaces. In a similar fashion, the manipulation of microbial communities on agricultural plants has been successful in suppressing populations of specific target microbes, especially plant pathogens (Chalutz and Wilson, 1990). More studies are needed to characterise differences in biotic and abiotic factors associated with *V. vulnificus*-contaminated and uncontaminated waters.

REFERENCES

- American Public Health Association (APHA), 1985. Standard Methods for the Examination of Water and Wastewater, 16th edition. *Amer. Publ. Health Assoc.*, Washington, DC.
- Bartley C.H., L.W. Slanetz, 1971. Occurrence of *Vibrio parahaemolyticus* in estuarine waters and oysters of New Hampshire. *Appl. Microbiol.*, **21**, 965-966.

- Canzonier W.J., 1971. Accumulation and elimination of coliphage S-13 by the hard clam, *Mercenaria mercenaria*. *Appl. Microbiol.*, **21**, 1024-1031.
- Chalutz E., C.L. Wilson, 1990. Biological control of postharvest diseases of fruits and vegetables through manipulation of epiphytic plant microflora. In ; Bills D.D., S. Kung (ed.) *Biotechnology and Food Safety*. Butterworth-Heinemann, Boston, MA. 255-266.
- Colwell R.R., J. Liston, 1960. Microbiology of shellfish. Bacteriological study of the natural flora of Pacific oysters (*Crassostrea gigas*). *Appl. Microbiol.*, **8**, 104-109.
- DuPont H.L., 1986. Consumption of raw shellfish-Is the risk now unacceptable ? *Lancet*, **314**, 707-708.
- Greenberg E.P., M. Dubois, B. Palhof, 1982. The survival of marine vibrios in *Mercenaria mercenaria*, the hardshell clam. *J. Food Safety*, **4**, 113-123.
- Howell T.L., L.R. Howell, 1989. *The Controlled Purification Manual*. New England Fisheries Development Association, Inc., Boston, MA. 77 p.
- Janda J.M., C. Powers, R.G. Bryant, S.L. Abbott, 1988. Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio spp.* *Clin. Microbiol. Rev.*, **1**, 245-267.
- Jones S.H., T.L. Howell, K.R. O'Neill, R. Langan, 1994. Relaying to eliminate bacteria from oysters in northern New England. Submitted.
- Jones S.H., T.L. Howell, K.R. O'Neill, 1991a. Bacterial evaluation of a commercial controlled purification plant in Maine. In: Otwell W.S., G.E. Rodrick, and R.E. Martin (ed.), *Molluscan shellfish depuration*. CRC Press, Inc., Boca Raton, FL. 181-187.
- Jones S.H., K.R. O'Neill, T.L. Howell, 1991b. Differential elimination of indicator bacteria and pathogenic *Vibrio sp.* from Maine oysters (*Crassostrea virginica*) in a commercial controlled purification facility. *J. Shellfish Res.*, **10**, 105-112.
- Kelly M.T., A. Dinuzzo, 1985. Uptake and clearance of *Vibrio vulnificus* from Gulf Coast oysters (*Crassostrea virginica*). *Appl. Environ. Microbiol.*, **50**, 1548-1549.
- de Mesquita M.M.F., L.M. Evison, P.A. West, 1991. Removal of faecal indicator bacteria and bacteriophages from the common mussel (*Mytilus edulis*) under artificial depuration conditions. *J. Appl. Microbiol.*, **70**, 495-501.
- Oliver J.D., R.A. Warner, D.R. Cleland, 1983. Distribution of *Vibrio vulnificus* and other lactose-fermenting vibrios in the marine environment. *Appl. Environ. Microbiol.*, **45**, 985-998.
- O'Neill K.R., 1991. Ecology and characterisation of *Vibrio vulnificus* from the Great Bay Estuary of Maine and New Hampshire. M.S. Thesis, Univ. of New Hampshire.
- O'Neill K.R., S.H. Jones, T.L. Howell, D.J. Grimes, 1991. Occurrence of *Vibrio vulnificus* in water and shellfish from Maine and New Hampshire. In : Otwell W.S., G.E. Rodrick, R.E. Martin (ed.), *Molluscan shellfish depuration*. CRC Press, Inc., Boca Raton, FL. 189-193.
- O'Neill K.R., S.H. Jones, D.J. Grimes, 1990. Incidence of *Vibrio vulnificus* in northern New England water and shellfish. *FEMS Microbiol. Lett.*, **72**, 163-168.
- Power U.F., 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.
- Prieur D., 1987. A review of the relationship between bivalve molluscs and bacteria in the marine environment. *Symbiosis*, **4**, 37-50.
- Richards G.P., 1988. Microbial purification of shellfish : A review of depuration and relaying. *J. Food. Prot.*, **51**, 218-251.

- Rippey S.R., 1991. Shellfish-associated disease outbreaks. Shellfish sanitation program technical report. U.S. Food and Drug Administration, Northeast Technical Services Unit, North Kingston, R.I., *Internal Technical Report*.
- Sobsey M.D., L. Jaykus, 1991. Human enteric viruses and depuration of bivalve molluscs. In: Otwell W.S., G.E. Rodrick, R.E. Martin (ed.), Molluscan shellfish depuration. CRC Press, Inc. Boca Raton, FL, 7-114.
- Son N.T., G.H. Fleet, 1980. Behavior of pathogenic bacteria in the oyster, *Crassostrea commercialis*, during depuration, re-laying, and storage. *Appl. Environ. Microbiol.*, **40**, 994-1002.
- Xu S., N. Roberts, F.L. Singleton, R.W. Attwell, D.J. Grimes, R.R. Colwell, 1982. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. *Microb. Ecol.*, **8**, 313-323.