FISHING INDUSTRY RESEARCH TRUST ACCOUNT

FINAL REPORT

Project Title

The occurrence and significance of pathogenic vibrios in oysters.

Grant Recipients

CSIRO and Department of Health, NSW.

Division

Division of Food Research, CSIRO; Division of Analytical Laboratories, Department of Health.

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SUMMARY

A method for the enumeration of *Vibrio parahaemolyticus* in oysters that is more reliable than the Australian Standard most probable number (MPN) method was developed. The improved MPN method was also shown to be superior to a plate count on a widely used selective agar medium.

Studies of the incidence and ecology of *V. cholerae* and *V. parahaemolyticus* in oysters and oyster-growing areas showed that both vibrios are common contaminants of unpurified Sydney rock oysters. An overall average of about 20% of oyster samples contained *V. cholerae* during autumn, the season of

highest incidence. The peak incidence of V. parahaemolyticus was in summer and early autumn, when almost all samples were positive and counts were highest. V. cholerae was undetectable in oysters for long periods during winter and spring, whereas V. parahaemolyticus was detectable throughout the year. The factors influencing the presence of the vibrios were different from those affecting bacteria of enteric origin (e.g. E. coli). The numbers of V. cholerae detected (<10/g in oysters from all sources) were well below the levels considered usually necessary to cause human illness. V. parahaemolyticus counts were much higher, sometimes approaching 10^5/g. Purification of oysters had no significant effect on V. parahaemolyticus counts. The process did not eliminate V. cholerae but appeared to achieve some reduction in V. cholerae contamination. Both vibrios were detectable in oysters at all stages of production, distribution and retail marketing.

Substantial growth of both vibrios occurred in oysters under certain conditions. The results indicated that storage and distribution of oyster shellstock at ambient temperatures does not lead to growth of vibrios to hazardous levels if the oysters are protected from excessively high temperatures. Growth of V. parahaemolyticus and V. cholerae (O1 and non-O1 serotypes) was limited during storage of unopened oysters at moderate temperatures. At elevated temperatures (37°C) vibrios were able to grow to unacceptable levels. Opened oysters provided an environment more suitable than unopened oysters for growth of both vibrio species. The results showed that correct refrigeration of opened oysters is necessary to prevent growth.

TABLE II

Behaviour of *Escherichia coli* in unopened oysters during storage.

Temp. (°C)	Time (days)	*E. coli*/g			
		Unpurified oysters [a]		Purified oysters	
	0	6	4	5	1
15	2	4	5	<1	3
	7	2	<1	<1	6
30	2	13	2	2	44
	7	2	1	<1	<1

[a] Duplicate experiments.

well in oysters under these conditions (Table I). Changes in *V. parahaemolyticus* counts were relatively small at 15°C. *V. parahaemolyticus* counts often increased at 30°C but high counts (> 10^4/g) were not observed. Extension of the storage period at 15°C to 14 days did not lead to growth of *V. parahaemolyticus*. When *E. coli* was present, *E. coli* counts did not usually change substantially during storage under these conditions (Table II). The batches of unpurified oysters used were not related to the batches of purified oysters in these experiments. Therefore, conclusions cannot be drawn about the effectiveness of the purification process from the initial *E. coli* counts recorded in Table II. Consistent differences were not observed between unpurified and purified oysters in their ability to support survival and growth of *V. parahaemolyticus* or *E. coli*. Purified oysters, in which *E. coli* was not detected, were used in all following experiments.

V. parahaemolyticus grew more readily in stored unopened oysters at 37°C than at 15 or 30°C. Counts approached or exceeded 10^6/g in samples held at 37°C either continuously or intermittently (Table III). The storage period that unopened oysters could withstand varied with the storage temperature. Oysters stored at 30°C for 7 days were near the end of their storage lives and dead and gaping oysters were

TABLE III

Growth of *Vibrio parahaemolyticus* in unopened oysters during storage under severe conditions.

Temp. (°C)	Time (days)	*V. parahaemolyticus*/g
37	0	7.0×10^1
	1	2.3×10^4
	2	1.5×10^5
	3	7.0×10^5
37/15 [a]	0	1.1×10^3
	6	2.0×10^6
42	0	1.1×10^3
		1.5×10^4

[a] Oysters stored at 37°C for 8 h each day and at 15°C for the remainder.

TABLE IV

Growth of *Vibrio parahaemolyticus* in opened oysters during storage.

Temp. (°C)	Time (h)	*V. parahaemolyticus*/g
	0	5.0×10^{2}
10	168	$< 3.0 \times 10^{1}$
15	44	5.0×10^{4}
	168	4.0×10^{5}
30	20	1.4×10^{6}
	44	6.5×10^{5}
37	20	1.5×10^{6}

occasionally found among the samples at this time. At 37°C this limit decreased to about 4 days, while at 15°C it was increased to beyond 14 days.

V. parahaemolyticus grew more readily in oysters which had been opened and stored on the half shell than in live oysters. *V. parahaemolyticus* counts were $> 10^{6}$/g after overnight storage of opened oysters at 30 or 37°C (Table IV). Growth occurred more slowly at 15°C and was not detected at 10°C. Storage of unopened and opened oysters in parallel clearly demonstrated the differing ability of live and opened oysters to support growth of *V. parahaemolyticus*. Oysters which contained 1.5×10^{3} *V. parahaemolyticus*/g before storage contained 2.2×10^{6}/g after opening and storage at 30°C overnight. Unopened oysters stored under the same conditions contained only 9.0×10^{2}/g. After storage of opened oysters for 20 h a slight off-odour had developed at 30°C and a quite strong off-odour had developed at 37°C. The appearance of the oysters was good. Oysters stored at 15°C for 44 h also appeared good but had a slight off-odour.

The survey of wholesale and retail oyster samples was conducted during summer and early autumn of 1984. Thirty samples were tested. Sixteen samples of unopened purified oysters originating from 6 estuaries were collected at the wholesale level. *V. parahaemolyticus* was detected in all 16 samples at concentrations from 0.4/g to 2.3×10^{4}/g with a median of 1.1×10^{3}/g. Three samples from different estuaries contained $> 10^{4}$/g. Fourteen samples of refrigerated opened oysters on the half shell were collected from 6 retailers. *V. parahaemolyticus* was detected in 13 samples. Counts were in the range 4.3/g to $> 1.1 \times 10^{3}$/g with a median of 90/g. Three samples contained $> 10^{3}$ *V. parahaemolyticus*/g. *E. coli* was detected in 4 samples of unopened oysters (range 1–16/g) and in 3 samples of opened oysters (range 1–3/g). There was no apparent correlation between high *V. parahaemolyticus* counts and the presence of *E. coli*.

Discussion

Shellstock of the Sydney rock oyster is frequently handled, transported and stored at ambient temperatures. The results indicate that this practice does not lead to

growth of *V. parahaemolyticus* to hazardous levels in *C. commercialis* if the oysters are protected from extremes of temperature. Growth of *V. parahaemolyticus* was not observed during storage of unopened oysters at 15°C. Moderate increases in numbers occurred at 30°C, but counts did not become unacceptably high. However *V. parahaemolyticus* was able to grow to potentially hazardous levels within a few days at 37°C, before the oysters would necessarily have been rejected.

These results are in general agreement with the limited information available from earlier studies, which suggests that *V. parahaemolyticus* does not grow well in oyster shellstock stored at moderate temperatures. Storage of *C. commercialis* or the American oyster, *C. virginica*, at 20–25°C does not usually cause large increases in *V. parahaemolyticus* counts (Hood et al., 1983; Son and Fleet, 1980; Thomson and Vanderzant, 1976). Both *V. parahaemolyticus* and *E. coli* are capable of growth in various foods at these storage temperatures and at temperatures as low as 10°C (Joseph et al., 1982; Michener and Elliott, 1964). For example, substantial increases in *V. parahaemolyticus* counts have been observed in irradiated oyster homogenates stored at 10 or 12°C for 1 week (Thomson and Thacker, 1973).

The results of earlier studies of the growth of *V. parahaemolyticus* in oyster shellstock stored at higher temperatures are contradictory. Semi-quantitative trials suggested that substantial growth of *V. parahaemolyticus* can occur in *C. virginica* at 35°C (Johnson et al., 1973), whereas growth was not observed in later, quantitative studies (Hood et al., 1983). The results presented here have important implications for the oyster industry. In warm climates, sacks of oyster shellstock left in the sun during summer may experience high temperatures for many hours. *V. parahaemolyticus* counts may approach a human infective dose if the oysters are exposed to such conditions for several days in succession.

The storage trials using oysters on the half shell demonstrated that once Sydney rock oysters are opened, and thus killed, they provide a more suitable environment for growth by *V. parahaemolyticus*. *V. parahaemolyticus* grew in opened oysters at 15, 30 and 37°C, and at each temperature grew considerably more readily than in unopened oysters. The rapid growth of *V. parahaemolyticus* in oysters after opening is probably a result of the destruction of the natural defences of the oyster against microbial invasion. These results contrast with those of Thompson and Vanderzant (1976), who found that *V. parahaemolyticus* counts did not usually increase substantially during storage of shucked *C. virginica* at 25°C for up to 36 h. They suggested that *V. parahaemolyticus* may have been inhibited by other bacteria, e.g. *Pseudomonas* species. Such inhibition does not appear to be operating in *C. commercialis* under the conditions studied here. The results of the present study show that opened Sydney rock oysters must be handled with the same precautions against temperature abuse as any other potentially hazardous flesh food which is to be consumed without further cooking.

V. parahaemolyticus counts of oysters examined during the survey were higher than have generally been observed previously in *C. commercialis* and other oyster species (Eyles and Davey, 1984; Joseph et al., 1982; Son and Fleet, 1980). This difference is probably partly due to the use of different selective enrichment media and partly because the samples tested during this study were collected at various

stages during marketing rather than directly from estuaries. Similar levels of contamination with *V. parahaemolyticus* have been observed in market samples of other types of shellfish (Sakazaki et al., 1979). The results suggest that *V. parahaemolyticus* is almost always present in Sydney rock oysters purchased by consumers during the warmer months of the year. Since counts were as high as 10^4/g, very little temperature abuse may be required in some instances to create a public health hazard. The degree of contamination with *V. parahaemolyticus* of the refrigerated retail samples indicates that, as suggested by Johnson and Liston (1973), the well-known cold sensitivity of *V. parahaemolyticus* provides very little practical protection against health hazards associated with this organism.

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Microbiological hazards associated with fishery products*

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Introduction

In those countries that maintain useful records of food-borne disease, fishery products account for a significant proportion of the outbreaks reported. The proportion varies from one country to another, depending on climate, dietary customs and other social differences. In the USA about 11% (233) of food-borne disease outbreaks reported between 1970 and 1978 were transmitted by fish, shellfish or marine crustacean products (Bryan 1980). In Japan, where dishes based on raw seafood are extremely popular, about 70% of the cases of food poisoning that occur in summer months are caused by a single bacterial pathogen derived from fishery products, *Vibrio parahaemolyticus* (Joseph *et al.* 1982). The limited information available on food-borne disease in Australia indicates that seafoods cause about 20% of confirmed food poisoning incidents (Sutton 1973; Davey 1985).

Many substances or organisms hazardous to health can be ingested with fishery products. These include various parasites (e.g. *Anisakis*), toxic chemical pollutants (e.g. mercury, pesticides) and a variety of toxins found in fish (e.g. tetrodotoxin in puffer fish). However, diseases caused by micro-organisms, especially bacteria, constitute the largest proportion of fish and shellfish-borne diseases.

This paper will consider some of the more significant diseases caused by micro-organisms that can be transmitted by fishery products. Fish-borne and shellfish-borne diseases are frequently divided into three categories on the basis of the major source of the responsible agent (Bryan 1980): 1. agents naturally present in aquatic environments, 2. agents derived from pollution of aquatic environments, 3. agents derived from workers, equipment or the environment of food handling, processing or service establishments.

Agents native to aquatic environments

Clostridium botulinum

Toxins produced by the various types of the bacterium *Clostridium botulinum* cause botulism, the well-known neuroparalytic disease affecting humans and animals. Although modern medical techniques have brought about a marked decline in mortality in outbreaks of botulism, it remains a very serious, potentially fatal disease. Its prevention is one of the most important considerations in assuring the microbiological safety of a wide range of foods. Many procedures used for the processing and storage of fishery products are designed specifically to prevent the growth of *C. botulinum*. Food-borne botulism is now relatively rare, but history has shown repeatedly that food processors who use improper procedures and cause an outbreak of botulism face severe, often ruinous, economic problems. These problems are frequently not restricted to the responsible processor, but affect a whole sector of the food industry.

The species *C. botulinum* includes a heterogeneous collection of anaerobic, spore-

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forming bacteria which have in common the production of one of the characteristic protein neurotoxins that cause botulism (Hobbs 1981). The species is divided into types A to G on the basis of the antigenic specificity of the toxins. Types A, B, E, F and G cause human botulism. These types are divided into two groups, proteolytic (A,B,F) and non-proteolytic (B,E,F) on the basis of physiological characteristics. The non-proteolytic types are of particular interest in the present context because of their low minimum growth temperatures (3°-4°C). Proteolytic types will not grow below 10°-15°C. *C. botulinum* has been shown to grow well in a variety of foods. Toxin production usually accompanies growth. The toxins are heat labile and destroyed readily by many cooking processes.

C. botulinum is widely distributed in soil, aquatic environments and other habitats. It is a natural contaminant of fish, including shellfish, and may be found in both ocean and freshwater fish at the time of catching or harvest (Hobbs 1976). All *C. botulinum* types may be present in aquatic environments, but type E has been isolated from aquatic sources most frequently and is most commonly implicated in outbreaks of botulism caused by fishery products. The number of *C. botulinum* spores or vegetative cells present in freshly harvested fish is believed to be low, no more than a few per gram of flesh, although few studies have been quantitative.

The incidence of *C. botulinum* and the types present in aquatic environments depend on a variety of ecological factors and vary markedly in different geographical regions (Hobbs 1981). The cold-tolerant (psychrotrophic) type E is present in a high proportion of samples collected from some cooler areas, such as parts of the North Sea, the US Great Lakes and around the Northern Japanese Islands, but is found infrequently in tropical and sub-tropical waters. Tanasugarn (1979) examined over 2000 fish samples from the Gulf of Thailand and found type E in only 5 samples and type D in 10 samples. *C. botulinum* was found in 2.4% of 3433 sediment and seafood samples from Indonesian waters (Suhadi *et al.* 1981). Type E was not detected. The levels of contamination of Australian soils and waters with *C. botulinum* are also relatively low (Murrell and Stewart 1983) and type E appears to be quite uncommon. This may be one reason why Australia has experienced only a handful of food-borne botulism outbreaks. Although the risk that fishery products will cause botulism varies markedly from place to place, it can never be assumed that *C. botulinum* spores are not present in raw fishery products.

For food-borne botulism to occur, the following events must coincide (Eklund 1982). 1. A food must become contaminated with *C. botulinum,* usually from the environment. 2. The processing to which the food is subjected must be inadequate to inactivate the *C. botulinum* present, or the product must be recontaminated after processing. 3. The food must be held under conditions which allow *C. botulinum* to grow and produce toxin. 4. The toxic food must be acceptable to the consumer and must be eaten without cooking or after insufficient heating to inactivate the toxin. The non-proteolytic types in particular have little effect on the odour or flavour of food.

The techniques used to prevent food-borne botulism fall into two broad categories:

- The complete destruction of the spores, usually by heating, accompanied by measures to prevent recontamination of the product (e.g. canning of low-acid foods).
- Inhibition of the growth of *C. botulinum* by physical or chemical means or a combination of these (e.g. salting, smoking, etc.).

The historical record shows that fresh or frozen fishery products are low risk foods with respect to botulism, largely because of two important safety factors. The first of these is the activity of the spoilage microflora. Although some *C. botulinum* types can grow and produce toxin at refrigeration temperatures, toxin production in unprocessed fish is usually so slow at temperatures below 10°C that the fish is rejected because of spoilage before detectable amounts of toxin are produced. In general, there is believed to be an increased safety margin between spoilage and toxin production as temperatures are reduced below 10°C. The second safety factor is cooking, since normal cooking of raw fish substantially inactivates botulinum toxins. Nearly all of the outbreaks in which fishery products have been implicated have been due to preserved products, i.e. smoked, salted, canned or fermented, usually eaten without further cooking.

Clearly the risk of botulism can be increased by preservation processes that selectively destroy or inhibit the spoilage flora while having little lethal effect on *C. botulinum* spores and possibly enhancing future growth of *C. botulinum*. Treatments such as smoking, irradiation or modified

atmosphere storage eliminate or modify the type of spoilage typical to raw products, extend the refrigerated shelf-life of the product, and interfere with the first of the safety factors mentioned above (Eyles and Warth 1981; Eklund 1982; Genigeorgis 1985). For example, experiments performed using fish artificially inoculated with *C. botulinum* spores have shown that the radurization process can increase the potential hazard from *C. botulinum* if irradiation doses over 100 Krad (1 kGy) are employed and products are stored above 3.3°C (Eklund 1982). Toxin production in advance of spoilage has been demonstrated experimentally in several species of fish from temperate waters.

There has been considerable debate in recent years over whether this increased hazard is sufficient to preclude the use of some potentially valuable techniques, particularly radurization and modified atmosphere storage, for extension of the storage life of fresh fish. A decision on whether new techniques for preservation of fishery products create an unacceptable botulism hazard will vary with the circumstances surrounding each application. The degree of contamination of raw materials with *C. botulinum,* the nature of the product, the integrity of the cold chain, and many other factors must be considered. There is certainly no hazard if fish are held at or below 3°C, but the integrity of the cold chain cannot be assured, particularly at the retail and domestic levels of distribution and storage. Similarly, consumers cannot always be relied upon to use sensible cooking procedures.

Vibrio parahaemolyticus and other vibrios

Vibrio parahaemolyticus was first isolated from cases of gastroenteritis in Japan in the 1950s. Since then it has become widely recognized as a food-borne enteric pathogen, with reports of infection coming from all over the world. Diarrhoea is the main clinical sign of infection. The incubation period may be between a few hours and one or two days. The illness usually subsides within a few days, but in a proportion of cases persists for a week or more. Some victims can be affected quite severely.

V. parahaemolyticus infections of man usually occur as a result of the consumption of contaminated and incorrectly handled fishery products. Some outbreaks caused by commercially prepared fishery products have been very large, involving hundreds of cases. Other types of food which have become contaminated from seafood or aquatic sources have also acted as a vehicle for the illness. The organism is a part of the normal microflora of estuarine and coastal waters throughout the world. It is found in water, sediment, plankton, fish and shellfish. The level of contamination usually follows a seasonal cycle. In cooler areas the highest counts are recorded in summer and autumn and the lowest counts in winter. Several investigators have observed such a cycle in New South Wales waters. In tropical countries the seasonal cycle has been correlated with rainy and dry seasons. *V. parahaemolyticus* is found infrequently in fresh water or the open ocean (Joseph *et al.* 1982).

Since it is impossible to prevent the presence of *V. parahaemolyticus* on raw fish and shellfish, various measures must be taken by those who handle and process fishery products to prevent outbreaks of food-borne illness. Fortunately, the number of *V. parahaemolyticus* cells present in freshly harvested animals is usually well below the large number required to cause illness. In addition, the organism is readily destroyed by cooking. Therefore, the most important means of controlling infection of man is the use of appropriate hygienic procedures to prevent growth of the organism in seafoods and to prevent recontamination of cooked foods from raw seafoods. Refrigeration and freezing are the most important methods of preventing growth of *V. parahaemolyticus.* Growth ceases at about 9°-10°C and below. At higher temperatures, particularly between about 20° and 40°C, it grows very rapidly in suitable foods and can reach an infective dose within a few hours (Fig. 1). Cross-contamination between raw fishery products and cooked foods must be avoided by good sanitation in food handling establishments and strict separation of raw and cooked products. Cooling, rinsing or thawing of cooked products with sea water is also hazardous.

The incidence and pattern of infection with *V. parahaemolyticus* in different parts of the world reflects the ecology of the micro-organism and national dietary habits (Joseph *et al.* 1982). In Japan, 24% of many thousands of cases of food poisoning reported in a large survey were attributed to *V. parahaemolyticus* (Blake *et al.* 1980). Most of the cases in Japan occur in the warmer months, when the level of contamination of seafood with *V. parahaemolyticus* is highest. The high incidence of the illness in Japan is

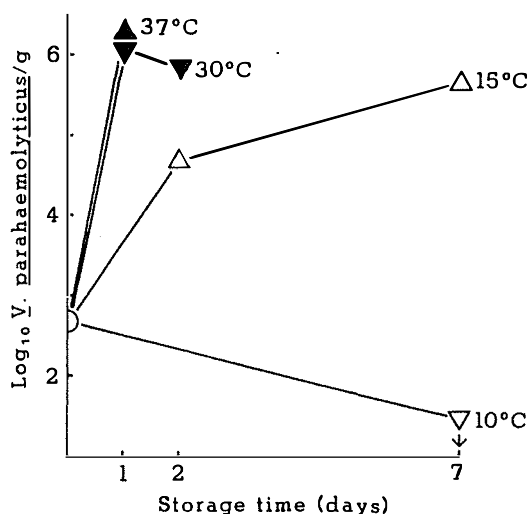

Fig. 1. Growth of *V. parahaemolyticus* during storage of opened Sydney rock oysters.

due to the national preference for raw fish and shellfish (Sakazaki 1979).

V. parahaemolyticus is known to be a common cause of gastroenteritis in several other Asian countries, such as Thailand and the Philippines (Sakazaki 1979). In countries like the USA and Australia, *V. parahaemolyticus* infections are much less frequent and constitute a minor proportion of food-borne disease. Infection is most often caused by cooked fishery products, especially shellfish such as crab, shrimp and lobster, which have been improperly cooked or recontaminated after cooking, then held at temperatures allowing growth of *V. parahaemolyticus* (Joseph *et al.* 1982).

Various other members of the genus *Vibrio* that are native to aquatic environments also appear to cause human illness when ingested with food, especially fishery products. Research on several of these organisms is at an early stage and our understanding of their ecology and pathogenicity is far from complete. *V. cholerae* is a common part of the normal microflora in brackish surface waters and may be present in oysters and other foods harvested from estuaries, etc. Gastro-intestinal illness caused by *V. cholerae* has been associated with the consumption of raw seafoods and other foods. *V. cholerae* is well known, as the species includes the strains that produce cholera toxin and cause cholera. The distinction between pathogenic and non-pathogenic strains of *V. cholerae,* as with *V. parahaemolyticus,* is not clear. The public health significance of strains of both of these species isolated from environmental or food sources is often difficult to establish. Infections with *V. vulnificus* have been attributed to consumption of raw seafoods, especially oysters. *V. vulnificus* infections are not common but can be particularly dangerous. In persons with pre-existing illnesses (e.g. liver disease) the infection may result in an often fatal septicaemia.

V. vulnificus appears to be relatively common in the estuarine and coastal environments that have been studied. These and other vibrios capable of causing enteric infections (e.g. *V. mimicus, V. fluvialis*) are present in Australian waters and cause human infections in Australia on occasions (Desmarchelier 1984). Recent reviews (Blake *et al.* 1980; Joseph *et al.* 1982; Blake 1983; Desmarchelier 1984) have discussed the characteristics and ecology of the pathogenic vibrios in detail.

Other pathogenic members of the family Vibrionaceae that are present in aquatic environments, fish and shellfish include *Aeromonas hydrophila, A. sobria* and *Plesiomonas shigelloides.* These organisms are receiving increasing attention as causative agents of gastroenteritis, including food-borne gastro-enteritis. *P. shigelloides,* for example, appears to have caused several recent outbreaks of oyster-borne gastroenteritis in North America. The importance of these organisms as food-borne pathogens is unresolved.

Scombroid poisoning

Scombroid poisoning occurs world-wide and has been reported on several occasions in Australia and New Zealand (Taylor *et al.* 1984). Fresh, canned, dried, smoked and salted fish have all been implicated. The symptoms of scombroid poisoning mimic those of allergic reactions and histamine toxicity. They may include an oral burning sensation, flushing of the face and neck, headache, palpitations, rash, itching, gastro-intestinal symptoms, and others. Both the incubation period (several minutes to a few hours) and the duration of the illness (<1 day) are usually brief.

Scombroid poisoning is caused by the consumption of scombroid fish (tuna, bonito, mackerel, saury, etc.) and some non-scombroid fish in which bacterial growth has taken place. The flesh of these fish contains large amounts of the free amino-acid histidine, which can be decarboxylated to histamine by certain bacteria (e.g. several members of the Enterobacteriaceae). The physiological basis for scombroid poisoning is still unclear. There is strong evidence that

histamine is important in producing the symptoms, although other compounds are also involved (e.g. cadaverine), possibly as potentiators of histamine action (Taylor *et al.* 1984). Fish incriminated in outbreaks usually contain high levels of histamine, up to several hundred mg/100 g of flesh. A level of 50 mg/100 g is considered hazardous by authorities in the USA (Taylor *et al.* 1984). Storage temperature is the critical factor influencing histamine formation (Sun Pan and James 1985). Scombroid poisoning can be prevented by rapid and uninterrupted refrigeration of susceptible fish after catching.

Dinoflagellate toxins

Certain marine dinoflagellates (photosynthetic microorganisms) produce toxins that cause disease in man. The dinoflagellates (Fig. 2) form part of the food supply of higher marine organisms and cause human disease when fish or shellfish containing hazardous quantities of the toxins are consumed. Ciguatera and some types of shellfish poisoning are diseases caused by dinoflagellate toxins that have significant public health and economic consequences. Many features of these diseases and their aetiology are still poorly understood.

The symptoms of ciguatera poisoning are complex and may appear in various combinations, with involvement of the digestive, cardiovascular and nervous systems (Ragelis 1984). Distinctive features of the illness include reversal of hot and cold sensations, tingling and numbness of the extremities, and severe pruritis. The neurological symptoms may persist for months or years. Fatalities occur in severe cases.

Ciguatera is caused by the consumption of toxic individuals of over 400 species of tropical and sub-tropical fish, predominantly from waters around coral reefs and islands. The dinoflagellate *Gambierdiscus toxicus* has been identified as an important source of ciguatoxin, the toxin believed to be the principal agent of ciguatera (Ragelis 1984). In general, outbreaks of ciguatera are sporadic and unpredictable, both in geographic distribution and time. Not all fish of the same species caught at the same time and place are toxic.

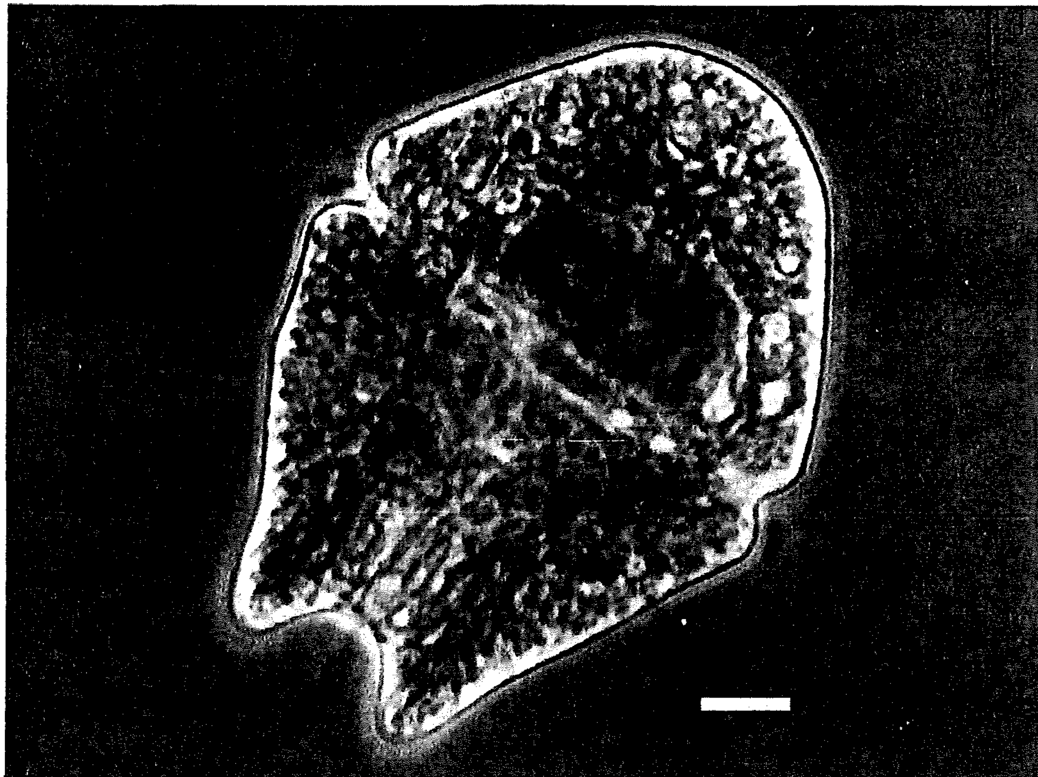

Fig. 2. A marine dinoflagellate. Dinoflagellates show considerable morphological variation from species to species. This species is not known to be toxic. (Photo J.C. Eyles). (Bar = 10 μm).

In Australia, toxic fish are frequently caught on the Great Barrier Reef (Gillespie 1980). Ciguatera has also been caused by fish from further south, principally by Spanish mackerel and other species from the Hervey Bay area. From 1976-80, at least 38 toxic Spanish mackerel, which caused 217 poisonings, came from this area (Lewis and Endean 1983). Ciguatera has also been reported in New South Wales, caused by fish from Queensland waters (Davey 1985). At present, control can be achieved only by preventing the marketing of fish likely to be hazardous. However, the value and practicality of this approach are limited in Australia and elsewhere (Gillespie 1980). There is no convenient test for identifying toxic fish, nor is the toxin destroyed by cooking, freezing. smoking, salting or drying.

Many species of shellfish may become poisonous through feeding on toxic dinoflagellates. A number of dinoflagellate species may cause illness in this manner, creating a significant public health problem in some parts of the world. Paralytic shellfish poisoning, often associated with "red tides" caused by blooms of dinoflagellates, is an important example. These toxins will not be discussed in detail in this presentation (see Bryan 1980; Ragelis 1984). Prevention of the illnesses caused by these toxins is usually achieved, where possible, by monitoring waters known to present a hazard and preventing the harvesting of shellfish at times when the toxic algae are present in the water in high concentrations.

Agents derived from pollution of aquatic habitats

Persons suffering from enteric infections excrete pathogenic microorganisms in their faeces in vast numbers. Thus, as a result of pollution of rivers, lakes and coastal waters by human wastes, aquatic animals harvested for food may become contaminated with a wide range of pathogenic microorganisms. Pathogens are frequently introduced into watercourses or coastal waters by the intentional or accidental discharge of treated or untreated sewage and by runoff from the land during rain. Although sewage treatment processes reduce pathogen concentrations in domestic sewage by varying degrees, very few of the processes in current use produce pathogen-free effluent. Agricultural wastes can also be a source of hazardous micro-organisms, for example *Salmonella* species. Many outbreaks of hepatitis A, typhoid fever, cholera and viral or bacterial gastro-enteritis, some involving hundreds of cases, have been attributed to the consumption of fish or shellfish from polluted waters (Bryan 1977, 1980; Eyles 1986). Hepatitis and several large outbreaks of viral gastroenteritis have been transmitted by oysters or mussels from Australian waters (Eyles 1986).

Several factors have made shellfish, especially bivalves, the major source of problems of this nature. Shellfish are frequently cultivated in or harvested from estuarine or coastal waters subject to pollution. Bivalves (oysters, mussels, clams) collect their food by filtration of large volumes of water, so that the contents of the bivalve digestive tract closely reflect the material suspended in the water. Bivalves may accumulate human pathogens to concentrations well above those in the surrounding water. This problem is exacerbated by the common custom of eating bivalves raw. Even when they are cooked, the cooking procedures are often too mild to inactivate all the pathogens that might be present. Cooked bivalves have transmitted both hepatitis A and gastroenteritis viruses.

Mis-handling of food after harvest is not necessarily required for disease transmission to occur by this route. The enteric viruses, such as those which cause hepatitis and gastroenteritis, do not multiply outside their human host. The dose of these agents necessary to initiate an infection is very small and the contamination acquired initially from polluted water is sufficient to cause disease. Some of the bacterial pathogens mentioned above (e.g. *Salmonella*) usually have a much larger infective dose and disease can occur only if contaminated fishery products are subjected to temperature abuse between harvest and consumption, thereby allowing growth of the pathogen.

Control of disease transmission by this route can be achieved by harvesting only from waters which are not unacceptably polluted or by subjecting shellfish or fish harvested from doubtful waters to a process that will inactivate or remote pathogens (e.g. effective heat treatment). Many countries have established procedures for controlling the quality of waters from which bivalves are harvested. These procedures involve sanitary surveys of waterways to identify sources of pollution, followed by classification of areas into various categories of acceptability. These surveys should be supported by continued monitoring of the bacteriological quality of water and shellfish.

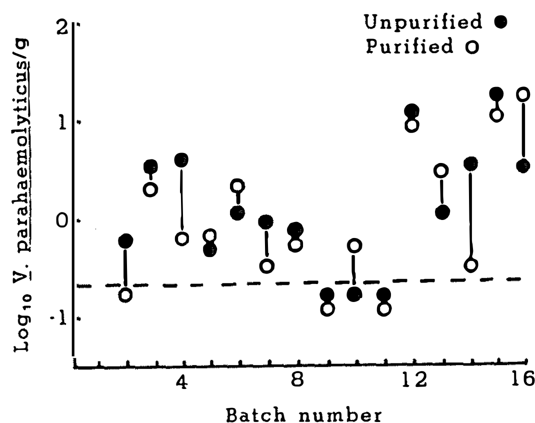

Fig. 3. *V. parahaemolyticus* in unpurified and purified Sydney rock oysters (Eyles and Davey 1984).

Control measures of this type have been introduced recently in the Tasmanian oyster industry.

Purification is a process used to remove human pathogens from bivalves. Human pathogens derived from pollution are believed to be associated transiently with bivalves. Thus, in several parts of the world, including New South Wales, live shellfish are held in unpolluted water for a short time before marketing to allow any pathogens present to be excreted by the animals. The purification process is referred to as depuration if carried out in man-made tanks of water, or relaying if an unpolluted natural waterway is used. The process does not remove some pathogens (e.g. *V. parahaemolyticus*) that are native to aquatic environments (Fig. 3) (Eyles and Davey 1984) and its ability to remove viruses reliably is doubtful (Eyles 1986).

Agents derived from contamination after harvest or catching

During processing, storage, distribution and preparation for consumption, fish or shellfish may become contaminated with a number of potentially pathogenic micro-organisms. Pathogens which cause some serious diseases, such as typhoid fever, hepatitis and cholera, can be introduced to fishery products by infected workers or polluted water in food processing or preparation establishments. Such contamination is not acceptable and, fortunately, is not common. Fishery products become contaminated much more frequently with bacteria capable of causing food poisoning, i.e. *Staphylococcus aureus, Salmonella, Clostridium perfringens, Bacillus cereus,* etc.

Bacterial food poisoning is characterized by a sudden onset of gastrointestinal symptoms, such as vomiting, nausea, diarrhoea and stomach cramps, usually within a day or two of eating contaminated food. The illness is usually caused by the consumption of food in which food poisoning bacteria have been permitted to grow to large numbers. Moist, high protein foods that do not contain excessive concentrations of acid, salt or other inhibitors (i.e. many fishery products and dishes prepared from them), provide ideal conditions for growth of food poisoning bacteria. Some contamination with food poisoning bacteria is almost inevitable during the handling, processing and preparation of fishery products for consumption. A substantial proportion of the food poisoning outbreaks attributed to fishery products is caused by food poisoning bacteria derived from utensils or equipment, the bodies of workers, food ingredients, etc. Nevertheless, good hygienic practices and temperature control can keep the degree of contamination within acceptable limits, prevent the growth of pathogens, and prevent outbreaks of illness (Davey 1985).

Staphylococcus aureus is a food poisoning organism of particular interest in the present context, since it has been one of the main causes of rejection of crustacean shellfish products by the health authorities of importing countries. *S. aureus* is a part of the normal microbial flora of the skin, nose and throat of man and can be found in particularly large numbers in skin eruptions and inflammations (e.g. boils, acne) and wounds. Staphylococci most commonly enter fishery products during processing and preparation from the bodies of workers. The small numbers of staphylococci found in food as a result of the initial contamination cannot cause illness. Staphylococcal food poisoning is caused by enterotoxins produced by the microorganisms during growth in food. Thus, illness can occur only if contaminated food is held under conditions which allow the staphylococci to grow to large numbers and produce harmful quantities of toxin.

S. aureus may be found on some raw seafoods at the time of catching or may enter raw products during primary handling and processing, e.g. filleting. Nevertheless, the staphylococci compete poorly with the normal spoilage flora of raw products and it is only if they are allowed to contaminate cooked products that they usually present a hazard. Because such a high proportion of

humans carry staphylococci on their bodies, heat-processed seafoods are very likely to become contaminated during various handling operations and final preparation for consumption. Contamination of pre-cooked crustacean products (e.g. frozen prawns) is considered to present a particularly significant hazard because these products are commonly eaten without further cooking in salads, cocktails, etc., that may be exposed to elevated temperatures before and during serving. They also provide an excellent environment for staphylococcal growth.

Control of staphylococcal contamination of food requires a high standard of personal hygiene among processing personnel, the use of procedures designed to minimize direct or indirect human contact with food, and stringent control of the temperature of the product at all stages of processing. *S. aureus* will not grow at temperatures below 6°C or above 46°C.

Conclusion

The preceding overview has demonstrated that a variety of public health problems can be caused by fishery products produced under unsatisfactory conditions. The key element in control of all these illnesses is effective education and training. Managers and supervisors must be aware of the food-borne disease problems that confront the industry and must realize that the implementation of effective hygiene and other control measures makes good economic sense. Examples of economic benefits derived from good sanitation and hygiene include: (1) prevention of the considerable losses incurred by processors who cause food-borne disease outbreaks, (2) production of a high quality product with good storage life and minimal spoilage losses, (3) elimination of rejections of unsatisfactory products by importing countries and customers, thereby expanding potential markets. Managers and supervisors must be capable of recognizing unsatisfactory situations and must be able to take appropriate remedial action or obtain professional assistance.

Workers should be trained in safe procedures for handling and processing fishery products and in proper sanitation of equipment. They should understand that fishery products can be a source of hazardous microorganisms and should know which steps in the operations they perform are critical in controlling pathogens. Managers and supervisors must motivate their employees to follow the procedures laid down. Fishermen should be able to identify potentially toxic fish in their areas and should be aware of the hazards associated with fish or shellfish taken from waters subject to pollution or growth of toxic micro-algae. Regulatory personnel often have an important part to play in the education and training of those in the industry. They must be given sufficient knowledge to enable them to carry out this role effectively and to perform their regulatory functions intelligently.

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1. INTRODUCTION

Oysters and other molluscan shellfish are filter feeders, concentrating within their bodies microorganisms from the surrounding water. Thus, if the water in which oysters are grown contains microorganisms that are pathogenic for man, consumption of the oysters can lead to outbreaks of disease. Widespread outbreaks of oyster-borne gastroenteritis occurred in Australia in 1978. The outbreaks were caused by viruses, and possibly bacteria, present in oysters as a result of sewage pollution of oyster-growing areas in NSW. Compulsory purification was introduced subsequently for all oysters produced in NSW, considerably reducing the risk that oysters will transmit pathogens introduced to estuaries as a result of sewage pollution.

After the introduction of purification, another microbial hazard emerged. *Vibrio cholerae* O1, the bacterial type that includes the causative agent of cholera, was detected in purified oysters. The strains detected did not produce the toxin responsible for typical cholera but may have been capable of causing less severe gastroenteritis. The reaction of the public and health authorities caused serious difficulties for the oyster industry. Other vibrios possibly capable of causing gastroenteritis, including *V. parahaemolyticus* and non-O1 serotypes of *V. cholerae*, were also present in purified oysters. These vibrios are unlike most pathogens introduced to estuaries with sewage in that they are native to estuarine environments and form part of the normal microflora of various estuarine organisms. Therefore the vibrios may be much more

difficult to remove from oysters during purification.

At the time that this project commenced very little was known about the ecology of these vibrios in Australian oyster-growing areas. There was little quantitative information on the occurrence of V. cholerae in Australian estuaries or in purified or unpurified oysters. Little was known about the ability of vibrios to persist in oysters during purification or to survive or grow in oysters during storage and transport. This lack of information caused dilemmas for health authorities and the industry. The bacteriological standards commonly applied to oysters and the bacteriological criteria used in the development of purification processes provided no indication of the occurrence or behaviour of potentially pathogenic vibrios. Some of the information that was available on the behaviour of vibrios during purification was known to be misleading.

The purpose of this research project was to provide information that would: (a) allow a realistic assessment of the hazard presented by these vibrios in purified oysters, and (b) help the industry to minimise the risk of oyster-borne outbreaks of illness caused by vibrios. The degree of hazard associated with vibrios in seafoods is closely related to the numbers of vibrios present. Therefore, the major objectives of the project were to determine: (a) the frequency and level of contamination of oysters with V. cholerae and V. parahaemolyticus before and after purification, (b) the factors affecting the presence of the vibrios, and (c) the factors influencing their persistence in oysters after harvest and purification. It was also necessary to conduct a study of methods for the

enumeration of *V. parahaemolyticus* in oysters, since existing accepted methods were found to be inadequate.

In addition to our studies on the microflora of commercially-purified oysters, we proposed initially to study the behaviour of vibrios during laboratory-scale purification. These experiments were abandoned for a variety of reasons. A reliable estuarine source of oysters contaminated with *V. cholerae* was not available. The low incidence of *V. cholerae* in oysters put meaningful experiments beyond the resources of the project. Purification experiments using oysters contaminated in the laboratory were considered undesirable, since our studies showed that vibrios added to oysters in the laboratory and vibrios acquired in an estuary behaved differently during purification. In addition, there is substantial evidence that the control of hazards associated with vibrios cannot be achieved by purification. The nature of the association between oysters and vibrios is fundamentally different from the association between oysters and pathogens derived from pollution. Our studies with commercially-purified oysters showed clearly that *V. parahaemolyticus* was not affected by purification.

2. DEVELOPMENT OF METHODS FOR THE DETECTION OF *V. parahaemolyticus* IN OYSTERS

The method used initially in our laboratories for the enumeration of *V. parahaemolyticus* in oysters was the most probable number (MPN) method specified in Australian Standard 1766. It became apparent that there were significant deficiencies in the method. The complexity of the method also limited the number of samples that could be examined. Therefore a study of alternative media and procedures was performed with the aims of improving the reliability of the method and decreasing the workload associated with the enumeration of *V. parahaemolyticus*. A detailed description of this study is contained in Attachment A. The findings are summarised below. As a result of this study the Australian Standard method for detection of *V. parahaemolyticus* in foods will be revised shortly.

The major deficiency in the Australian Standard method was related to the selective enrichment medium used, glucose salt Teepol broth (GSTB). GSTB did not give optimal recovery of *V. parahaemolyticus* and yielded erratic results on occasions. Three selective enrichment media were evaluated for use in the MPN procedure, alkaline peptone water (APW), arabinose ethyl-violet broth, and GSTB. APW was the most effective medium, yielding more positive samples and significantly higher counts than the other media. The performance of the MPN method with APW as the enrichment medium was considered satisfactory and it was used in all studies described in this report, unless otherwise stated.

Plate count methods have important fundamental advantages over MPN methods, which are labour-intensive, slow and expensive to perform. Thiosulphate citrate bile-salts sucrose (TCBS) agar is a selective, differential agar medium that is widely used for the isolation of pathogenic vibrios. A plate count on TCBS agar was evaluated as an alternative to the MPN procedure. In a series of comparative trials, the counts of *V. parahaemolyticus* in oysters were lower by the plate count than by the MPN method, over a wide range of contamination levels. Overall mean counts were about five-fold higher by the MPN method. The plate count was not adopted as a routine procedure.

The identification procedure specified in Australian Standard 1766 may mis-identify some strains of *V. harveyi* or *V. vulnificus* as *V. parahaemolyticus.* Tests to eliminate this potential error were devised and the frequency with which isolates from oysters are identified incorrectly by the Australian Standard method was assessed. Errors in identification were found to be likely with only a small proportion of isolates from Australian oysters.

Finally, a medium designed to provide rapid, presumptive identification of *V. parahaemolyticus* (VP medium) was assessed. The identification of potential *V. parahaemolyticus* isolates is a very time-consuming process. VP medium was found to reduce the number of isolates requiring full characterization by eliminating many non-*V. parahaemolyticus* isolates at an early stage. The medium also usefully extended the range of biochemical tests applied to cultures.

3. ECOLOGY AND INCIDENCE OF V. cholerae AND V. parahaemolyticus IN OYSTERS

Accurate information on the incidence of *V. cholerae* and *V. parahaemolyticus* in oysters and oyster-producing waterways is required before the public health significance of these vibrios in oysters can be assessed realistically. Such information is also essential to the development of appropriate measures for controlling any hazard. It is necessary to know the frequency and level of contamination with vibrios and to understand the factors affecting their presence in oysters before and after purification.

Three complementary studies designed to provide the necessary ecological data are described below. The aim of the first study was to establish the pattern of occurrence of *V. parahaemolyticus* and *V. cholerae* in oysters and oyster-growing areas. The second study obtained accurate quantitative information on the levels of contamination with these vibrios. The third was a broad survey of the incidence of *V. cholerae* in oysters from a large number of purification plants.

The first of these studies has been described in part in the final report on an earlier FIRTA-funded project (Viral and Bacterial Contamination and Decontamination of Oysters). The present project was commenced as a result of the findings of the earlier project. The purpose of the earlier project was to gain basic information about the contamination of oysters with a range of microorganisms, including vibrios, that have public health significance. Some aspects of the earlier project that

were concerned with the vibrios were concluded during the present project and were not discussed in full in the previous final report.

A) PATTERN OF OCCURRENCE OF *V. parahaemolyticus* AND *V. cholerae* IN OYSTERS AND OYSTER-GROWING AREAS

During this study, samples from selected areas were analysed to determine the frequency and extent of contamination of oysters with relevant microorganisms and the factors that influence microbial contamination of oysters. The nature and degree of contamination with which oyster purification processes must cope and with which they are able to cope were assessed.

Methodology

Three estuarine sampling stations were established, two in the Georges River (Woolooware Bay and Neverfail Bay) and one in Brisbane Water (Murphys Bay). Five oyster samples and water and sediment samples were collected from each of these sites about every two weeks for one year. Samples were collected from the Georges River sites on 25 occasions and from Brisbane Water on 21 occasions. The sites selected could be expected to yield results representative of a large number of other oyster-producing areas. Oyster samples were also collected from purification plants which purified oysters from Woolooware Bay and Neverfail Bay.

In addition to *V. cholerae* and *V. parahaemolyticus*, oysters were examined for indicator microorganisms (aerobic

plate count, coliforms, Escherichia coli) and pathogens (Salmonella, viruses). The same analyses, except for the aerobic plate count and coliform count, were performed on water and sediment. Physicochemical measurements (temperature, salinity, turbidity and pH) were performed on the water. The methods used are described in detail in Attachments B and C. Note that this work was performed before methods for the isolation of V. parahaemolyticus were modified.

Results

Both vibrios were detected frequently at all 3 sampling stations. The presence of the vibrios was related to broad climatic influences rather than short-term influences, such as rainfall.

V. cholerae was detected in a total of 20/211 (9.5%) unpurified oyster samples, 21/70 (30%) water samples and 8/71 (11%) sediment samples from the three stations. V. cholerae 01 strains were isolated from water twice, once from each of Woolooware Bay and Brisbane Water. Neither of the 01 isolates produced heat-labile cholera toxin. There were no marked differences between the stations in rates or patterns of isolation of V. cholerae.

The major influence on the presence of V. cholerae was seasonal. V. cholerae was detected most often in autumn (Figure 1). Unlike the enteric bacteria (E. coli, Salmonella), whose presence in oysters was clearly related to rainfall in the days preceding sampling, isolations of V. cholerae did not appear to be related to high rainfall or reduced salinity (Table 1). It was expected that V. cholerae isolations might

have been increased by rainfall, since this species is generally believed to be an inhabitant primarily of waters of lower salinity (i.e further upstream) than those studied. E. coli and other enteric bacteria enter estuaries during periods of rainfall as a result of urban runoff, sewer discharges, etc. V. cholerae did not persist in the environment of the sampling stations throughout the year. Oysters, water and sediment were all negative at 44/77 samplings.

V. parahaemolyticus was isolated much more frequently than V. cholerae. V. parahaemolyticus was detected in 64% of oyster samples, 51% of water samples and 71% of sediment samples. The peak occurrence of V. parahaemolyticus in oysters and water was in summer, in terms of both numbers of isolations (Figure 1) and counts. Maximum counts of V. parahaemolyticus at all three stations were about 10^2/g oyster. Although the levels of V. parahaemolyticus in oysters and water declined during the colder months, it was present consistently in the environment of the 3 stations. A high proportion of sediment samples contained V. parahaemolyticus throughout the year.

The effects of purification on the microflora of oysters are discussed in detail in Attachment C. Purification had no significant effect on the level of contamination with V. parahaemolyticus. In contrast, contamination with E. coli was reduced significantly by purification. V. cholerae was present in purified oysters on occasions. Its incidence in purified oysters was slightly lower than in oysters taken directly from the estuary.

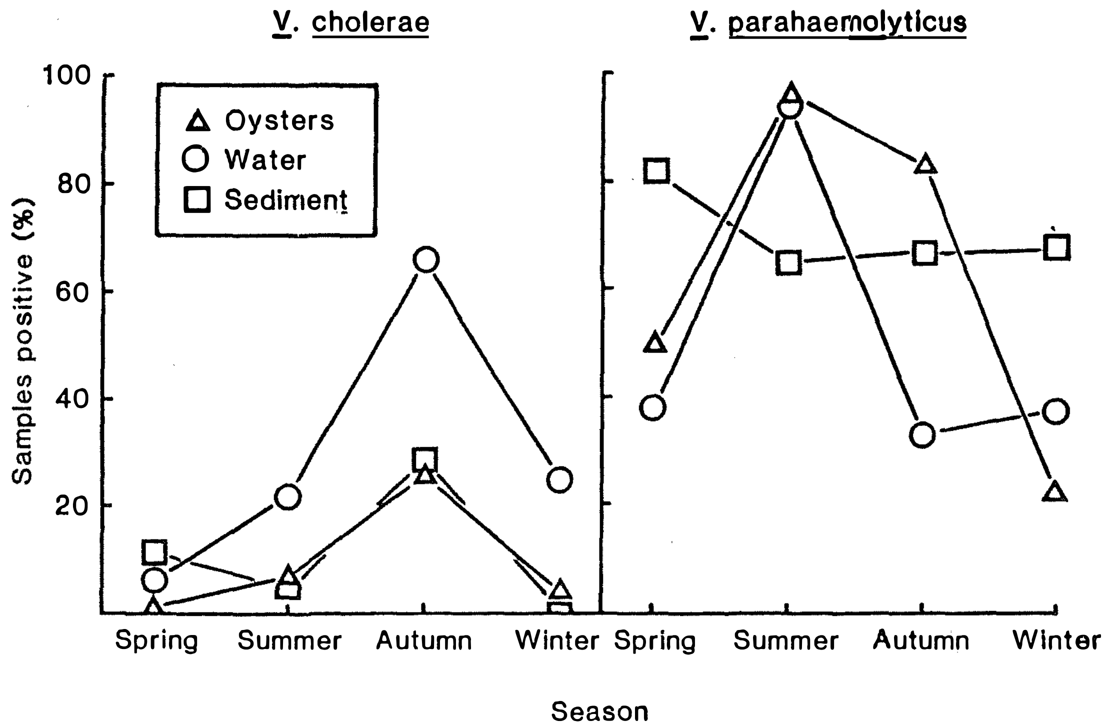

FIGURE 1 Incidence of V. cholerae and V. parahaemolyticus at the three sampling stations.

TABLE 1 Rainfall, E. coli counts and the presence of V. cholerae at the sampling stations

	Rainfall (mm)#		
	Woolooware Bay	Neverfail Bay	Brisbane Water
E. coli count (oysters)*			
High	34.9	43.1	59.1
Low	1.5	6.9	16.7
V. cholerae (all samples)			
Detected	17.4	26.4	20.4
Not detected	25.3	20.5	38.6

Total rainfall in the 7 days before sampling

* High - counts were above the limit specified in NH&MRC standards

Low - counts were within the NH&MRC limit

B) QUANTITATIVE STUDY OF GEORGES RIVER SITES

Our initial studies on the incidence of *V. cholerae* in oysters, described above, were based on qualitative analyses. Further studies were performed at the Georges River sampling sites examined earlier to acquire quantitative data on the incidence and pattern of occurrence of *V. cholerae.* It was also necessary to acquire more accurate quantitative data on the occurrence of *V. parahaemolyticus.* The earlier studies employed the Australian Standard method for enumeration of *V. parahaemolyticus* which our subsequent comparative trials have shown to yield artificially low counts.

Methodology

The two sampling sites were studied during summer and autumn. Previous work had shown that potentially pathogenic vibrios are most likely to be detected during these seasons. At each sampling, three samples of 12 oysters each were collected. Samples were collected from the estuary at the Neverfail Bay site on 8 occasions (total number of samples = 24). Oysters that had been harvested from Neverfail Bay then purified commercially were also sampled 8 times (total number of samples = 24), usually at the same time as samples were collected from the estuary. Samples were collected from the estuary at the Woolooware Bay site on 4 occasions (total number of samples = 12). Purified oysters from Woolooware Bay were not available for analysis. The total number of oyster samples examined during this part of the study was 60. Microbiological methods were as described in Attachments A and B. Quantitative detection of *V. cholerae* was achieved by using the enrichment

technique described in Attachment A as a multiple-tube dilution (MPN) method.

It would have been desirable to collect samples a little more frequently, especially from Woolooware Bay. However, sampling was somewhat inhibited by some oyster farmers' wariness of microbiological studies of oyster growing areas and purification plants.

Results

The microbiological findings are summarised in Figure 2. V. cholerae was isolated from a total of 6 samples, 1/12 from Woolooware Bay and 5/24 from Neverfail Bay. The counts were above the quantitative limit of detection in only 2 positive samples, which yielded 0.7 and 0.4 V. cholerae/g. V. cholerae levels were <0.3/g (i.e. detectable only by enrichment of 10g of sample) in the remaining 4 positive samples. One positive sample was collected in January, the remainder in March and April. All V. cholerae isolates were non-O1 serotypes. There was no observable correlation between the presence or absence of E. coli and the presence of V. cholerae. V. cholerae was isolated from samples with both very high (340/g) and very low (<1/g) E. coli counts. The presence of V. cholerae was also unrelated to high or low V. parahaemolyticus counts.

V. parahaemolyticus was detected at every sampling. All samples from Woolooware Bay and 23/24 samples of both purified and unpurified oysters from Neverfail Bay contained V. parahaemolyticus. As expected, V. parahaemolyticus counts were higher than recorded in previous studies. The maximum count in

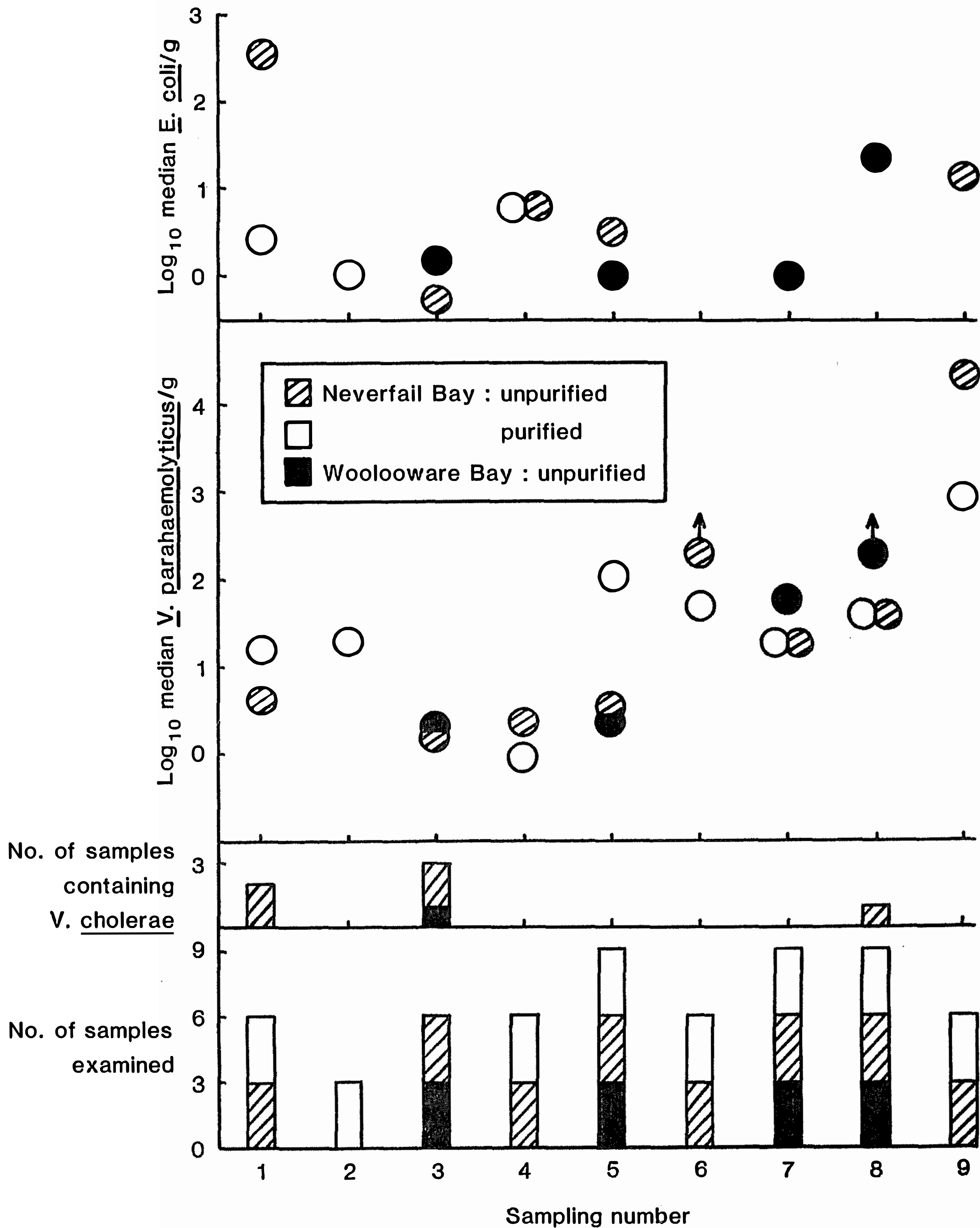

FIGURE 2 V. cholerae, V. parahaemolyticus and E. coli in oysters from Neverfail Bay and Woolooware Bay.

individual samples of unpurified oysters was $9.3x10^4$/g (Neverfail Bay) and >$2x10^2$/g (Woolooware Bay). The maximum count recorded in purified oysters was $4.3x10^3$/g.

E. coli was detected in 9/24 samples of purified oysters, with a maximum count of 9.5/g. *E. coli* was present in 13/24 (maximum count 700/g) and 12/12 (maximum count 29/g) samples of unpurified oysters from Neverfail and Woolooware Bays respectively.

The comparative patterns of contamination of purified and unpurified oysters from Neverfail Bay were quite different for the two vibrios. *V. cholerae* was detected in 5/24 samples of unpurified oysters and not at all in purified oysters, whereas *V. parahaemolyticus* was present in 23/24 samples of both purified and unpurified oysters. The geometric mean *V. parahaemolyticus* counts were 32 and 37/g in unpurified and purified oysters respectively. The results indicate that purification is reducing the incidence of contamination with *V. cholerae,* while having little or no effect on *V. parahaemolyticus.*

C) SURVEY OF PURIFIED OYSTERS FOR THE PRESENCE OF *V. cholerae*

The results discussed above were limited in that a relatively small number of oyster purification plants was examined. A series of samples obtained from a large number of different purification plants was examined to assess the incidence of *V. cholerae* in purified oysters on a broader scale.

Fifty-five samples of oysters were examined over a period of one year. The samples had been harvested originally from 6 different estuaries. Samples were obtained from a total of 21 purification plants. Standard plate counts, E. coli counts and V. cholerae counts were performed on the samples. V. cholerae was found in one sample and was detected in that sample only by qualitative enrichment. The isolate was a non-O1 serotype. The sample was obtained from the Macleay River in early February. E. coli was not detectable in the sample. The aerobic plate count, $5.2x10^2$/g, was not notably high or low. E. coli was detected in 7/55 samples tested, at levels from 0.5/g to 30/g.

D) DISCUSSION

V. cholerae and V. parahaemolyticus are both present regularly in Sydney rock oysters harvested from NSW estuaries. The two species behave quite differently in several important respects.

V. cholerae was detectable in a small proportion of oyster samples collected from estuaries during the warmer months of the year. The results show that V. cholerae is detectable most often in the autumn, when an average of 20% or more of oyster samples might be expected to yield V. cholerae. V. cholerae was undetectable for long periods during winter and spring, although positive samples were obtained occasionally during these seasons. The number of V. cholerae present in positive samples was low. It is generally accepted that V. cholerae is adapted to waters further upstream than oyster-producing areas.

Small numbers of V. cholerae probably appear in oyster-growing areas at certain times of the year as a result of increases in numbers in areas of lower salinity. Its presence appeared to be affected by factors different from those that influence pathogens introduced to estuaries by pollution. The behaviour or presence of conventional enteric indicator organisms (e.g. E. coli) appears to provide little useful information about the behaviour or presence of V. cholerae.

V. parahaemolyticus is detectable in oysters much more frequently and in much higher numbers than V. cholerae. The peak incidence of V. parahaemolyticus was in summer and early autumn. However it persists in the environment of oyster-growing areas and can be detected in oysters throughout the year. The number of V. parahaemolyticus present in oysters immediately after removal from the estuary is quite high at times. Using our improved method for enumeration of V. parahaemolyticus, counts approaching 10^5/g were observed in individual oyster samples.

These studies have shown quite clearly that V. parahaemolyticus counts in oysters are not significantly affected by purification. V. cholerae is also detectable in purified oysters on occasions, as demonstrated by both repeated sampling from individual purification plants and a broader survey of a large number of plants. Although V. cholerae is not eliminated entirely by purification, the level of contamination with V. cholerae does appear to be reduced.

The inability of purification to eliminate potentially

pathogenic vibrios from oysters should not be seen as a failure of the process. Purification was designed and introduced as a means of removing from oysters pathogenic microorganisms that are not native to aquatic environments. Vibrios are now known to live in close association with components of higher aquatic organisms. In the light of present knowledge of the ecology of the vibrios, it would be unreasonable to expect purification processes to remove all vibrios from oysters. On the other hand, the regularity with which we have detected *E. coli* in purified oysters during these studies is cause for concern.

4. SURVIVAL AND GROWTH OF V. parahaemolyticus AND V. cholerae IN OYSTERS

An important factor determining the ability of V. cholerae and V. parahaemolyticus to cause human illness is the number of cells of the pathogen that are ingested. The numbers of V. parahaemolyticus and V. cholerae detected in oysters immediately after harvest or purification are well below the high counts considered necessary to cause human illness in most circumstances. V. parahaemolyticus must be present at levels of 10^6/g or more to cause gastroenteritis, and the human infective dose of V. cholerae is believed to be similarly high in most cases. Thus, the ability of these vibrios to survive and grow in oysters during storage and transport is an essential consideration in assessing the public health hazards associated with their presence. Information of this kind is required for the establishment of safe handling practices for oysters.

At the wholesale level, Sydney rock oysters are usually transported and marketed alive at ambient temperatures. Live oysters may be held for days or weeks at temperatures at which V. cholerae and V. parahaemolyticus will grow in suitable foods. At the retail level, the oysters are most commonly sold refrigerated in the half shell. Opportunities for temperature abuse of the product occur frequently at the retail and domestic levels of distribution and storage. The ability of V. parahaemolyticus and V. cholerae to survive and grow in Sydney rock oysters during storage under a variety of conditions was examined during this study. The storage conditions included

conditions of temperature abuse.

A) SURVIVAL AND GROWTH OF V. parahaemolyticus

Experiments designed to determine whether V. parahaemolyticus can survive or grow in Sydney rock oysters are described in detail in Attachment D. The findings are summarised below. Oysters that were naturally contaminated with V. parahaemolyticus were used throughout the experiments.

V. parahaemolyticus survived but did not grow well in unopened (i.e. live) oysters stored at moderate temperatures. During storage at 15°C and 30°C for up to 14 or 7 days respectively, limited increases in V. parahaemolyticus counts often occurred but high counts (above 10^4/g) were not observed in any samples. When present, E. coli was also able to survive but was unable to grow in oysters under these conditions. In these experiments the behaviour of V. parahaemolyticus and E. coli was similar in unpurified and purified oysters.

When unopened oysters were stored under more severe conditions substantial growth of V. parahaemolyticus did occur. Counts approached or exceeded 10^6/g in samples stored intermittently or continuously at 37°C for several days.

Opened oysters provided a much more favourable environment than live oysters for growth of V. parahaemolyticus. When oysters were opened and stored in the half shell, V. parahaemolyticus counts increased to more than 10^6/g at 30°C or 37°C overnight. Growth occurred slowly at 15°C but not at 10°C.

B) SURVIVAL AND GROWTH OF V. *cholerae*

The survival and growth of V. *cholerae* was assessed in experiments performed in the same manner as those discussed above (see Attachment D). It was necessary to use oysters that had been contaminated with V. *cholerae* in the laboratory for some experiments. Oysters that were naturally contaminated with V. *cholerae* were available only infrequently. In addition, it was considered desirable to include strains of V. *cholerae* serotype O1 in the storage trials. Oysters that were naturally contaminated with serotype O1 were not available.

Live oysters were contaminated with *V. cholerae* by placing them for 4-5 hours in an aquarium containing freshly-collected estuarine water to which V. *cholerae* had been added at the rate of about 20 colony forming units/ml water. The aquarium was a flow-through aquarium, i.e. there was a continuous inflow of freshly-contaminated water throughout the contamination period. There was also a recirculation system which ensured good aeration and mixing of the water in the aquarium. The strain of V. *cholerae* used was a serotype O1 isolate obtained recently from a NSW estuary.

The behaviour of *V. cholerae* in oysters was broadly similar to that of V. *parahaemolyticus.* When unopened oysters contaminated with V. *cholerae* were stored at moderate temperatures, *V. cholerae* was capable of remaining viable but substantial growth did not occur and V. *cholerae* counts remained very low. This pattern was observed with both naturally-contaminated oysters containing non-O1 serotypes of

V. *cholerae* (Table 2) and oysters contaminated in the laboratory with serotype O1 (Table 3). Opened oysters stored in the half shell supported rapid growth of V. *cholerae* O1 at 30°C and 37°C (Table 4). The growth of *V. cholerae* O1 in opened oysters was restricted to a higher temperature range than growth of *V. parahaemolyticus.* V. *cholerae* O1 was able to survive but did not grow in opened oysters at 15°C.

TABLE 2 Behaviour of V. *cholerae* in naturally contaminated unopened oysters during storage.

Temperature (°C)	Time (Days)	V. *cholerae* (MPN/g)		
		Trial A	Trial B	Trial C
	0	+[a]	+	+
15	2	[b]		
	6	50		
30	2			
	6	4		

[a] *V. cholerae* detected by qualitative enrichment of 3x10g of oyster flesh; MPN <0.3/g.

[b] V. *cholerae* not detected.

TABLE 3 Behaviour of V. *cholerae* O1 in laboratory-contaminated unopened oysters during storage.

Temperature (°C)	Time (Days)	V. *cholerae* (MPN/g)
	0	50
15	2	2.3
	6	4
30	2	2.3
	6	4

TABLE 4 Behaviour of V. cholerae O1 in laboratory-contaminated opened oysters during storage.

Temperature (°C)	Time (Hours)	V. cholerae (MPN/g)
	0	$4x10^1$
37	20	$>2x10^4$
30	20	$>2x10^4$
15	48	$2x10^1$
	144	$4x10^0$

C) DISCUSSION

The prevention of public health hazards associated with pathogenic vibrios in oysters depends upon control of the conditions under which oysters are transported and stored. Shellstock of the Sydney rock oyster is frequently transported and stored at ambient temperatures. Our studies of the survival and growth of vibrios have indicated that this practice does not lead to growth of V. parahaemolyticus or V. cholerae to hazardous levels if the oysters are protected from excessively high temperatures. Growth of V. parahaemolyticus or V. cholerae was not observed during storage of unopened C. commercialis at 15°C. Moderate increases in numbers occurred at 30°C, but unacceptably high counts were not observed during these experiments. However, potentially pathogenic vibrios were able to grow to hazardous levels within a few days at 37°C, before the oysters would necessarily have been rejected. In warm climates, sacks of oyster shellstock left in the sun during summer may experience high temperatures for many hours. Counts of pathogenic vibrios may approach a human infective

dose if the oysters are exposed to such conditions for several days in succession.

The storage trials using oysters on the half shell demonstrated that once Sydney rock oysters are opened, and thus killed, they provide a much more suitable environment for growth by *V. cholerae* and *V. parahaemolyticus.* Both vibrios grew in oysters in the half shell under conditions of temperature abuse (30 or 37°C). *V. parahaemolyticus* also grew at 15°C. The rapid growth of the vibrios in oysters after opening is probably a result of the destruction of the natural defences of the oyster. The results show that opened Sydney rock oysters must be handled with the same precautions against temperature abuse as any other potentially hazardous flesh food that is to be consumed without cooking.

5. EXAMINATION OF MARKET SAMPLES OF OYSTERS

A market survey was conducted to assess the degree of contamination of oysters from wholesale and retail sources with V. cholerae and V. parahaemolyticus. During our previous studies, most samples had been obtained either directly from estuaries or immediately following purification. Several factors operating during the marketing of oysters could cause substantial changes in counts of vibrios. As demonstrated in our studies of survival and growth of vibrios in oysters, temperature abuse of oysters can lead to markedly increased levels of contamination with vibrios. On the other hand, the well-known cold-sensitivity of V. parahaemolyticus could lead to substantially lower V. parahaemolyticus counts in refrigerated oysters.

The examination of market samples of oysters is discussed in part in Attachment C. The samples were collected during summer and autumn of 1984. Thirty nine samples were examined quantitatively for V. cholerae, V. parahaemolyticus and E. coli. Sixteen samples of unopened purified oysters originating from 6 estuaries were collected from wholesale sources. Eighteen samples of refrigerated oysters on the half shell were obtained from 6 retailers. Five samples of bottled oysters were obtained from 5 retailers. The results are summarised in Table Q. Three samples of unopened oysters, from different sources, contained $>10^4$ V.parahaemolyticus/g and 3 samples of opened oysters, again from different sources, contained $>10^3$ V. parahaemolyticus/g.

E. coli counts of the samples that contained V. cholerae were 1, <1 and 23/g for unopened, half-shell and bottled oysters respectively.

Table Q Bacteriological status of market samples of oysters

	Wholesale unopened (16 samples)	Retail	
		Half-shell (18 samples)	Bottled (5 samples)
V. cholerae			
Samples positive	1	1	1
Count (/g)	0.7	<0.3	3
V. parahaemolyticus			
Samples positive	16	18	3
Maximum count (/g)	2.3×10^4	$>2.0\times10^3$	4.3
Median count	1.1×10^3	23	0.9
E. coli			
Samples positive	4	3	2
Range of counts (/g)	1-16	1-3	0.4-23

The results indicate that V. cholerae is present in a small proportion of samples of Sydney rock oysters at all stages of marketing. As in samples collected from other sources (i.e. directly from estuaries etc.), the counts of V. cholerae are very substantially below the counts considered necessary to cause illness in normal individuals. V. cholerae was detected in both the presence and absence of E. coli.

The analyses show that V. parahaemolyticus is almost always present in Sydney rock oysters purchased by consumers during the warmer months of the year. Since counts were above

10^4/g and 10^3/g in several samples of unopened and half-shell oysters respectively, relatively little temperature abuse may be required in some cases to create a public health hazard. The degree of contamination of refrigerated oysters indicates that the cold sensitivity of *V. parahaemolyticus* provides very little practical protection against the health hazards associated with this organism.

The results of the analyses of bottled oysters are interesting, although firm conclusions are impossible because of the small number of samples tested. *V. parahaemolyticus* counts in bottled oysters were well below the counts in other types of oyster sample collected at about the same time of year. This might indicate that the packaging of bottled oysters in fresh water and/or the longer periods for which bottled oysters are stored under refrigeration create unfavourable conditions for the survival of *V. parahaemolyticus*. *V. parahaemolyticus* is halophilic (requires salt).

The detection of *E. coli* in nearly a quarter of the samples is interesting, since all samples should have been purified.

6. CONCLUSION

This project has demonstrated that potentially pathogenic vibrios, *V. cholerae* and *V. parahaemolyticus*, are common and unavoidable contaminants of oysters harvested in NSW, especially during the warmer months of the year. *V. cholerae* and *V. parahaemolyticus* are present in oysters at all stages of production, distribution and retail sale. The isolates of *V. cholerae* obtained from oysters are occasionally of the O1 serotype. The O1 isolates that we have obtained have not produced cholera toxin and therefore would not be capable of causing typical cholera. However toxigenic strains of *V. cholerae* O1 are present in some rivers on the East coast of Australia and there is no reason to believe that toxigenic isolates cannot be found in oysters in the future.

The purification process is ineffective in reducing the degree of contamination of oysters with *V. parahaemolyticus* and has a limited ability to remove *V. cholerae*. This is not a failure of the purification process used in Australia, but is simply a consequence of the substantial differences between the ecology of the estuarine vibrios and that of the sewage-associated enteric pathogens. The vibrios must be accepted as part of the natural microflora of oysters. All raw foods carry potentially harmful microorganisms as part of their normal flora. The risk of food-borne illness associated with the presence of pathogens in raw foods can be minimised by handling and storage procedures that prevent excessive dissemination and growth of the pathogens. Because oysters are commonly eaten raw, the hazards associated with oysters may be

somewhat greater than those associated with most other raw foods of animal origin.

Like many other food-borne pathogens, the ability of these vibrios to cause illness is linked closely to the number of viable cells of the pathogen that are ingested with food. The numbers of V. cholerae that were detected in oysters during this study were low. The highest V. cholerae count observed in any oyster sample was 3/g. This is several orders of magnitude below the counts considered necessary to cause illness in normal consumers. The numbers of V. parahaemolyticus detected were considerably higher, but there was usually a margin between the numbers detected and the numbers required to cause illness. However an improved method for enumerating V. parahaemolyticus showed that, on occasions, counts are above 10^4/g when oysters are removed from the estuary, substantially higher than had been observed in previous studies. A relatively small amount of growth would be required to raise V. parahaemolyticus counts in such samples to levels considered hazardous.

Some members of the community, particularly those with certain pre-existing medical conditions, appear to have increased susceptibility to vibrio infections. The infective dose of vibrios may be lower for these individuals. Some authorities have suggested that such persons should be warned of the possible hazards of eating raw seafoods, especially during the warmer seasons.

The project has shown that temperature control is an

essential element in avoiding oyster-borne illness caused by vibrios. The ability of these vibrios to grow in unopened oysters is limited if the oysters are not stressed by exposure to high temperatures. Therefore, those involved in the handling of oysters must ensure that shellstock is protected from temperature extremes.

Once oysters are opened they must be handled in the same manner as any other potentially hazardous food that will be consumed without further cooking. Unless opened oysters are refrigerated effectively, they will support rapid growth of vibrios. Although opened oysters are frequently chilled inadequately during distribution and sale at present, effective refrigeration is more important for opened oysters than for many other foods because oysters are known to contain significant numbers of potential pathogens.

The frequent presence of *E. coli* in purified oyster samples is probably cause for concern. *E. coli* was detected in about 15-30% of the samples tested during the various phases of the project. The counts suggest that commercial purification may not be operating with optimum effectiveness. History has shown clearly that the industry and associated regulatory authorities cannot afford to become complacent about the microbiological safety of oysters.

Attached are scientific papers associated with this project that have been published so far (Attachments A,B,D,E).

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ATTACHMENT A

Evaluation of methods for enumeration and identification of *Vibrio parahaemolyticus* in oysters

M.J. EYLES, G.R. DAVEY, G. ARNOLD and H.M. WANE*

Alkaline peptone water (APW) was compared with glucose salt Teepol broth (GSTB) and arabinose ethyl violet broth (AEB) for enumeration of *Vibrio parahaemolyticus* in oysters by the most probable number (MPN) technique. APW gave greater recovery of *V. parahaemolyticus* than did GSTB or AEB. The combinations of positive tubes observed in MPN determinations performed using GSTB were often statistically unacceptable. The MPN method, with APW as the enrichment medium, gave greater recovery of *V. parahaemolyticus* than did a spread plate count on thiosulphate citrate bile-salts sucrose (TCBS) agar. VP medium was found to be useful for biochemical screening of presumptive *V. parahaemolyticus* isolates. The frequency with which other vibrios are likely to be misidentified as *V. parahaemolyticus* by the identification procedures in Australian standard methods for the microbiological examination of oysters was found to be low.

Australian Standard (AS) 1766 (SAA 1976) describes two methods for the isolation of *Vibrio parahaemolyticus* from food, a qualitative enrichment method and a quantitative most probable number (MPN) method. The most widely used of the two selective enrichment broths specified in both of these methods is glucose salt Teepol broth (GSTB). Comparative studies have indicated that GSTB is not the best medium available for the isolation of *V. parahaemolyticus* from seafoods (Beuchat 1976, Peterson 1979, Sakazaki *et al.* 1979). Furniss, Lee and Donovan (1978) recommended alkaline peptone water (APW) for the isolation of *V. parahaemolyticus* and other vibrios from aquatic environments and seafoods. Arabinose ethyl violet broth (AEB), also known as Horie broth, has also been used successfully, yielding higher MPN values than GSTB (Peterson 1979). During the present study APW was compared with GSTB and AEB for enumerating *V. parahaemolyticus* in oysters using the MPN technique. Although Furniss *et al.* (1978) suggested that APW inoculated with seafoods should be incubated overnight, the work of Dupray and Cormier (1983) and observations in the authors' laboratories indicated that a shorter period was more appropriate. Thus, a primary enrichment for 6 h followed by a secondary enrichment overnight, as is commonly used in the isolation of *V. cholerae*, was used with APW during this study. The usefulness of this enrichment procedure was evaluated.

V. parahaemolyticus is often present in uncooked seafoods at concentrations which would permit the use of a plate count rather than an MPN determination for its enumeration. A plate count has significant advantages over the MPN method which is extremely time-consuming and expensive to perform. The selective, differential solid medium used most widely for the isolation of *V. parahaemolyticus* is thiosulphate citrate bile-salts sucrose (TCBS) agar. TCBS agar is specified in AS 1766 for plating of enrichment broths. Following the comparison of selective broths, in which APW proved the superior medium, the MPN method using APW was compared with a plate count on TCBS agar for enumerating *V. parahaemolyticus* in oysters.

Two aspects of procedures for identification of *V. parahaemolyticus* were examined. First, a medium designed to provide rapid, presumptive identification of *V. parahaemolyticus*, VP medium (Kaper, Remmers & Colwell 1980), was evaluated. Secondly, the identification procedure specified in AS 1766 may mis-identify *V. harveyi* or *V. vulnificus* as *V. parahaemolyticus* (Desmarchelier 1984); accordingly the frequency with which this error is likely to occur was assessed.

Materials and methods

Oyster samples

Oysters were obtained unopened or in the half shell from commercial oyster leases or from wholesalers or retailers in New South Wales. A proportion of the samples examined for the comparison between the MPN and plate count procedures had been incubated in the laboratory at temperatures between 15° and 37°C before testing, allowing *V. parahaemolyticus* to grow to high counts. Homogenates of the oyster samples were prepared as described in AS 1766 (SAA 1976). For comparisons between media or methods, the media being compared were inoculated from a single homogenate and series of dilutions.

Comparison of selective enrichment broths

The MPN method specified in AS 1766 was followed. Briefly, tubes of GSTB (SAA 1976) or AEB (Beuchat 1977) were inoculated with 1 mL of appropriate tenfold dilutions of the samples and incubated (37°C, 18 h). Each tube was streaked onto a plate of TCBS agar (Oxoid, England) which was incubated (37°C, 18 h). Typical *V. parahaemolyticus* colonies were tested for indole and lysine decarboxylase production, reaction in triple sugar iron agar, salt tolerance (0, 80 and 110 g/L NaCl), growth at 42°C and reaction in oxidase and Voges–Proskauer tests. The only modification made to the standard method was a reduction in the number of tubes inoculated with each dilution from 5 to 3. The same procedure was followed with APW, with the following modifications. Tubes of APW containing 10 g/L NaCl (Furniss *et al.* 1978) were incubated for 6 h then 1 mL was transferred from each tube to a fresh tube of APW which was incubated overnight. Plates of TCBS agar were streaked from both primary and secondary enrichment tubes at the completion of their respective incubation periods.

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Comparison of MPN and plate count methods

The MPN method with APW as the enrichment broth was compared with a spread plate count on TCBS agar performed according to the general method of AS 1766. Briefly, 0.1 mL volumes of appropriate dilutions of the sample were spread in duplicate on TCBS agar plates. The plates were incubated at 37°C overnight. Typical colonies were counted, confirmed as *V. parahaemolyticus* as described above, and the number of colony forming units (CFU) of *V. parahaemolyticus*/g of sample was calculated.

Evaluation of identification procedures

VP medium was prepared and used according to Kaper *et al.* (1980). Typical *V. parahaemolyticus* colonies selected from TCBS agar plates were confirmed using both the AS 1766 procedure and VP medium and the results were compared.

To assess the frequency with which the AS 1766 procedure might misidentify *V. harveyi* (sucrose negative) or *V. vulnificus* as *V. parahaemolyticus*, cultures which the AS 1766 method had identified as *V. pararhaemolyticus* were tested for their ability to grow on cellobiose, ethanol and leucine as sole organic carbon sources (Furniss *et al.* 1978, Brayton *et al.* 1983).

Results

APW was the most effective of the selective enrichment media evaluated (Tables 1,2), giving significantly greater recovery of *V. parahaemolyticus* than GSTB (sign test, $p<0.001$) or AEB ($p<0.01$). The difference between APW and AEB was less marked than the difference between APW and GSTB. A further deficiency of GSTB was that the combinations of positive tubes obtained in MPN determinations were frequently so improbable as to be statistically unacceptable (De Man 1975). During the comparative trials and related studies, 26 MPN determinations were performed in which *V. parahaemolyticus* was detected using GSTB. Unacceptable combinations of positive tubes were obtained on 6 occasions.

When APW was used as the enrichment medium, use of both primary and secondary enrichments gave maximum recovery of *V. parahaemolyticus*. However, secondary enrichment was often superfluous, since most positive tubes were detectable at the primary enrichment stage. The MPN calculated from the combined result of primary and secondary enrichments was higher than that calculated from the primary enrichments alone in 20 of 51 MPN determinations in which *V. parahaemolyticus* was detected. On 12 of these occasions the secondary enrichments increased the number of positive tubes by only one. Most of the additional positive tubes had been inoculated with the higher dilutions. Secondary enrichments more often failed to detect *V. parahaemolyticus* in the lower dilutions. Statistically unacceptable combinations of positive tubes were not encountered when the combined results were used. Results derived from the primary enrichments alone fell into De Man's (1975) unacceptable category on 2 occasions.

The results of the comparison of the MPN method and the spread plate count are shown in Figure 1. Thirty four samples were tested for the purpose of this comparison. All of the samples contained >100 MPN *V. parahaemolyticus*/g. The geometric mean counts on samples which gave positive results by both methods were 1.2×10^4 MPN/g and 2.8×10^3 CFU/g. The mean of the counts obtained by the MPN method was significantly higher than that obtained by the plate count (t test, $p<0.001$). The plate count gave a higher count than the MPN determination for only three samples. There was a statistically significant linear relationship between the MPN and plate count results ($r^2=0.764$, $p<0.001$).

The usefulness of VP medium as a screening medium was evaluated with 303 cultures derived from 62 oyster samples, 12 water samples and 10 sediment samples. The results are shown in Table 3. Subsequently, VP medium was used routinely as a screening medium in testing cultures derived from oyster samples. Cultures that gave a negative result in VP medium were discarded, while positive cultures were tested by the AS 1766 procedure. Of 973 cultures that gave a positive result in VP medium, 877 (90%) were confirmed as *V. parahaemolyticus* by the AS 1766 procedure.

Table 1. Enumeration of *V. parahaemolyticus* in 21 oyster samples using APW and GSTB as enrichment media

	APW	GSTB
Number of samples positive (>0.3/g)	19	9*
Most efficient medium† (no. of samples)	19	0
Geometric mean MPN/g in samples positive by both methods	7.3	1.2
Range of counts (MPN/g)	<0.3–50	<0.3–9

Two of the 9 positive samples yielded statistically unacceptable combinations of positive tubes
†Defined as the medium yielding the highest count

Table 2. Enumeration of *V. parahaemolyticus* in 24 oyster samples using APW and AEB as enrichment media

	APW	AEB
Number of samples positive (>0.3/g)	23	18
Most efficient medium (no. of samples)	18	6
Geometric mean MPN/g in samples positive by both methods	7.0	2.4
Range of counts (MPN/g)	<0.3–>110	<0.3–110

Table 3. Comparison of VP medium with the SAA procedure for biochemical identification of *V. parahaemolyticus*

	SAA procedure	
	Positive	Negative
VP medium positive	81*	2.7
VP medium negative	0.7	16

**Proportion of cultures tested (303) that gave the indicated results (%)*

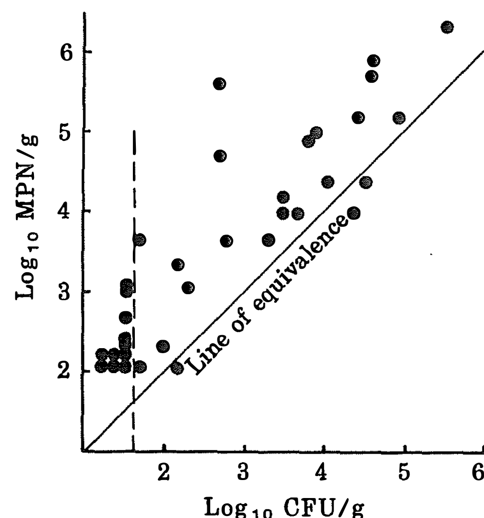

Figure 1. Comparison of the MPN method, with APW as the enrichment medium, and a plate count on TCBS agar for enumeration of V. parahaemolyticus *in oysters. The vertical dashed line indicates the limit of detection of the plate count; points to the left of this line represent samples in which the plate count failed to detect* V. parahaemolyticus

Carbon source utilisation tests were performed on 151 cultures identified as *V. parahaemolyticus* by the AS 1766 procedure. These cultures were derived from 96 oyster samples. Only three cultures gave reactions not typical of *V. parahaemolyticus*. Each of these cultures gave one atypical result, none gave results typical of *V. harveyi* or *V. vulnificus*.

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ATTACHMENT B

Detection of *Vibrio cholerae* in oysters, water and sediment from the Georges River

*G.R. DAVEY, J.K. PRENDERGAST AND M.J. EYLES**

Vibrio cholerae biotype *eltor* serotype Ogawa was detected in purified oysters which had originally been harvested from Woolooware Bay, a major oyster-producing area in the Georges River estuary, and in water in Woolooware Bay. Neither of these isolates was toxigenic. Non-01 strains of *V. cholerae* were detected in sediment and unpurified oysters collected from the same area. The average salinity in the area from which the isolates were obtained is 32.5‰. *Vibrio cholerae* has been considered to be an inhabitant of waters with a much lower salinity. *Vibrio parahaemolyticus* was also detected in the same series of samples of unpurified and purified oysters.

Vibrio cholerae may be subdivided into many serotypes on the basis of its 0 antigens, with the aetiological agent of pandemic cholera belonging to 0 group 1. Serotypes other than 01, once called non-agglutinable (NAG) vibrios or non-cholera vibrios (NCV), are generally considered to be much less significant to public health, although they can cause cholera-like diarrhoea. The pathogenicity of *V. cholerae* is not fully understood. Typical cholera is due to the production by *V. cholerae* of an enterotoxin, cholera toxin. Strains of *V. cholerae* which do not produce cholera toxin cannot cause typical cholera, but some appear able to cause a milder diarrhoea by some other mechanism. Water has long been accepted as the major vehicle for transmission of cholera. However, it was usually assumed that the cholera vibrios did not persist for long periods in natural waters and that they were present in water only as a result of relatively recent contamination by infected humans. Reports from several countries have indicated that *V. cholerae* serotypes other than 01 are widespread in aquatic environments and are probably often present in shellfish (Desmarchelier 1978, Kaper *et al.* 1979, De Paola 1981). It has also been suggested that *V. cholerae* 01 might be much more widely distributed in the environment than was believed previously, having a natural habitat in estuarine and brackish waters (Colwell *et al.* 1981).

This report describes the detection of *V. cholerae*, including 01 strains, in oysters *(Crassostrea commercialis)* from the Georges River estuary in New South Wales (NSW), Australia, and in other samples from the Georges River. The Georges River estuary is the most important of the many estuarine systems used for cultivation of *C. commercialis* in NSW. The Georges River catchment includes large areas of bushland, rural areas and expanding urban areas. The estuary, approximately 50 km long and including several large bays, lies within the southern metropolitan area of Sydney. Like many estuaries close to large cities it suffers from various types of pollution from time to time. Sources of microbial pollution and geographical features in the area from which samples were taken during the present study have been described by Qadri, Buckle & Edwards (1975).

Vibrio cholerae 01 has not been isolated from environmental samples collected in NSW nor has the presence of *V. cholerae* in shellfish from Australian waters been reported previously. The results presented were obtained during a study of the microbial ecology of oyster-growing areas and oyster purification processes in NSW. Thus, the samples were also examined for the presence of other pathogenic and indicator bacteria, including *V. parahaemolyticus*, another potentially pathogenic *Vibrio* species indigenous to estuarine environments.

Materials and methods

Sampling

Samples were collected from two points in late April - early May 1981. The first of these sampling points was a tray of oysters on a commercial oyster lease in Woolooware Bay (a major oyster-producing area approximately 10 km from the mouth of the Georges River estuary). The second was a commercial oyster purification plant in which oysters from Woolooware Bay are purified. The purification plant is of the recirculating type and employs ultraviolet light for sterilisation of the water. The following samples were collected from the oyster lease: 60 oysters divided into 5 sub-samples of 12 oysters each, 200 g of sediment, 1 L of water and 2 Moore swabs. The Moore swabs (Anon. 1976) had been suspended in the water beneath the tray for 24 h before the samples were collected. A second sample of 60 oysters, subdivided as above, was collected from the purification plant. The latter oysters had been harvested from Woolooware Bay two days after the first samples were collected from the lease and had subsequently been purified for 48 h.

The temperature, salinity, turbidity and pH of the water at the time the samples were collected from the oyster lease were measured. Turbidity was determined by the nephelometric method (Anon. 1976) using a Hach Laboratory Turbidimeter, Model 2100A. Salinity was measured by determining the density of a 100 mL sample of water and referring to density/salinity conversion tables (Wolf & Collins 1979).

Bacteriological analyses

A homogenate of each sub-sample of oysters was prepared as follows. The oysters were scrubbed thoroughly under running potable water to remove mud, barnacles and other material attached to the shell. The shell surface was flooded with 70% ethanol and excess ethanol was allowed to drain from the shell

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surface before the oysters were opened. The flesh of the oysters was removed from the shell, placed in a stainless steel blender jar with an equal weight of nutrient broth (Oxoid) and homogenised for 30 sec at setting 7 on a Sorvall Omni-Mixer. An aerobic plate count and an *Escherichia coli* count were performed on each homogenate. Three of the sub-samples from each sample were each tested for the presence of *V. cholerae*, *V. parahaemolyticus* and salmonellae.

Escherichia coli counts were performed on the water and sediment samples. The sediment sample was also tested for the presence of *V. cholerae*, *V parahaemolyticus* and salmonellae. One Moore swab was tested for the presence of *V. cholerae*, the other for the presence of salmonellae.

Aerobic plate counts, tests for the presence of salmonellae, and the enumeration of *V. parahaemolyticus* by the most probable number (MPN) method were performed according to the methods described in Australian Standard 1766 (Standards Association of Australia 1976). When testing the Moore swab for the presence of salmonellae the swab was placed in 900 mL of pre-enrichment medium. The MPN test for *V. parahaemolyticus* was modified by a reduction from five to three in the number of tubes inoculated with each dilution. *Escherichia coli* was enumerated in oyster homogenates and sediment using the rapid method of Anderson and Baird-Parker (1975) and in water using a membrane filtration method (Anon. 1969).

To detect *V. cholerae*, 50 g of oyster homogenate, 25 g of sediment or one Moore swab were placed in a glass jar containing 200, 225 or 900 mL, respectively, of pre-warmed alkaline peptone water (Oxoid). The pH was adjusted to 8.6–9.0 and the culture was incubated at 37°C. A loopful of broth was removed from the surface of the culture after 6–8 h incubation and streaked on thiosulphate citrate bile salts sucrose agar (TCBS Oxoid). After 6–8 h incubation 1 mL of this broth was also transferred to 10 mL of fresh alkaline peptone water which was incubated at 37°C for 18–24 h then streaked on TCBS. All TCBS plates were incubated at 37°C for 18–24 h. Typical *V. cholerae* colonies on TCBS were subcultured and checked for purity using CLED medium (Oxoid) then screened for Gram reaction, motility, oxidase reaction, salt tolerance (growth in peptone water containing 0, 3, 5, and 7% NaCl), lysine decarboxylase, ornithine decarboxylase and arginine dihydrolase activity, indole production, and reaction in triple sugar iron agar. The identity of cultures giving results typical of *V. cholerae* was checked using the API 20E system (Analytab Products). Cultures of *V. cholerae* were serotyped by the slide agglutination method using *V. cholerae* polyvalent, Inaba, and Ogawa agglutinating sera (Wellcome).

Isolates of *V. cholerae* 01 were sent to the Cholera Reference Laboratory at the Commonwealth Institute of Health, Sydney for confirmation and biotyping. The cultures were forwarded by the Commonwealth Institute of Health to (a) Dr N. Ryan, Enteric Pathogenicity Laboratory, Latrobe University, Bundoora, Vic. for testing for toxin production using the ligated rabbit ileal loop technique, the Y-1 mouse adrenal tumour cell assay, and an ELISA assay based on that of Back *et al.* (1979): (b) Dr J. Kaper, University of Maryland School of Medicine, Baltimore, MD, USA for examination for the presence of genes homologous to the genes encoding *E. coli* heat labile enterotoxin, a toxin which possesses a high degree of homology with cholera toxin: and (c) Dr J.V. Lee, Public Health Laboratory, Preston Hall Hospital, Maidstone, Kent, England for phage typing.

Results

Vibrio cholerae 01 serotype Ogawa was isolated from one sub-sample of purified oysters and from the Moore swab. These findings were confirmed by the Cholera Reference Laboratory. The biotype of both isolates was *eltor*. Neither of the isolates was shown subsequently to produce cholera toxin by the assays performed by the Enteric Pathogenicity Laboratory. The genetic studies performed by Dr Kaper suggested that the isolates did not possess genes enabling them to produce cholera toxin. The phage types of the isolates from the two sources were not significantly different. The phage type of the isolates reported here is different from the phage types of isolates of *V. cholerae* 01 obtained from some rivers in Queensland from time to time and of isolates obtained early in 1981 from a locally-acquired case of cholera in Lismore, NSW (P.M. Desmarchelier, personal communication).

Non-01 strains of *V. cholerae* were detected in one sub-sample of unpurified oysters and in the sediment sample. *Vibrio parahaemolyticus* was detected in three sub-samples of unpurified oysters at a level of 4.6/g, in one sub-sample of purified oysters at a level of 0.8/g, and in the sediment sample.

Aerobic plate counts and *E. coli* counts of the five sub-samples of unpurified oysters ranged from 1.8×10^3 to 4.0×10^3 and from <1 to 2/g, respectively. Aerobic plate counts and *E. coli* counts of the purified oysters were from 3.2×10^2 to 7.6×10^2 and from <1 to 2/g, respectively. *Escherichia coli* counts on water and sediment samples were 1.1/mL and 30/g, respectively. No salmonellae were detected. At the time the samples were collected the temperature, pH, salinity and turbidity of the water at the sampling station in Woolooware Bay were 19.4°C, 7.6, 32‰ and 3.7 nephelometric turbidity units, respectively.

Discussion

The original source of the *V. cholerae* strains detected during this study remains unknown. However, the results are consistent with the hypothesis (Colwell *et al.* 1981) that *V. cholerae*, including 01 strains, is a normal part of the microflora of many estuarine systems. Although the Georges River estuary is subjected to pollution with sewage from time to time, especially after prolonged heavy rainfall, faecal contamination could be considered unlikely to have been the source of the *V. cholerae* isolated. We are not aware of any isolations of *V. cholerae* from human sources in the region surrounding the Georges River in recent years, yet a mixture of strains of *V. cholerae* was isolated from a single area over a short period of time. In addition, *E. coli* counts on the oysters indicated that they had not been subjected to serious pollution in the few days before samples were collected.

Although many aspects of the ecology of *V. cholerae* are poorly understood, the available data increasingly indicate that non-toxigenic and perhaps also toxigenic strains of both 01 and non-01 serogroups of *V. cholerae* are indigenous to many waterways. Studies by Kaper *et al.* (1979) suggested strongly that *V. cholerae* was an autochthonous estuarine bacterial species resident in Chesapeake Bay, USA. None of their isolates was agglutinable in 0 group 1 antisera, but most yielded positive results in several toxigenicity tests. *Vibrio cholerae* 01 has been isolated from waterways or shellfish in the UK (Bashford *et al.* 1979) and USA (Colwell *et al.* 1981, Hood, Ness & Rodrick 1981, Twedt *et al* 1981). These isolations have demonstrated the presence of *V. cholerae* 01 in relatively unpolluted waterways in cholera-free areas. Most of these *V. cholerae* 01 isolates were non-toxigenic, as were the 01 strains isolated during the present study. Toxigenic strains of *V. cholerae* 01 have persisted in rivers in south-east Queensland for five years in the absence of evidence of contamination (Rogers *et al.* 1980, Anon. 1981).

It is interesting to note that the salinity of the water in the area in which *V. cholerae* was detected in the present study was 32‰ at the time the samples were collected from the river. The salinity in this area rarely falls below 20‰, with the average being 32.5‰ (Wolf & Collins 1979). During their studies in Chesapeake Bay, Kaper *et al.* (1979) noted a distinct relationship between salinity and the presence of *V. cholerae*, with *V. cholerae* being found only at stations where the salinity was 17‰ or less.

The isolation of *V. cholerae* and *V. parahaemolyticus* from purified oysters is of particular interest and illustrates the need for further studies of the behaviour of potentially pathogenic vibrios during the purification of oysters. Compulsory purification of oysters is presently being introduced in NSW. The purification plant from which oysters were taken during the

January). On each occasion 5 samples of 12 oysters each were taken from the estuary at about the same time that oysters were harvested commercially from the same area for purification. After purification a further 5 samples were taken from the depuration plant. This area of the estuary has an annual mean water temperature of 20°C and an annual mean salinity of 29‰. Like most urban estuaries the area is subject to pollution, especially during wet weather, when urban drainage and emergency sewage overflows influence the water quality (*11*).

During the second part of the study single samples of 12 oysters each were collected from depuration plants in various parts of NSW. The samples were collected over a 1-year period.

Microbiological analyses

The oysters were scrubbed thoroughly under running potable water, surface-disinfected in sodium hypochlorite solution, allowed to drain, then opened. Their flesh was homogenised with an equal weight of nutrient broth (Oxoid, England). The microbiological analyses made on each of the 5 samples taken from each batch of oysters during the first part of the study and the methods used are listed in Table 1. The MPN test for *V. parahaemolyticus* was modified by reducing the number of tubes inoculated with each dilution from 5 to 3. A selection of the *V. cholerae* strains isolated was sent to the Cholera Reference Laboratory, Commonwealth Institute of Health, Sydney, NSW for confirmation. The virus detection technique listed in Table 1 was modified by the deletion of MRC-5 cell cultures from the virus isolation procedure. Therefore the technique was able to detect human enteric viruses which are cytopathogenic in primary cultures of Cynomolgus monkey kidney epithelial cells. The analysis of variance was used to evaluate statistically the overall differences between microbial counts in unpurified and purified oysters.

During the second phase of the study, the following examinations were made on each individual sample: aerobic plate count, *E. coli*, *V. parahaemolyticus*, *V. cholerae*. The methods above were modified in that nutrient broth was omitted from the initial homogenate and the sensitivites of the methods for detection of *E. coli* and *V. parahaemolyticus* were increased to 0.5/g and 0.03/g, respectively.

RESULTS

Table 2 compares the overall microbiological status of unpurified and purified oyster samples examined during the first part of the study. The overall mean aerobic plate counts of batches of purified oysters (geometric mean 4.8×10^2/g) were significantly lower ($P<0.01$) than those of unpurified oysters (geometric mean 1.2×10^3/g), although the differences were usually not large. Coliforms and *E. coli* were detected considerably less frequently and at generally lower levels in purified oysters. The overall mean *E. coli* and coliform counts were significantly lower in purified oysters than in unpurified oysters ($P<0.001$). The change in mean *E. coli* counts achieved by the purification process in each batch of oysters is shown in Fig. 1. Three batches (numbers 3,6,13) contained unacceptably high concentrations of *E. coli* after purification, failing to comply with recommended standards for *E. coli* in Australian oysters. The standards state that when 5 samples are tested, no sample may have an *E. coli* count above 7/g and not more than one of the samples may contain more than 2.3/g (*10*). The operator of the plant attributed the first of these failures (batch 3) to a fault detected in the UV light units at about this time. The UV lamps had inadvertently not been replaced at the end of their operational life. The second failure (batch 6) occurred at a time when unpurified oysters contained very high concentrations of *E. coli*. Although the purified oysters contained significantly fewer *E. coli* ($P<0.05$), the levels remained unacceptably high. Each of these unacceptable batches of oysters had been harvested for purification within a few days of substantial rainfall (35-78 mm in the 4 d before harvesting). Only one of the other batches tested had been harvested within 4 d of >20 mm of rain. These three batches account for nearly all of the purified oyster samples listed in Table 2 as having contained *E. coli*.

V. parahaemolyticus was detected only slightly less frequently in purified than in unpurified oysters (Table 2). There was no statistically significant difference between the overall mean *V. parahaemolyticus* counts in purified and unpurified oysters ($P>0.1$). The change in mean *V. parahaemolyticus* count which occurred during purification of each batch of oysters is shown in Fig. 2. The first set of samples tested was not examined for *V. parahaemolyticus*. The maximum count detected in individual samples of both unpurified and purified oysters was 48 MPN/g. The purification plant was not in use during spring and early summer, when higher levels of *V. parahaemolyticus* might be expected in this estuary. The results suggest that *V. cholerae* may also persist in oysters throughout the purification process (Table 2). All the *V. cholerae* isolates were non-01 serotypes. *V. cholerae* was detected during autumn (5 samples positive), summer (1 sample) and winter (1 sample). There was no apparent relationship between the presence of

TABLE 1. *Microbiological examinations performed at each sampling.*

Examination	Oyster sample 1	2	3	4	5	Method type	Method reference[a]	Sensitivity or sample size[b]
Aerobic plate count	x	x	x	x	x	Pour plate	14	5/g
Coliforms	x	x				MPN[c]	14	0.4/g
E. coli	x	x	x	x	x	Spread plate	1	1/g
Salmonella			x	x	x	Qualitative	14	25g
V. parahaemolyticus			x	x	x	MPN	14	0.6/g
V. cholerae			x	x	x	Qualitative	3	25g
Viruses	x	x				Qualitative	4	10 oysters

[a]See the text for minor modifications to the methods listed.
[b]Expressed as theoretical maximum sensitivity/g of sample for quantitative methods and as sample size for qualitative methods.
[c]Most probable number.

TABLE 2. *Comparative microbiological status of 16 batches of unpurified and commercially purified oysters.*

Test	Batches positive (number)		Count range (MPN or cfu/g)	Percentage of samples in range[a]	
	Unpurified	Purified		Unpurified	Purified
Aerobic plate count	16[b]	16[b]	$<10^3$	49	76
			$1\text{-}9 \times 10^3$	46	18
			$1\text{-}9 \times 10^4$	5	6
Coliforms	16[c]	10[c]	ND[f]	3	56
			0.4-5	66	34
			5.1-50	22	6
			>50	9	3
E. coli	14[d]	6[d]	ND	45	81
			1-2.3[g]	21	6
			2.4-7	14	3
			7.1-50	16	5
			<50	5	5
V. parahaemolyticus	12[e]	12[e]	ND	31	47
			0.6-5	62	47
			5.1-50	7	7
V. cholerae	4	2	ND	90	94
			Present	10	6
Salmonella	1	0	ND	98	100
			Present	2	0

[a]The number of individual samples tested in each category was - aerobic plate count, *E. coli* 80; coliforms 32; *V. parahaemolyticus* 45; others 48.
[b,c,d]Overall mean counts significantly lower in purified than in unpurified oysters.
[e]Overall mean counts in unpurified and purified oysters not significantly different.
[f]Not detectable.
[g]Ranges based on Australian standards for *E. coli* in oysters (see text).

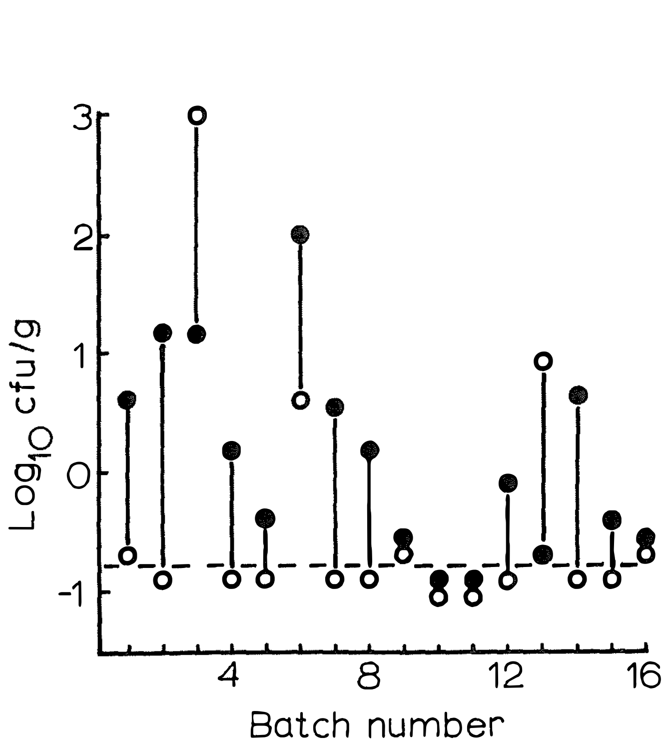

Figure 1. *Mean* Escherichia coli *concentration in unpurified and purified oysters; each point represents the mean count of five samples. Symbols:* ● *unpurified oysters,* ○ *purified oysters. The dashed line indicates the limit of detection of the test, points below this line represent batches in which* E. coli *was not detected.*

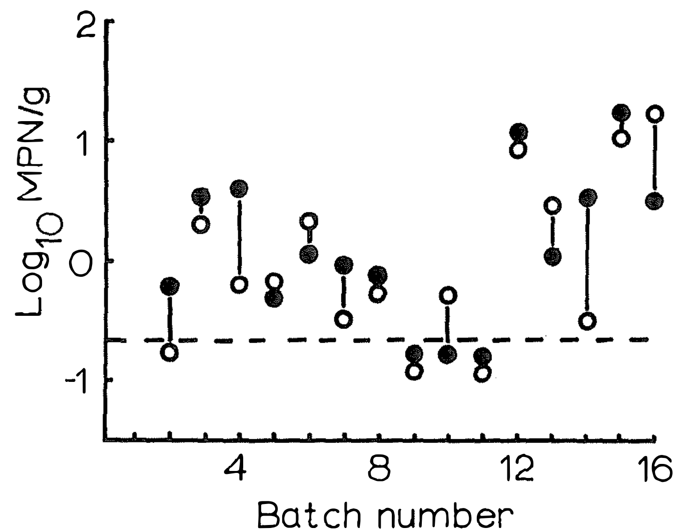

Figure 2. *Mean* Vibrio parahaemolyticus *concentration in unpurified and purified oysters; each point represents the mean count on 3 samples. Key as for Fig. 1.*

either vibrio and the presence or concentration of any of the other microbial groups.

Salmonella warragul was isolated from a sample of unpurified oysters collected during late autumn. Viruses were not isolated from any samples.

During the second phase of the study, 54 oyster samples from 25 purification plants and 5 different estuaries were tested. Twenty one (39%) of the samples contained

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ATTACHMENT D

Behavior and incidence of *Vibrio parahaemolyticus* in Sydney rock oysters (*Crassostrea commercialis*)

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Vibrio parahaemolyticus grew poorly or not at all during storage of unopened Sydney rock oysters (*Crassostrea commercialis*) at 15 and 30°C for 2 and 7 days. Although *V. parahaemolyticus* counts often increased at 30°C, counts above 10^4/g were not observed. *Escherichia coli* counts did not usually change substantially under these conditions. *V. parahaemolyticus* grew more readily during storage of unopened oysters under more severe conditions, with counts approaching or exceeding 10^6/g after continuous or intermittent storage at 37°C. Opened oysters provided a much more favourable environment than unopened oysters for growth of *V. parahaemolyticus*. Growth occurred at 15, 30 and 37°C, with counts $> 10^6$/g after overnight storage at 30 or 37°C. A survey of 30 market samples of oysters was conducted. Sixteen samples of unopened oysters were collected at the wholesale level and 14 samples of refrigerated opened oysters were purchased from retailers. *V. parahaemolyticus* was present in all samples of unopened oysters (range 0.4/g–2.3×10^4/g) and in 13 samples of opened oysters (range 4.3/g– $> 1.1 \times 10^3$/g).

Key words: Oysters; *Crassostrea commercialis*; *Vibrio parahaemolyticus*; *Escherichia coli*; Growth; Storage

Introduction

The Sydney rock oyster (*Crassostrea commercialis*) is cultivated extensively in estuaries along the East coast of Australia, particularly in the State of New South Wales (NSW). In Australia, oysters are usually eaten raw or after very mild cooking. Any pathogens present in oysters when they are marketed and which remain viable during storage are likely to reach the consumer. Because of pollution of several of the estuaries in which *C. commercialis* is cultivated, oysters produced in NSW are now purified using the depuration process to remove human pathogens (Souness et al., 1979; Son and Fleet, 1980). The food-borne pathogen *Vibrio parahaemolyticus* is part of the normal microflora of oysters and other foods harvested from estuaries, and oysters are among the many seafoods implicated as vehicles for *V. parahaemolyticus* food poisoning (Joseph et al., 1982). Unlike bacteria introduced into estuaries as a result of pollution, *V. parahaemolyticus* is not removed from filter-feeding shellfish during depuration (Eyles and Davey, 1984; Greenberg et al., 1982). The

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numbers of *V. parahaemolyticus* detected in oysters immediately after harvest or purification are considered to be usually well below the high levels (10^6/g or more) believed necessary to cause food poisoning (Blake et al., 1980).

At the wholesale level *C. commercialis* is usually transported and marketed live at ambient temperatures, as this species can remain alive out of the water for several weeks under good conditions (Korringa, 1976). At the retail level it is most commonly sold refrigerated in the half shell. Opportunities for abuse of the product occur at both levels. The conditions which permit growth of *V. parahaemolyticus* in oysters are not well defined. The purpose of this study was to examine the ability of *V. parahaemolyticus* to survive and grow during storage of *C. commercialis*, including storage under conditions of temperature abuse. Both live and opened oysters were studied, all naturally contaminated with *V. parahaemolyticus*. The behaviour of the enteric indicator organism *Escherichia coli* was also examined. Both *V. parahaemolyticus* and *E. coli* are capable of growth in foods at temperatures above 10°C (Joseph et al., 1982; Michener and Elliott, 1964; Thomson and Thacker, 1973). A survey was also conducted to assess the level of contamination of *C. commercialis* in the marketplace with *V. parahaemolyticus*. Unopened oysters collected at the wholesale level and opened oysters on the half shell obtained from retailers were examined.

Materials and Methods

Storage trials

Undamaged Sydney rock oysters of marketable size were used in all experiments. Unpurified oysters were collected from a commercial oyster farming area. Purified oysters were collected from a commercial oyster purification plant on the day purification was completed. Oysters were held at 15°C and used within 24 h of collection. Oysters used in storage trials were placed in plastic boxes to prevent excessive drying then placed in constant temperature rooms. Samples were removed after various periods for microbiological examination. For storage trials involving opened oysters, the oysters were cleaned and opened as described below, excess shell liquor was removed and he oysters were left in the cupped valve of the shell.

Survey

Samples of unopened oysters were collected from various wholesale sources and samples of refrigerated oysters in the half shell were purchased from retailers. The samples were examined quantitatively for the presence of *V. parahaemolyticus* and *E. coli* as described below. Where more than one sample was obtained from a single source the samples were collected from distinct batches of oysters, usually on different days.

Microbiological examination of oysters

Each sample consisted of the pooled flesh of 10 oysters. Unopened oysters which were damaged, gaping or otherwise abnormal were excluded. The oysters were scrubbed under running tap water to remove surface mud and marine life, immersed in 70% ethanol, allowed to dry, then opened aseptically. The flesh was transferred to a sterile blender jar and blended for 30 s. It was sometimes necessary to add 10–15 ml of peptone diluent (peptone 1 g, NaCl 30 g, water 1 l, pH 7.0) to the oysters to obtain a satisfactory homogenate. *V. parahaemolyticus* was enumerated in each sample using both the most probable number (MPN) technique and the surface plate count procedure specified in Australian Standard 1766 (Standards Association of Australia, 1976) with the following modifications. The number of MPN tubes inoculated with each dilution was reduced from 5 to 3 and the selective enrichment medium used was alkaline peptone water (peptone 10 g, NaCl 10 g, water 1 l, pH 8.6–9.0) rather than glucose salt Teepol broth. Alkaline peptone water tubes were incubated for 6 h at 37°C then 1 ml was transferred from each tube to a fresh tube of alkaline peptone water which was incubated at 37°C for 18 h. Both primary and secondary enrichment tubes were plated. The effectiveness of this procedure for enrichment of *V. parahaemolyticus* in oysters will be the subject of a separate communication. The plate count procedure proved unsatisfactory for enumeration of low numbers of *V. parahaemolyticus* in oysters ($< 10^3$/g). Therefore, unless otherwise stated, all *V. parahaemolyticus* counts are expressed as MPN/g of oyster flesh. In addition to these modifications, tests for growth on ethanol, cellobiose and leucine as sole organic carbon sources (Lee et al., 1979) were added to the biochemical identification procedures to avoid the problem of misidentification of *V. harveyi* as *V. parahaemolyticus* (Desmarchelier, 1984). *E. coli* was enumerated using the modified Anderson Baird-Parker membrane method (Holbrook et al., 1980).

Results

During the first series of storage trials both unpurified and purified oysters were stored unopened at 15 and 30°C for 2 and 7 days. *V. parahaemolyticus* did not grow

TABLE I

Behaviour of *Vibrio parahaemolyticus* in unopened oysters during storage.

Temp. (°C)	Time (days)	*V. parahaemolyticus*/g			
		Unpurified oysters [a]		Purified oysters [a]	
	0	1.1×10^2	2.2×10^1	3.5×10^1	5.7×10^1
15	2	9.0×10^1	2.0×10^2	9.0×10^0	9.0×10^0
	7	2.0×10^2	9.0×10^1	2.3×10^1	9.0×10^0
30	2	5.0×10^2	1.1×10^3	2.3×10^1	1.1×10^3
	7	1.1×10^3	4.0×10^1	2.0×10^3	2.0×10^3

[a] Duplicate experiments.