Depuration of the Sydney Rock Oyster with Particular Reference to *Vibrio vulnificus*

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SUMMARY

Vibrio vulnificus is a highly virulent pathogen that occurs naturally in the estuarine environment of coastal waters of countries including the USA and Australia. The organism can cause a severe form of gastroenteritis as well as wound infections that often require limb amputations, and mortality rates can exceed 50% in persons who are immuno compromised or who suffer from long term liver degeneration (e.g. cirrhosis). The organism is present in some oysters, including the Sydney rock oyster (SRO, *Saccostrea commercialis)*, and in water and sediments of some oyster- growing areas, especially during warmer months.

Although research on the removal of *V. vulnificus* from contaminated oysters has been conducted in the USA, no such depuration studies have been undertaken on the SRO with respect to this microorganism.

Peptone (0.1%) solution containing 3% sodium chloride (PS) was shown to be a more suitable diluent than phosphate buffered saline (PBS) solution in methods for the enumeration of *V. vulnificus* in both broth cultures and oyster homogenates. PBS caused significant underestimation of the viable population of the species by plate counts on either selective or non-selective media, and is not recommended.

The culture medium cellobiose polymyxin B colistin (CPC) agar used for the isolation of *V. vulnificus* from SRO was modified to improve its sensitivity. Modified CPC (m CPC) contained marine agar (MA) instead of the original basal medium and the colistin content was reduced.

Laboratory studies were conducted on the uptake and removal of *V.vulnificus* and *Escherichia coli* from SRO. Shellstock oysters were artificially contaminated or depurated in a perspex tank with water at temperatures of 15°-25°C and salinities of 20-40 ppt circulated through a UV light. Survival of *V.vulnificus* cells in UV - sterilised water was greater as the water temperature increased, while *E. coli* survival was inversely related to temperature. Survival of both microorganisms varied inversely with water salinity. Depuration of *V.vulnificus* from oysters at 25°C was more effective than at 20°C, while at 15°C microbial numbers were reduced substantially, possibly due to cold shock. Higher salinities increased the elimination of both microorganisms. While reductions in numbers of *V.vulnificus* occur during depuration for 48 h, complete removal of the organism is generally not achieved. Levels of *E. coli* in depurated oysters were always less than statutory levels.

Freezing of shucked oysters or storage at 5°C caused rapid drastic decreases in numbers of viable *V.vulnificus*, but some cells survived after 20 days storage. Thus chill or frozen storage, irrespective of temperature, does not appear to completely eliminate *V.vulnificus* from oyster meat. Storage of shucked oyster meats at temperatures greater than 10°C caused increases in *V.vulnificus* cell numbers until overtaken by the spoilage microflora. The pathogen persisted in live oysters for up to three weeks and in shucked and bottled oysters for up to 2 weeks, even at chilled and frozen temperatures.

Laboratory studies were conducted on the ozone destruction of *V.vulnificus* in sea water and SRO. At low (0.5-1.0 mg/L) levels of ozone residual in sea water, the initial number of *V.vulnificus* cells was reduced by 5 to 6 log cycles when water was ozonated

for 2 min. When SRO were depurated in a system using recirculated seawater ozonated at levels of 0.2 to1.5 mg/L, reductions in the level of *V.vulnificus* cells were of the order of only 2 log cycles, and complete destruction was not achieved.

A limited survey of the presence of *V.vulnificus* in oysters, water and sediments was conducted at one site in Port Stephens for 3 months and two sites in Georges River for 6 months. One water sample taken from Lime Kiln Bay, Georges River, contained *V.vulnificus* at a level of 0.15 MPN cells/mL. The identity of the organism was confirmed by the Biolog GN and ATP computerised microbial identification systems.

Fluorescence microscopy with acridine orange staining was used to assess the viablity of non-culturable cells of *V.vulnificus*. Characteristic microscopic changes (rod-shaped to miniature coccoid cells) were observed by image analysis, but the technique needs further modification for routine use.

BACKGROUND

The Sydney rock oyster (*Saccostrea commercialis*) is a molluscan bivalve of high quality that has been cultivated in New South Wales for decades. Cultivation techniques have traditionally used elevated racks, sticks or trays positioned at mid tide in a wide variety of estuaries along coastal areas both north and south of Sydney.

The public health safety of this shellfish, however, has been called into question several times in the past 25 years as the result of several major food poisoning outbreaks. In 1978 over 2000 persons across Australia succumbed to illness from a Norwalk virus ingested with polluted oysters following periods of heavy rainfall. Additional outbreaks have occurred since 1989.

Following the 1978 outbreak the New South Wales Government legislated in the early 1980s for the compulsory depuration (purification) of oysters offered for sale in New South Wales. The depuration technology introduced following these problems was based overseas on research and the research findings obtained by Fleet and his co-workers in the Department of Food Science and Technology, The University of New South Wales (UNSW). Buckle (1994) has summarised research and commercial development work at UNSW over the period 1968-1994. While commercial depuration is able to remove or reduce to significant levels organisms of faecal origin such as *Escherichia coli* and *Salmonella*, it is unable to remove significant bacteria that are indigenous to the estuarine environment, including some vibrios.

In recent years another bacterial pathogen of considerable public health importance has become identified with shellfish and other marine organisms. *Vibrio vulnificus* is a highly virulent pathogen that occurs naturally in estuarine environments in many regions of the world. In recent years it has killed at least four persons in Australia and about 100 in the USA, principally from shellfish consumption. It is able to cause illness in humans either from consumption of contaminated seafoods or from muscle penetration through wounds.

Although the behaviour of other *Vibrio* spp during the depuration of oysters including the SRO has been studied (eg. Son and Fleet 1980, Eyles and Davey 1984, Eyles *et al.* 1985), the behaviour of *V.vulnificus* during the depuration of the SRO is unknown, although the incidence of this organism in shellstock and shucked Pacific (*Crassostrea gigas*) and Eastern (*C. virginica*) oysters and other shellfish in the USA has been reported (eg. Kaysner *et al.* 1989, Tamplin, Murphy and Oliver 1992, Ruple and Capers 1992, Kaspar and Tamplin 1993, Klontz *et al.* 1993).

The principal method of water disinfection in commercial depuration is UV light, although ozone (O_3) has been used in Australia and overseas for the depuration of oysters and other shellfish (e.g. Blogoslawski *et al.* 1975, Richards 1988, Schneider *et al.* 1991). It is becoming increasingly apparent that ozone may have benefits for the reduction in concentration or elimination of *V.vulnificus* and paralytic shellfish poisoning (PSP) toxin and other microalgal toxins in shellfish or seawater (e.g. Blogoslawski *et al.* 1979, Gacutan *et al.* 1984, Blogoslawski 1988, Schneider *et al.* 1990). Such possibilities emerged during a feasibility study on molluscan shellfish hygiene and sanitation in ASEAN countries (Buckle *et al.* 1993) and were reinforced during discussion at the *First International Conference on Molluscan Shellfish Safety* hosted by the author at UNSW

on 13-17 November 1994. Since studies on the ozone destruction of *V.vulnificus in vitro* or in the SRO have not been conducted in Australia, it was decided to undertake preliminary laboratory studies on ozone as a comparison to UV depuration during 1993-94. These preliminary results are included in this final report.

No comprehensive surveys of the distribution of *V.vulnificus* in oysters, waters and sediments have been conducted in Australia, although the NSW Department of Health Division of Analytical Laboratories has examined oysters from many oyster growing areas in NSW for *E.coli* and faecal coliforms during the periods 1978-86 (Bird 1990), 1987-90 (Bird 1991) and 1991-92 (Bird *et al.* 1993). During the period 1987-90, *V.vulnificus*, *V.parahaemolyticus, Salmonella* and *Campylobacter* were examined while in 1991-92 standard plate counts were also examined in addition to *E.coli* and faecal coliforms. It was intended in the present study that the incidence of *V.vulnificus* and other marine vibrios and traditional microbiological contaminants be examined in the marine environment of major oyster growing areas in NSW. However, the unavailablity of appropriate students led to a limited (3-6 months) preliminary survey of oysters, water and sediments in one area of Port Stephens and two areas of Georges River.

NEED

During the past 17 years large numbers of oyster consumers have become ill as the result of consumption of pathogen-contaminated product, and at least 4 persons have died from the consumption of raw oysters contaminated with *V.vulnificus*. Such outbreaks and deaths have a devastating effect on the viability of the oyster industry, since declines in oyster consumption during such episodes are rarely recovered when the event has passed. The commercial viability of the NSW oyster industry will be determined by a combination of its marketing success relative to other seafood and food products, and the public health safety of consumed products. Because of the virulent nature of *V.vulnificus* and its known threat to raw oyster consumers, knowledge of the behaviour of this pathogen during its uptake and elimination by the SRO is of importance if the microbiological adequacy of depuration is to be determined. Discussion at the *First International Conference on Molluscan Shellfish Safety* reaffirmed the need for further depuration studies with respect to *V.vulnificus* in raw shellfish.

OBJECTIVES

The original objectives were as follows:

* Optimise methodology for the identification and enumeration of *V.vulnificus* and other marine vibrios of public health importance in the Sydney rock oyster.
* Assess the incidence of *V.vulnificus* and other marine vibrios and traditional microbiological contaminants (faecal coliforms, *Escherichia coli*) in the marine environment of major oyster growing areas in NSW.
* Determine the rate and extent of accumulation in and removal of *V.vulnificus* from the Sydney rock oyster during laboratory and commercial depuration.

Changes to the above objectives include the following:

* Methods for *V.vulnificus* identification and enumeration were assessed and a paper has been submitted to the *International Journal of Food Microbiology*. Other marine vibrios were not examined due to the significant additional work required, and the much greater public health importance of *V.vulnificus*. The methodology developed in this study is a considerable improvement on that previously available.

* Only a limited survey of the incidence of *V.vulnificus* in the marine environment was carried out as previously described. The author plans to continue research on *V.vulnificus* when funds are available.

* The depuration behaviour of *V.vulnificus* in the SRO was examined in detail in laboratory studies. Commercial depuration was not conducted because of the public health danger of this organism.

In addition to the original objectives, further studies were conducted of significant relevance to the NSW oyster industry. Some of these studies followed the findings of Buckle *et al.*(1993) and were justified by discussion at the *First International Conference on Molluscan Shellfish Safety*. They include:

* Examination of the destruction of *V.vulnificus* by ozone in model systems.
* Examination of the reduction in the numbers of *V.vulnificus* in SRO during ozone depuration.
* Determination of the effects of time and temperature of postharvest storage on the persistance of *V.vulnificus* in shellstock, half shell and bottled SRO.
* Examination of viable but nonculturable (VBNC) cells of *V.vulnificus* by fluorescence microscopy and image analysis.

METHODS

All methods used in these studies are described in detail in the theses of Murden (1993), Wongchinda (1993), Tay (1994) and Azanza (1995). A summary of the methods used has been provided in the Preliminary Report and is provided here in more detail.

Isolation and Enumeration of *V.vulnificus*

Published media for the isolation and enumeration of *V.vulnificus* (CPC, mCPC , TCBS and SPS) from water and oysters were examined, and further modifications made to improve recovery. The effects of the diluent (0.1% peptone solution containing 3% NaCl =PS, and phosphate buffered saline =PBS) on cell recovery from both cultures and oyster homogenate were examined. Four commercial computer-based methods for organism identification (API ZONE strip, Biolog GN Microplate, Baxter MicroScan Gram Negative, ATB 1B 32 GN) were assessed against traditional biochemical test procedures.

Fluorescence Microscopy

Preliminary studies were conducted on the detection of viable but nonculturable (VBNC) cells of *V.vulnificus* by fluorescence microscopy using acridine orange staining and image analysis. Staining techniques using malachite green-iodonitrotetrazolium reduction previously proposed were difficult to conduct and interpret, and were replaced by more acceptable techniques. The acquisition by the Department of Food Science and Technology of sophisticated image analysis equipment enabled high quality fluorescence microscopy to be undertaken. FRDC funding enabled higher quality lenses and filters to be obtained for a modern instrument, rather than updating an older instrument (Zeiss) as originally proposed.

Uptake and Elimination of *V.vulnificus* by SRO

SRO (approx. 60/batch) were maintained in a laboratory aquarium (60x40x40cm) containing 50-70 L aerated seawater at 20°C, and were fed suspensions of *Dunaliella salina* to maintain activity, and after 30 min. cultures (18-24 h) of *V.vulnificus* grown on marine broth were introduced to the tank. This feeding / inoculation scheme was repeated after 12, 24 and 36 h. After this inoculation regime, concentrations of *V.vulnificus* in the oyster meat were 10^5 -10^6 cells/g. Depuration was conducted by circulating water with a cent rifugal pump through a UV light sterilising system at temperatures from 15° to 25°C and salinities from 20 to 40 ppt. Survival of *V.vulnificus* and *E.coli* was assessed in both water and oysters. Distribution of *V.vulnificus* within the SRO was assessed after organ isolation from oysters sampled during 48 h depuration.

Oyster Storage

Shellstock oysters were stored at 5°, 10°, 20°and 30°C, while half-shell shucked and bottled oysters were assesed at -20°, 5°, 10°, 20°and 30°C during 20 days storage or until spoiled.

Isolation of *V.vulnificus* from Estuarine Environments

Samples of undepurated oysters, water and sediments were obtained from Mr Robert Bailey, The Cove Oysters, Oyster Cove, Lemon Tree Passage, Port Stephens, and Mr Andrew Derwent, Neverfail Bay, Oatley, Georges River, and examined between July and September 1993 using US Food and Drug Administration (US FDA 1992) most probable number (MPN) methods. Water and sediment samples were taken from Lime Kiln Bay, Georges River, NSW between April and September 1993 and examined using a MPN technique. This latter study was part of a larger study conducted by Australian Water Technologies Pty Ltd on pollution of the marine environment.

Ozone Destruction of *V.vulnificus* in Seawater

V.vulnificus levels of 10^3-10^7 cells/mL were exposed to direct ozonation for up to 2 min and with continued contact for up to 4 min, or were exposed to previously ozonated sea water for up to 4 min. Aliquots were taken and residual levels of *V.vulnificus* and ozone were measured.

Ozone Depuration

SRO were maintained in a laboratory aquarium containing 50-70 L aerated seawater at 20°C, and inoculated with varying levels of *V.vulnificus* by natural feeding. Oysters were fed dried algae (1 mg/g oyster) to maintain oyster viability and condition. After 24 h, inoculated oysters were transferred to a depuration tank and subjected to ozone levels of 0.2-1.0 mg/L for 8 h. Samples were removed periodically for determination of ozone and *V.vulnificus* were also kept for 48 h in a dry container to assess natural die-off of *V.vulnificus*.

RESULTS

Isolation and Enumeration of *V.vulnificus*

Plating Medium

The principal media used for the isolation of *V.vulnificus* from seawater or marine products include cellobiose polymyxin B colistin (CPC) agar (Massad and Oliver 1987), modified CPC agar (Kaysner and Tamplin 1988), thiosulphate citrate bile salts sucrose (TCBS) agar and sodium dodecyl sulphate polymyxin B sucrose (SPC) agar (Kitaura *et al.*1983) Further modifications to CPC were made by replacing the original basal medium (peptone 10 g, beef extract 5 g, NaCl 20 g, agar 15 g) with marine agar (55.1 g) and reducing the colistin content from $1.36x10^6$ to $4x10^5$ units/L medium (imCPC). The improved recoveries of *V.vulnificus* on imCPC are shown in Tables 1 and 2. mCPC gave recoveries of up to 65% relative to marine agar, while imCPC increased the recovery of *V.vulnificus* cultures to >87% for both broth cultures and oyster meat. This improved formulation was used in all subsequent studies.

TABLE 1. Recoveries (%) of *Vibrio vulnificus* on selected media

Strain	TCBS	SPS	CPC	mCPC	imCPC
Vv 59961/89	55	43	8	65	95
Vv 1283/88	61	37	19	61	93
C7164	28	ND	2	9	87
ATCC 27562	1	4	$<<10^{-2}$	$<10^{-2}$	90
*Vv*148	74	6	1	3	95

Mean recoveries of 9 determinations relative to values from Marine Agar
ND = not determined

TABLE 2. Counts (log cfu/g) of *Vibrio vulnificus* in oyster homogenate on imCPC and TCBS

Strain	TCBS	imCPC
ATCC 27562	5.48 ± 0.06	7.27 ± 0.08
Vv 148	7.54 ± 0.04	7.68 ± 0.03

* *Means of 3 trials ± standard deviation*

Effect of Diluents

Dilution of *V.vulnificus* cultures in phosphate buffered saline (PBS) produced a significant reduction in cell numbers compared to dilution in peptone saline (PS) solution. Typical results are shown in Table 3 for two cultures of *V.vulnificus* (Azanza, Buckle and Fleet 1995).

TABLE 3. A comparison of plate counts of *Vibrio vulnificus* using peptone saline and phosphate buffered saline diluents

Strain	Plating medium	Mean count (log cfu/g)	
		PS	PBS
Vv 148	PCA	9.7 ± 0.11a	9.05 ± 0.48a
	TCBS	9.3 ± 0.01a	8.92 ± 0.01b
ATCC 27562	PCA	8.6 ± 0.12a	7.58 ± 0.14b
	TCBS	6.4 ± 0.12a	6.18 ± 0.04b

Mean of 3 trials ± standard deviation. Values in the same line followed by the same letter are not significantly different (P>0.01)

Fluorescence Microscopy

Detailed results are provided in Azanza (1995). Considerable effort was expended developing a technique to enable VBNC cells to be identified in a variety of cultures, with considerable assistance provided by the School of Pathology, UNSW. Typical VBNC cells (small, rounded to coccoid) were detected in cultures which showed no growth upon broth or agar inoculation. Changes in cell morphology were examined as affected by inoculation temperature, salinity and UV exposure time.

Effect of Salinity

The numbers of culturable *V. vulnificus* cells in sterile estuarine water exposed to UV light for 10 min decreased as the water salinity increased from 20 to 40 ppt. Counts obtained from the non-selective marine agar were slightly higher than those obtained from the selective medium (imCPC). The total bacterial count as indicated by the acridine orange count did not vary with salinity. Viability as monitored by the INT count followed the same trend as the plate count , however there existed a large discrepancy in the numbers of culturable cells on agar plates compared to the INT count. This difference in count suggests the existence of a nonculturable sub-population of VBNC cells stressed by the combined effects of UV treatment and elevated salinity.

Effect of Temperature

The effects of temperature (15° - 25°C) on the number of *V. vulnificus* cells in estuarine water exposed to UV light for 10 min were not as distinct as the effect of water salinity. All the test temperatures caused about a 3 log decrease in plate count obtained on imCPC. Based on the INT counts, there was a 1 log decrease in viable count from the initial viable population after 10 min exposure to UV light for cells held at 15°C. Viable counts of cells held at 20° and 30°C showed less than 1 log decrease under the same conditions. The acridine orange count used to monitor total bacterial numbers showed a slight decrease for cells held at 15°C but not for those held at 20° and 25°C.

Uptake and UV Elimination of *V.vulnificus* and *E.coli* by SRO

Levels of *V.vulnificus* in SRO increased rapidly in hemolymph and soft tissues in the first 6 h, and more slowly during a further 42 h. Aerobic plate counts also increased, but more slowly. Typical results are shown in Table 4 (Azanza 1995).

TABLE 4. Levels of *V.vulnificus* and total microorganisms in SRO soft tissues and hemolymph during laboratory contamination

Inoculation time (h)	*V.vulnificus* (MPN/g or mL)		Aerobic plate count (cells/g or mL)	
	Hemolymph	Soft tissue	Hemolymph	Soft tissue
0	<0.3	<0.3	$1.5x10^3$	$2.4x10^5$
6	$4.6x10^3$	$1.5x10^5$	$6.5x10^4$	$9.9x10^5$
24	$1.1x10^4$	$1.1x10^7$	$9.1x10^4$	$1.5x10^7$
48	$4.6x10^4$	$7.5x10^5$	$3.5x10^5$	$4.0x10^6$

When the contaminated SRO were UV depurated for 48 h at 18°-22°C, levels of *V.vulnificus* in both hemolymph and soft tissues showed 3-4 log decreases, with levels in

the hemolymph lower than in the soft tissue. The decreases in *V.vulnificus* counts were more rapid during the first 36 h of UV depuration and thereafter clearance slowed down. Total microbial loads decreased 2-3 log counts over 48 h depuration. Minimal reduction in numbers occurred during the last 12 h. The tissue distribution of *V.vulnificus* during uptake and depuration studies is seen in Figures 1 and 2.

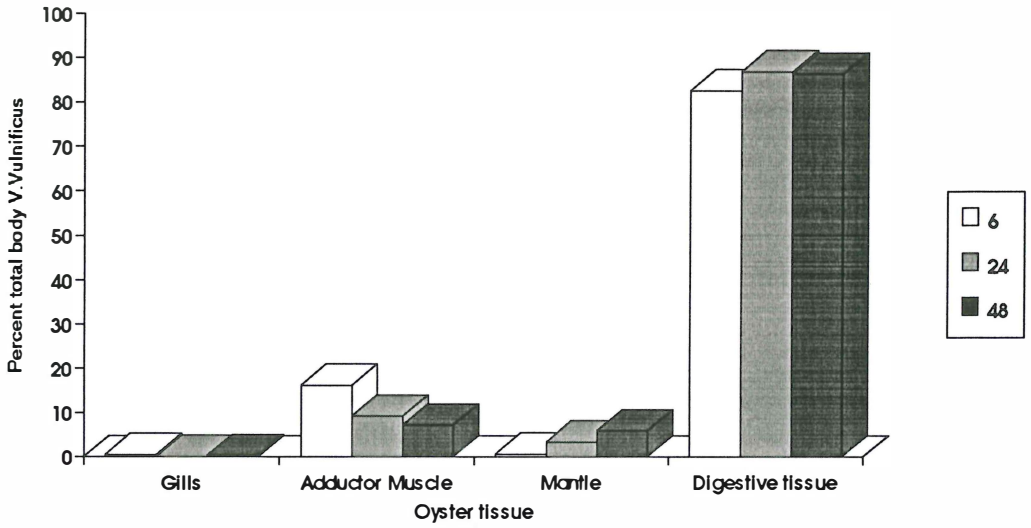

Figure 1. Tissue distribution of *Vibrio vulnificus* during 48 h inoculation of SRO

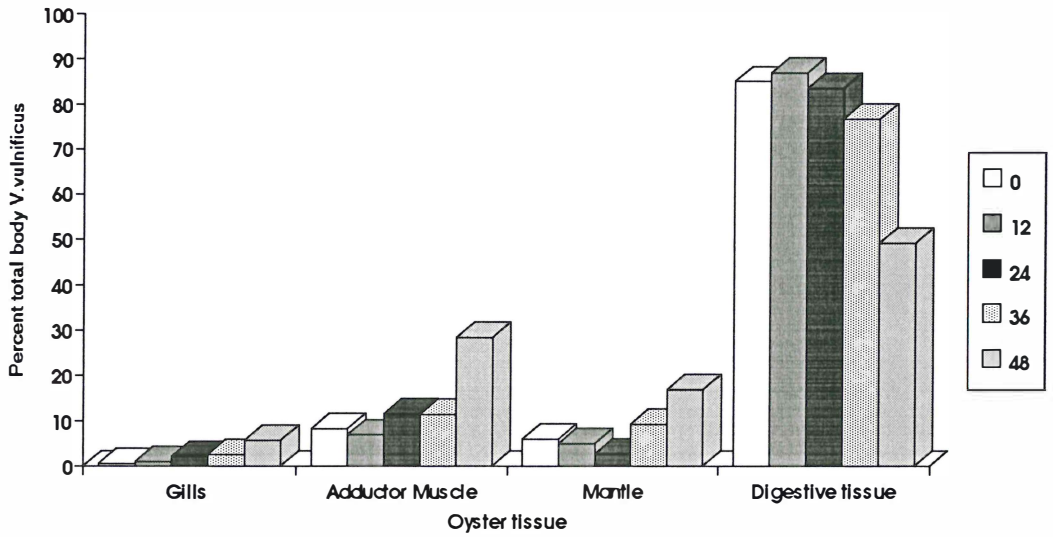

Figure 2. Tissue distribution of *V.vulnificus* during 48 h depuration of SRO

The relative distribution of *V.vulnificus* in oyster tissues during uptake and elimination were monitored (Figures 1 and 2). The digestive tissues contained the highest proportion of the pathogen compared to the other soft tissues, and levels of the pathogen increased in the gills, adductor muscle and mantle but decreased in the digestive tissues during depuration. However, during 48 h depuration, *V.vulnificus* was not eliminated from the oysters examined.

The effects of water temperature (15°, 20°, 25°C) and water salinity (20, 30, 40 ppt) on removal of *V.vulnificus* during depuration of SRO were examined. A reduction of more than 3 log cycles occurred when SRO were depurated at 15°C and 20 ppt, while reductions were less at 20 C (1.5 log) and 25°C (2.5 log). *E.coli* levels decreased by about 2.5-3 log cycles over the temperature range 15°-25°C, with reductions slightly higher at 15°C. Levels of total aerobic bacteria decreased by only 0.5-2 log cycles, with reductions greater at 25°C than at 15°C.

During depuration at 20°C, levels of *V.vulnificus* decreased by 3-3.5 log cycles at 20 and 30 ppt, and by 6-7 log cycles at 40 ppt. Reductions in *E.coli* levels were significantly greater at 20 ppt (4-5 log cycles) and 30 ppt (5-6 log cycles), than at 40 ppt (3-4 log cycles). Again, reductions were more rapid during the first 12-24 h of depuration and then slowed dramatically.

Thus it is clear that levels of *V.vulnificus* do decrease during depuration, and more rapidly at lower temperatures possibly due to cold shock, but the organism was never reduced to undetectable levels in these studies. Considerably more research is required to evaluate a wider range of environmental conditions during depuration to determine whether it is possible to convert cells of this pathogen into the VBNC state under conditions in which they are unable to recover their viabilty and hence pathogenicity and hence public health importance.

V.vulnificus and *E.coli* Survival in UV-treated Seawater

The survival of *V.vulnificus* and *E.coli* in UV-treated seawater at 15°, 20° and 25°C and 20, 20 and 40 ppt was examined. *E.coli* reductions were similar (5-6 log cycles) over the temperature range 15°-25°C and 30 ppt salinity while *V.vulnificus* reductions were greater at 15°C (5-6 log cycles) than at 20°C (3-4 log cycles) or at 25°C (2-3 log cycles). At 20°C, reductions in *E.coli* were greater at 20 ppt (4-5 log cycles) and 30 ppt (5-6 log cycles) than at 40 ppt (3-4 log cycles).

Reductions in levels of *V.vulnificus* at 20°C were lower at 20 ppt (3 log cycles) than at 30 ppt (3-4 log cycles) and at 40 ppt (6-7 log cycles). Thus more significant reductions in levels of *V.vulnificus* occur in highly saline, colder waters than under conditions more optimal for oyster depuration. Again, more research is needed to ascertain the range of conditions required to maximise the loss of viability of the pathogen in the water in which depuration is undertaken, since this will reduce the final level of the bacterium in the depurated oyster.

Oyster Storage

Live oyster shellstock were stored at 5°, 10°, 20° and 30°C, and oysters in the half shell or bottled oysters were stored at -20°, 5°, 10°, 20° and 30°C for 20 days or until spoiled. Generally, freezing at -20°C or storage at 5°C caused immediate and drastic reductions in numbers of viable *V.vulnificus* cells, and then the decrease became more gradual. Surviving cells were still isolated after 20 days. Thus low temperatures decrease but do not eliminate *V.vulnificus* cells in stored oyster meats. Storage temperatures at ≥ 10°C increased cell numbers during the first 3 days of storage, but numbers eventually decreased as total bacterial levels increased rapidly with the onset of spoilage. These results are in general agreement with those of Kaspar and Tamplin (1993) and Cook (1994). Typical results for shellstock and half shell oysters are shown in Table 5.

TABLE 5. Changes in *Vibrio vulnificus* cell numbers in stored SRO

Oyster sample	Storage temperature (°C)	Trials no.	*V.vulnificus* (MPN/g oyster) Storage time (days) 0	3	7	14	21
Shell	5	1	4300	23	36	43	24
stock		2	240	46	15	24	21
	10	1	150	46	91	93	75
		2	240	93	43	46	46
	20	1	9300	2400	1500	930	240
		2	240	1400	930	700	460
	30	1	3600	2400	9300	7500	2400
		2	4600	46000	2300	1500	2400
Half	-20	1	9300	11	24	75	46
shell		2	2400	24	24	24	11
	5	1	4600	150	46	24	11
		2	9300	750	110	75	46
	10	1	2400	750	4600	7500	1100
		2	4600	2300	1100	2400	9300
	20	1	4600	11000	S		
		2	2300	4600	S		
	30	1	4300	24000	S		
		2	2400	11000	S		

S=spoiled

V.vulnificus persisted in live shellstock for 3 weeks at ambient temperatures (Table 5), and in both open (half shell) and bottled oysters for up to 2 weeks even at frozen (-20°C) and refrigerated (5°C) temperatures. Thus traditional storage conditions cannot be relied upon to reduce or eliminate cell numbers to harmless levels.

Isolation of *V.vulnificus* from Estuarine Environments

An extensive survey of oyster- growing areas in New South Wales for the organism *V.vulnificus* was not possible because of the unavailability of appropriate undergraduate or postgraduate students. Consequently, a limited examination was conducted of water, sediment and oysters from one site in each of Port Stephens and Georges River, and of water and sediments from a second site in Georges River (Murden 1993).

Recovery studies on inoculated water, sediments and oyster homogenate highlighted significant methodological problems with sediment and some water samples. Recoveries for sediment varied from 38-125% using an MPN technique, with greater discrepancies at higher inoculation levels (10^4-10^5 cells/g sediment). The chemistry of adsorption in sediments is complex and further research is required to unravel the reasons for the low recoveries. Recoveries from oyster samples were satisfactory (100-125%) except at very low inoculation levels (< 10 cells/mL) where minor errors are magnified.

V.vulnificus was not isolated from any samples at Port Stephens or Neverfail Bay, Georges River, by the method used (sensitivity <0.3/g or mL). Total aerobic counts varied from 10^2- $6x10^3$/mL (water), $3.3x10^5$-$1.1x10^6$/g (sediments) and $1.0x10^4$ - $5.4x10^4$/g (oysters), with levels generally higher in September than in July. Water temperatures varied from 11° to 16°C, and salinity from 27-33 ppt. Clearly, a comprehensive survey beyond the scope of the present study would be required to determine the influence of temperatures, salinity, location and other environmental factors on the seasonal presence and distribution of *V.vulnificus* in those locations important to SRO cultivation (Kaspar and Tamplin 1993). The original survey objective was overly optimistic and dependent on major collaboration with a range of oyster farmers and health and environment authorities that was not possible at that time.

One of five samples of water collected on 2 September 1993 from Lime Kiln Bay, Georges River (temperature 15.5°C, salinity 33 ppt) contained *V.vulnificus* at a low level (0.15 MPN cells/mL), and the organism was subsequently and positively identified by traditional and computer-based techniques. Of some interest was the fact that this isolate did not utilise citrate, and utilised adonitol and N-acetyl glucosamine, by comparison to the typical reactions of *V.vulnificus* reported in a variety of sources. Oliver *et al.* (1993) also reported that all of their 33 environmental isolates from US sources were unable to utilise citrate as sole carbon source, while 76% of clinical isolates were able to utilise citrate. However, West *et al.* (1986) reported that 31/33 environmental *V.vulnificus* isolates from the USA were able to utilise citrate. Such biochemical differences may be due to differences in media composition and sample treatment as much as real differences between the isolated organisms.

The ability to detect *V.vulnificus* in environmental samples is no doubt related to the myriad reactions involved in the conversion of normal to VBNC cells. The present surveys were conducted during autumn to spring, when water temperatures are lowest, hence the possibilities of temperature stress and the VBNC state are likely to be higher. Future studies should also examine samples at times when such stresses are lowest, i.e. late spring to early autumn.

Ozone Destruction of *V.vulnificus*

Two studies were undertaken of the destruction of *V.vulnificus* in model seawater systems by ozone (Wongchinda 1993, Tay 1994), and of the ozone depuration of the SRO (Tay 1994).

Destruction of V.vulnificus *by Ozone in Seawater*

The effects of ozonation time, temperature (15°, 20°, 25°C), salinity (10, 20, 30, 40 ppt) and pH (5-9) on residual ozone concentration (measured by iodometric titration) were assessed to standardise the ozonation process. Residual ozone levels were linear with ozonation time, decreased by about 30% with increasing temperature (15° to 25°C), and decreased significantly (by~ 40%) with increased salinity (10 to 40 ppt), while pH had no significant effect. Under standardised conditions (20° ±1°C, pH 8.2, 31 ppt), residual ozone levels obeyed Henry's Law.

V.vulnificus was not isolated from seawater samples used in this study. Levels of about 10^{9}/100 mL of *V.vulnificus* in inoculated seawater were reduced by over 7 log cycles in 30 sec contact with sea water that had been ozonated for 60 sec at a flow rate of about 1 L ozone/min. At initial *V.vulnificus* levels of 10^{5}-10^{6} cells/100 mL, complete destruction of the organism occurred at several ozonation time/ contact time combinations, e.g. 15 sec/240 sec, 30 sec/180 sec, 45 sec/ 120 sec, 60 sec/120 sec. Thus an ozone residual of about 1 mg/L and a contact time of more than 2 min would be sufficient to destroy levels of V.vulnificus is seawater much greater than those likely to be encounted in the environment.

When seawater containing *V.vulnificus* cells at levels of about 10^{6} - 10^{9} cells/100 mL were directly ozonated for up to 60 sec, reduction in cell numbers of up to 5 log cycles occurred, depending on ozonation time. Little further cell destruction occurred following the cessation of ozonation. The rate of destruction of *V.vulnificus* was slightly lower for direct ozonation than for cell contact with previously ozonated seawater.

In this study of ozonated seawater, cells of *V.vulnificus* were isolated from TCBS plates more frequently than from mCPC agar. However, TCBS agar does not permit the differentiation of *V.vulnificus* from sucrose-negative vibrios, especially *V.parahaemolyticus* which has a similar appearance to *V.vulnificus* on TCBS agar. However, *V.parahaemolyticus* rarely grows on mCPC agar. Thus, streaking cultures from alkaline peptone water (APW) enrichment onto both mCPC and TCBS is recommended. These results support similar findings by Oliver *et al.* (1992). TCBS agar was inoculated at 39°-40°C rather than 37°C since there was less interference from other organisms without hindering the isolation of *V.vulnificus*.

Tay (1994) confirmed the destruction kinetics of *V.vulnificus* in ozonated seawater as found by Wongchinda (1993).

Ozone Depuration of SRO

Tay (1994) examined in detail the residual ozone levels in a laboratory depuration tank connected to an ozonation system. Ozone concentrations were measured at different

locations in the tank without oysters and changes monitored with time. *V.vulnificus* levels (10^6/100 mL) within the tank decreased rapidly when the water was ozonated (residual ozone 0.2-1.5 mg/L), and were undetectable (MPN procedure) after 2 min exposure.

When oysters containing about 10^5 *V.vulnificus* cells/g were introduced into the depuration tank and ozonation commenced, cell levels were reduced by 2 log cycles in 3 h with an ozone residual of 0.9 mg/L. Over 48 h depuration, cell levels were reduced by about 3 log cycles but 10^2/g remained. At an initial level of 10^3/g oyster, ozone depuration (ozone residual 0.6-1.6 mg/L) produced a 2 log cycle reduction, but again failed to completely destroy the organism. At the end of the depuration runs, no viable *V.vulnificus* was detected in any seawater samples in the tank.

When oysters contaminated at a level of 10^3 *V.vulnificus*/g were introduced into the depuration tank and depurated without ozone treatment, a 2 log cycle reduction was achieved, similar to the ozonation trial. However, without ozone in the water, *V.vulnificus* levels rose to about 10^2-10^3/100 mL and remained at that level throughout depuration.

Contaminated but intact oysters decreased the level of *V.vulnificus* cells by about 0.75 log cycle when stored at 20 -25°C without depuration.

Overall, there was about a 2 log cycle additional reduction in levels of *V.vulnificus* in SRO depuration with ozone compared to those depurated without ozone or not depurated at all. However, no oysters that had been depurated by any method were free of *V.vulnificus*. Since the incidence of VBNC compared to culturable cells in depurated (ozone or UV) oysters is not known, the real public health concern may be greater than the results of this study demonstrate.

BENEFITS

The results of this research are not conclusive but nevertheless demonstrate the difficulties of destroying *V.vulnificus in situ*, i.e. in live SRO during UV or ozone depuration. Further detailed research is required and forms the basis of an application for funds from FRDC for 1995-98. This research will ultimately lead to results which are of direct benefit to all oyster consumers, and to oyster farmers and processors whose livelihoods will be severely compromised if further outbreaks of *V.vulnificus* infections or deaths from this organism occur. The benefits are related directly to public health which is difficult to quantify directly. However, previous outbreaks of oyster-associated food poisoning have resulted in severe and irreversible reductions in the purchase of oysters in the marketplace due to consumer concern and fear. Positive outcomes from this and subsequent research will enable strong marketing campaigns based on high public health safety to be launched with positive benefits for the industry and consumers.

INTELLECTUAL PROPERTY

No patents have been applied for or granted. Research requests have been decribed in detail in four UNSW student theses (see References). Some of these results have been and will be submitted for publication to international journals. Further work on ozone depuration will be undertaken if industry considers this work of high priority because of its public health implications.

FURTHER DEVELOPMENTS

The results of this and other research (e.g. Oliver 1993) clearly demonstrate the importance of the VBNC state in achieving complete destruction of *V.vulnificus* during depuration or other postharvest treatments (e.g. heat, cold; see Cook and Ruple 1992). If cells can be converted to the VBNC (avirulent) state and prevented from returning to a viable and virulent state (Stelma *et al.* 1992) by an appropriate treatment, problems from this organism caused by the consumption of oysters may be prevented. Thus further work on the VBNC state is warranted.

It would appear that conventional (UV, ozone) depuration of *V.vulnificus* contaminated SRO is unable to completely remove the organism. However, further more detailed research may enable mechanisms to be developed that will enhance the conversion of the organism into a non-infective state.

The present research is currently not at a stage where it can be exploited commercially.

STAFF

No staff have been employed on this project. Personnel associated with the research included:

* Professor K.A.Buckle (Project coordinator)
* A/Professor G.H.Fleet (co-supervisor of Ms Azanza)
* Ms M.P.Azanza (full-time PhD student, UNSW, funded by AIDAB Fellowship)
* Ms K.Murden (BSc student)
* Ms S-L. Tay (BSc student)
* Ms N. Wongchinda (MAppSc student, funded by AIDAB)
* Dr P.M. Cranston (Director, Food Industry Development Centre, UNSW - provided ozone generator)
  *Mr C. Taraborrelli, Mr Z. Suminski (technical assistance, equipment and facilities)

FINANCIAL REPORT

A financial report for this project prepared by the Ledgers Section, UNSW, is attached.

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DISTRIBUTION

This report should be distributed to the following organisations:

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* United Oyster Growers' Council of NSW (UOGC)
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  Karuah, NSW 2324

* NSW Fishing Industry Research Advisory Committee (NSW FIRAC)
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