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Opinion of the

Scientific Committee on Veterinary Measures relating to Public Health

on

Vibrio vulnificus and *Vibrio parahaemolyticus* (in raw and undercooked seafood)

(adopted on 19-20 September 2001)

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1. TERMS OF REFERENCE

The Scientific Committee on Veterinary Measures relating to Public Health is requested to assess the risk to health from the presence of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in raw and undercooked seafood and to examine the appropriateness of setting standards for these pathogens.

In doing this, the Committee is invited to take into account the principles for the development of microbiological criteria for animal products and products of animal origin intended for human consumption and to develop a risk assessment where appropriate.

Considering the common field of interest, the Committee is invited to set up a joint working group including external experts and experts from both the Scientific Committee on Veterinary Measures relating to Public Health and from the Scientific Committee for Food.

2. BACKGROUND

In the context of the SPS agreement, the Commission services have been informed about ongoing discussions in the United States concerning the establishment of standards for *Vibrio vulnificus* and *Vibrio parahaemolyticus.* In the US, the Center for Science of Public Interest (CSPI) has asked the Food and Drug Administration (FDA) to establish a performance standard on "non-detectable" for the marine bacterium *Vibrio vulnificus* in raw molluscan shellfish harvested from waters that have been linked to illness from this organism.

The EU-US equivalency agreement does not include such standards.

3. INTRODUCTION

Bacteria belonging to the genus *Vibrio* are straight or curved rods giving a Gram-negative reaction. They are capable of both fermentative and respiratory metabolism and are motile by polar flagella. Vibrios do not form endospores or microcysts. Members of this group are widespread in aquatic habitats of various salinities (Baumann *et al*., 1994). They are very common in marine and estuarine environments, and on the surfaces of marine plants and animals (Baumann *et al*., 1984). They also occur naturally in the intestinal content of marine animals (Baumann *et al*., 1984; Bergh *et al*., 1994; Sakata, 1990). Some species are also found in freshwater (Baumann *et al*., 1984; West, 1989).

Several species that are pathogenic to man or marine vertebrates and invertebrates have been identified. More than twenty *Vibrio*-species have been described as causing disease in animals, while twelve species have so far been reported to be pathogenic to humans (Oliver and Kaper, 1997). Eight of these may be associated with foodborne infections of the gastrointestinal tract (Oliver and Kaper, 1997; West, 1989). Table 1 provides an overview of the pathogenic *Vibrio* species associated with human infections, and gives references to known or suspected sites of infection.

Table 1. Overview of pathogenic *Vibrio*-species associated with human infections.

	***Vibrio* -species**	**Site of infection**						
		GI tract	Wound	Ear	Primary septicaemia	Bacteremia	Lung	Meninges
1	*V. cholerae* O1/O139	++	(+)	?	?	?	?	?
	V. cholerae non O1/ non O139	++	+	+	(+)	(+)	?	(+)
2	*V. parahaemolyticus*	++	+	(+)	?	(+)	(+)	(+)
3	*V. vulnificus*	+	++	?	++	+	(+)	(+)
4	*V. fluvialis*	++	?	?	?	?	?	?
5	*V. alginolyticus*	?	++	+	?	(+)	?	?
6	*V. damsela*	?	++	?	?	?	?	?
7	*V. furnissii*	(+)	?	?	?	?	?	?
8	*V. hollisae*	++	?	?	(+)	?	?	?
9	*V. mimicus*	++	+	+	?	?	?	?
10	*V. metschnikovii*	(+)	?	?	(+)	?	?	?
11	*V. cincinnatiensis*	?	?	?	?	(+)	?	(+)
12	*V. carchariae*	?	++	?	?	?	?	?

GI tract: gastro intestinal tract, Primary septicaemia: septicaemia with no apparent infectious focus, ++ : most common site of infection, + : other sites of infection, (+) : rare sites of infection, ?: infection site remains to be firmly established. (Table redrawn from West, 1989, and updated with information from Oliver and Kaper, 1997)

This overview might give the false impression that all these twelve species are equally important as human pathogens. In fact, three *Vibrio* species represent a serious and growing public health hazard: *V. cholerae* (toxigenic strains belonging to serogroups O1 or O139 causing cholera), *V. parahaemolyticus* and *V. vulnificus*. Human infections with the remaining species are less common and usually less severe, although deaths have been reported (Oliver and Kaper, 1997). Four *Vibrio* species, *V. cholerae* (strains belonging to serogroups other than O1 or O139), *V. fluvialis, V. hollisae* and *V. mimicus* have been associated with a significant number of infections arising from contaminated seafood elsewhere in the world. Most of these infections presents as gastroenteritis (Table 1). They should be considered if further risk assessments are undertaken on pathogenic vibrios in the future.

In contrast to most other foodborne pathogens, bacteria belonging to the genus *Vibrio* have the aquatic habitat as their natural niche. The growth of all *Vibrio* species is stimulated by concentrations of Na^+ greater than those of unsupplemented

laboratory media indicating the marine origin of these bacteria (Varnam and Evans, 1991). Another characteristic of these bacteria, showing their adaption to aquatic conditions, is their close interaction with protozoa.

The prevalence and density of human pathogenic vibrios in the environment and also in seafood products are shown to be highly dependent on the ambient temperature with the largest numbers occurring at high sea water temperatures (Baffone *et al*., 2000; Høi *et al.*, 1998; Oliver and Kaper, 1997; O'Neill *et al.*, 1992; West, 1989). This may at least in part be explained by the ability of these bacteria to respond to adverse environmental conditions by entering a viable, but non-culturable phase (Linder and Oliver, 1989; Oliver and Kaper, 1997; Varnam and Evans, 1991). In such a state cells are viable, but it is not possible to obtain growth on the media routinely employed for their isolation.

4. VIBRIO VULNIFICUS

4.1. Hazard identification

V. vulnificus infections seems to be rare in Europe but little data exist on their true incidence. However, *V. vulnificus* infections are usually severe and they will be diagnosed even without having this organism specifically in mind because the organism will grow on most ordinary media. Thus, if these infections were more common in Europe than they seem to be, this higher incidence would probably be reflected by more reports in the literature.

In the USA, most cases of infections with *V. vulnificus* are associated with the consumption of raw oysters. The major form of the disease is primary septicaemia, i.e., septicaemia with no apparent infectious focus (Levine and Griffin, 1993; Oliver and Kaper, 1997). Other presentations are wound infections or gastrointestinal infection. Gastrointestinal infection is very rare, only accounting for 3 % of the reported cases involving *V. vulnificus* in the USA (Evans *et al*., 1999). There are no reports of any gastrointestinal infections with *V. vulnificus* as the etiological agent from Europe.

The majority of publications on infections by *V. vulnificus* involves sporadic cases. Thus, this bacterium is usually not involved in typical foodborne disease outbreaks. There are so far no reported cases of more than one person developing infection with *V. vulnificus* after consumption of raw oysters from the same batch (Oliver and Kaper, 1997). However, Bisharat *et al.* (1999) report on the development of bactaeremia and wound infection in over 60 patients after contact with pond cultured fish. This is, according to the authors, the first documented case of an outbreak of invasive *V. vulnificus* infection from a single common source.

The number of reported cases of foodborne infections involving *V. vulnificus* in the USA reaches 50 on an annual basis (Linkous and Oliver, 1999). These figures only involve cases serious enough to require hospitalisation. According to Linkous and Oliver (1999), the number of unreported cases in the USA might on an annual basis be as high as 41000, indicating that most infections with *V. vulnificus* might be self-limiting.

As a result of low water temperatures, the number of reported clinical cases in the Nordic countries is very low. In Norway, wound infections caused by *V. vulnificus* have so far been reported in some sporadic cases (Digranes and Lassen, personal communication), as is the situation in Sweden (Melhus *et al*., 1995). During some unusual hot summers with high water temperatures for several weeks, some cases have been reported from Denmark (Andersen, 1991; Høi *et al.*, 1998).

4.2. Hazard characterisation

4.2.1. Characteristics of human disease

Infections with *V. vulnificus* as the etiological agent was for the first time described in 1970 when it occurred in a previously healthy man who developed a leg infection and diarrhoea after bathing and collecting shellfish in sea water (West, 1989). At that time the infection was thought to have been caused by *V. parahaemolyticus*. However, later work indicates that *V. vulnificus* was the responsible agent in this case.

Infections caused by *V. vulnificus* show different clinical presentations, of which primary septicaemia, wound infection, and gastroenteritis are the most important. In rare cases, *V. vulnificus* has been reported to cause infections in the meninges and lungs (West, 1989).

(a) Primary septicaemia

The major form of infection with *V. vulnificus* involves a rapidly progressing septicaemia with few gastrointestinal signs. Such infections have a reported incubation period of from seven hours to several days (Levine and Griffin, 1993). The most frequent symptoms are fever, chills, nausea and cardiovascular hypotension (Oliver and Kaper, 1997). This disease has a high fatality rate of up to 60% in reported cases, and is in the majority of cases affecting predisposed persons (Levine and Griffin, 1993; Tacket *et al.*, 1984; West, 1989). Several authors claim that primary septicaemia is predominantly associated with the consumption of raw bivalve shellfish (Chin *et al.,* 1987; Johnston *et al.*, 1985; Morris, 1988; West, 1989).

(b) Wound infections

Another form of infection with *V. vulnificus* is cutaneous infections. These are not foodborne in the strict sense as they usually occur in connection with puncture wounds after handling of raw seafood or trauma and exposure to saline environments (Blake *et al.*, 1980; Johnston *et al.*, 1985; Veenstra *et al.* 1993; West, 1989). Wound infections range from mild self-limiting lesions to rapidly progressing erythema, cellulitis and necrosis, occasionally developing to secondary septicaemia (Morris, 1995). This form is reported to affect both healthy and predisposed persons (Levine and Griffin, 1993; West, 1989). Wound infections have a reported mean incubation period of 12 hours (Blake *et al.*, 1980). Severe wound infections with this bacterium often require extensive surgery of affected tissue or even amputations, and have reported fatality rates ranging from 7 to 25 % (Levine and Griffin, 1993; Oliver, 1981; Oliver and Kaper, 1997).

(c) Gastroenteritis

Infections in the gastrointestinal tract presenting as diarrhoea seem to be relatively infrequent (Oliver and Kaper, 1997; West, 1989). Gastrointestinal infections with *V. vulnificus* were first described by Jonston *et al.*, (1986) who reported abdominal spasms and diarrhoea in three males after consuming raw oysters. The reported case/fatality rate is low if the infection is limited to the gastrointestinal tract (West, 1989). The pathogenic determinants involved in the enterotoxicity of this bacterium have not yet been characterised (Stelma *et al,* 1988).

Bean *et al.* (1998) reported on the transmission of *Vibrio* infections mediated by crayfish from two coastal areas in Louisiana and Texas, USA. In contrast to the positive correlation between infections by *V. vulnificus* and the consumption of raw bivalve shellfish, especially oyster, consumption of cooked crayfish was reported by patients infected with *V. parahaemolyticus*, *V. alginolyticus* or *V. mimicus*, but not by those infected with *V. vulnificus*. This might either indicate that the ecological niche of these bacteria are different, or that consumers at risk for *V. vulnificus* infection are more likely to eat raw oyster than cooked seafood.

(d) Route of infection

As mentioned earlier, primary septicaemia seems most often to be a result of ingestion of bivalve shellfish. The recent focus on *V. vulnificus* in Denmark stems from 11 clinical cases of *V. vulnificus* infections which occurred in the unusually warm summer of 1994 (Dalsgaard *et al.*, 1996). The route of infection in 10 of 11 cases was a pre-existing skin lesion. All patients had a history of exposure to seawater or handling of fish prior to infection and none had consumed seafood (Dalsgaard *et al.*, 1996). In France, among 5 cases of *V. vulnificus* infections which occurred between 1995 and 1998, direct contact with the sea was established for 4 cases (Geneste *et al.*, 2000). In a recent review on the epidemiology and pathogenesis of *Vibrio vulnificus*, it was mentioned that it is difficult to definitely state that *V. vulnificus* can cause seafood-related gastroenteritis (Strom and Paranjpye, 2000).

Since the consumption of seafood usually involves handling the products and often involves activities giving direct contact with estuarine water or sea water, the actual route of infection, whether ingestion or skin contact, is difficult to identify.

As has been shown for the occurrence of *V. vulnificus* in the environment, a marked seasonality correlated with high water temperature has also been reported for the incidence of human infections caused by this bacterium (Evans *et al.*,1999; Geneste *et al.*, 2000).

4.2.2. Pathogenicity

Three distinct biotypes of *V. vulnificus* have so far been described (Bisharat *et al.*, 1999; Oliver and Kaper, 1997).

Biotype 1 was originally described as "lactose-positive" vibrios. In recent studies, approximately 85% of clinical specimens associated with disease in humans have been reported to be lactose positives (Oliver and Kaper, 1997).

Strains belonging to biotype 2 are a major source of diseases in eels. This biotype has been reported sporadically as an opportunistic pathogen in human infections (Høi *et al.*, 1998; Veenstra *et al.*, 1992), but is not generally regarded as a human pathogen (Linkous and Oliver, 1999; Oliver and Kaper, 1997).

In a recent study Bisharat *et al.* (1999) described a third biotype of *V. vulnificus*. This biotype was associated with seafood mediated bacteraemia and wound infections in a total of 62 patients, which had been in contact with pond cultivated fish in Israel.

Oliver *et al.* (1983) employed DNA-DNA hybridisation techniques to compare environmental and clinical isolates of *V. vulnificus*. The environmental isolates, totalling 33 strains, were found to be practically identical to clinical specimens isolated from diseased persons. More than 80% of the environmental strains of *V. vulnificus* isolated from sea water in this study were found to be pathogenic to mice, with death resulting after intraperitoneal injection of between $5x10^7$ and $5x10^8$ cells. In contrast to the previous study, Jackson *et al.* (1997) found, applying pulsed-field gel electrophoresis, that only one strain of *V. vulnificus* could be isolated from each diseased patient, indicating a strong host-pathogen specificity.

Several factors are reported as affecting the pathogenicity of *V. vulnificus.* The capsule of the bacterium is essential to its ability to initiate infections, giving protection against host phagocytosis. Thus strains without a capsule seem to be non-pathogenic (Linkous and Oliver, 1999; Oliver and Kaper, 1997). Furthermore, a high concentration of host serum iron is essential for the multiplication of the bacterium, which only shows growth at elevated iron concentrations (Wright *et al.* 1981).

It has also been demonstrated that the lipopolysaccharide LPS layer of *V. vulnificus* contains several endotoxins involved in pathogenesis, typically inducing fever, tissue oedema, and haemorrhage. In addition, a wide range of extracellular cytolytic and cytotoxic components produced by *V. vulnificus* are described, including haemolysin, proteases, elastases, collagenases, and mucinases (Kreger and Lockwood, 1981; Linkous and Oliver, 1999; Oliver and Kaper, 1997; Wright and Morris, 1991). However, the precise role of all these factors in the pathogenesis of infection remains to be elucidated.

4.2.3. Infectious dose

There is a lack of detailed information regarding the infectious dose of *V. vulnificus* in humans. Some animal experiments have been done on mice, rats, hamsters, rabbits or guinea pigs, but the relevance of these experiments to human infections has yet to be established (Oliver, 1981). Jackson *et al.* (1997) have reported on the number of *V. vulnificus* cells found in shellfish associated with human infections. In their study, cell numbers above 10^3 were found to result in infection.

Taking into account the frequent isolation of *V. vulnificus* in seafoods from warm waters, especially in oysters, the incidence of infections is much lower than might have been expected (Morris, 1995). This suggests that the infectious dose is rather high, or that not all strains of *V. vulnificus* detected in the environment or seafoods are equally pathogenic (Linkous and Oliver, 1999).

4.3. Exposure assessment

It is important to note that different methods to detect *V. vulnificus* have different sensitivities. Thus, prevalence rates reported in different studies applying different detection methods may not be comparable.

4.3.1. Prevalence in the environment

While the isolation of *V. vulnificus* from blood samples in clinical situations is reported to be straightforward, isolation of the bacterium from the aquatic environment is more problematic (Oliver and Kaper, 1997). However, several studies describe the presence of *V. vulnificus* in environmental samples.

V. vulnificus is widely distributed in the estuarine and marine environment. The bacterium has been isolated from sea water, sediments, sand, plankton, fish and shellfish from tropical and temperate parts of the world (De Paola *et al.*, 1994; Ghinsberg *et al*., 1999; Oliver and Kaper, 1997; Veenstra *et al.* 1994). De Paola *et al.*, 1994 have reported on the distribution of *V. vulnificus* in the intestinal content of fish from the U.S. Gulf of Mexico. They found the bacterium in the intestines of bottom-feeding fish in concentrations of 10^6 cells/g, in particular in fish that feed on molluscs and crustaceans. In this study, *V. vulnificus* was found infrequently in offshore fish. Morris (1988) reports on the prevalence of *V. vulnificus* in oysters and crabs from the Atlantic coast of the United States during the summer months. Over 50% of the oysters and 11 % of the crabs examined were found to harbour the bacterium. In a study conducted by Arias *et al.* (1999) on the prevalence of vibrios in sea water and shellfish in the Spanish part of the Mediterranean sea, a comparatively low number of bacteria belonging to the genus *Vibrio*, especially *V. vulnificus*, were observed. According to the authors, the high salinity found in this area may explain the low numbers found. In a study conducted by Barbieri *et al.* (1999), the occurrence of vibrios from estuarine waters along the Italian Adriatic coast was examined. Of a total of 103 *Vibrio* strains, five were found to be *V. vulnificus*, showing a low relative prevalence.

In a large survey conducted by Oliver *et al.* (1983), the presence of *V. vulnificus* at 80 sites along the East coast of the United States was studied, and nearly 4000 strains of sucrose negative vibrios were described. Of these strains, 4 % were able to ferment lactose. Of the lactose positive strains, 35 % were identified as *V. vulnificus*. In this study, sea water was reported to have contained on average 7 colony forming units of *V. vulnificus* per ml, whereas the average number of bacteria found in oysters was reported to be $6x10^4$ CFU/g. Other publications indicates that the number of bacteria belonging to this species found in oyster outnumber those found in the

surrounding sea water by a factor of at least 100 (Morris, 1995). Tamplin *et al.* (1982) found counts of 0.3-7000 CFU/100 ml of seawater in Florida.

Correlation between season of the year and water temperature is very notable in *V. vulnificus*. Nevertheless, the bacterium has been isolated from waters at temperatures as low as 8ºC. Levels of *V. vulnificus* in surface waters and shellfish are markedly higher during months with high water temperatures (Ghinsberg *et al*., 1999; Høi *et al.*, 1998; Oliver and Kaper, 1997; West, 1989). Tilton and Ryan (1987) demonstrated a direct correlation between water temperature and the rate of isolation from water and seafood. According to Linder and Oliver (1989), cells of *V. vulnificus* exhibit a stress response to low ambient temperatures resulting in a reduction in size and a change in shape combined with alterations in the composition of membrane fatty acids. These dormant cells have reduced virulence as shown by their inability to infect mice. This response is thought to be part of a strategy for surviving cold periods.

These observations may at least in part explain why a minimum water temperature of 20ºC seems to be required if human infections with *V. vulnificus* are to occur (Høi *et al.*, 1998 and Dalsgaard *et al.*, 1996).

4.3.2. Prevalence in food

The prevalence of vibrios in seafood from markets in Hong Kong during the summer was investigated by Chan *et al.* (1989), who reported a 6% prevalence of *V. vulnificus* in raw oysters. Mussels had a prevalence of 2%, clams 4%, prawns 4% and crabs showed 3% positives when examined for this bacterium. A much lower prevalence of *V. vulnificus* was reported by Little e*t al.* (1997). In this study almost 2500 samples of cooked, ready-to-eat, out-of-shell mollusc products from UK were examined. *Vibrio* spp. was present in excess of 10^3 CFU/g in 2% of the samples, and *V. vulnificus* were found in 0.4 % of the samples. Oliver *et al.* (1983) reported the average number of bacteria found in oysters to be $6x10^4$ CFU/g in samples taken from the East Coast of the USA.

Berry *et al.* (1994) conducted a survey of the microbial quality of raw shrimp imported into the United States from China, Ecuador, and Mexico. The shrimps had been frozen during transportation. In this study, *V. vulnificus* could be detected in 17 % of the products under study. Over 50 % of the vibrios examined in this study also carried genes coding for resistance against two antibacterial agents, while 30 % were resistant to three or more agents.

During 1999 and 2000, the Norwegian School of Veterinary Science examined 152 *Vibrio* isolates submitted by local Municipal Food Control Authorities for verification. Among these strains 37% were found to be non-O1-non-O139 *V. cholerae,* 33% *V. alginolyticus,* 24% *V. parahaemolyticus,* 3% *V. vulnificus*, 2% *V. mimicus*, and 1% *V. harveyi.* The strains were mainly isolated from sea foods imported to Norway (B. Underdal, personal communication).

Under the Fishery Products Directive (91/493/EEC)[1], crustaceans and bivalve molluscs that are cooked may be cooled in either drinking water or clean seawater, defined as being free from microbiological contamination. The practice of using seawater for such cooling is widespread in the EU, especially where cooking is undertaken on boats or in shore side facilities. Where the quality of such seawater is presently checked, this is usually on the basis of faecal indicator bacteria such as faecal coliforms and/or *E. coli*.

In the Rapid Alert System *V. vulnificus* has since 1998 been notified in all five times in seafood imported to Europe from China, India, Indonesia, Thailand, and Vietnam.

4.3.3. Survival and growth in the environment

(a) Salinity

Survival of *V. vulnificus* has been studied by using strains inoculated into sterilised seawater, the salinity of which had been adjusted with deionised water (Kaspar and Tamplin, 1993). At 14°C there was a tendency for an increase in numbers over a 6 day incubation period at salinities of 5-25 ppt, while at higher salinities (30-38 ppt) there was a reduction over this period. At 21°C, the numbers of *V. vulnificus* had dropped by 1-2 logs by 6 days at all salinities, although those between 5 and 15 ppt contained the greatest numbers. *V. vulnificus* could not be recovered from deionised water after 2 days incubation. There is therefore an interaction of salinity and temperature on the survival of *V. vulnificus*.

The max. tolerance for NaCl is 5- 6% for *V. vulnificus* (FDA, 1998b; Mortimore and Wallace, 1994).

(b) pH

The minimum and maximum pH values for the growth of *V. vulnificus* have been reported to be 5 and 10, respectively (FDA, 1998b). In a survey of cooked, ready-to-eat shellfish undertaken in the UK, no samples with a pH <5 (shellfish pickled in vinegar post-processing) showed unsatisfactory levels of vibrios, including *V. vulnificus* (Little *et al*, 1997).

(c) Nutrients and suspended solids

A study regarding the occurrence of pathogenic vibrios in a US estuary recorded a correlation between salinity, dissolved nutrients, suspended solids, and chlorophyll A with high numbers of *V. vulnificus* and *V. parahaemolyticus* (Jones and Summer-Brason, 1998). The effect of environmental variables on vibrio concentrations was investigated using multiple regression analysis. When the *V. vulnificus* concentrations were determined using a culture technique, the most significant variable was the suspended solids concentration. Temperature and salinity effects were also significant, but to a lesser extent. When a gene probe detection method were

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used instead, the most significant variable was dissolved organic carbon. In this case the concentration of suspended solids was not significant. The method of detection/enumeration may therefore have a marked effect on the outcome of such studies.

(d) Plankton

V. vulnificus has been isolated from small zooplankton (up to 200μm) in nutrient rich waters in Italy, with a significant correlation being noted between plankton abundance and bacterial density (Montanari *et al*, 1999). The effects of nutrients found in other studies (noted above) could therefore be due to the increase in plankton numbers rather than a direct effect on *V. vulnificus*.

4.3.4. Survival and growth in food

The post-harvest multiplication of *V. vulnificus* has been examined by Cook (1994; 1997) who found *V. vulnificus* capable of multiplying in oysters at temperatures above 13ºC underlining the importance of short post-harvest storage times and low storage temperatures.

Oliver (1981) reported on the survival of *V. vulnificus* in experimentally contaminated raw oysters stored on ice. He found a rapid reduction in the original numbers indicating either a rapid die-off or transformation to a viable, but non-culturable state as has been seen in cells transferred to cold water.

Several experiments in which pure cultures of the bacterium itself or contaminated oysters were kept at various temperatures have been reported (Cook and Ruple, 1992; Cook, 1997; Parker *et al.*, 1994). Even though freezing pure cultures of *V. vulnificus* results in a reduction of the number of culturable cells, the organism can still be detected in oysters kept at – 20ºC for 12 weeks. Thus, freezing alone does not seem to eliminate *V. vulnificus* from seafood products.

An adaptive response to low temperatures of *V. vulnificus* found in oysters has been described by Bryan *et al.* (1999). When contaminated oysters were transferred from a temperature of 35°C directly to a temperature of 6°C, the present *V. vulnificus* cells entered a viable but not culturable state. In contrast to this, cells adapted to a temperature of 15°C prior to 6°C, remained viable and culturable. According to the authors, this cold adaptive response is due to alterations in the cell proteins, and may give implications for oyster handling procedures.

4.3.5. *Estimation of growth, predictive models*

No predictive models on survival and growth of *V. vulnificus* have been published.

4.3.6. Correlation between indicator organisms and V. vulnificus

In a study performed by Oliver *et al.* (1983) on the distribution of *V. vulnificus* and other lactose-fermenting vibrios in the marine environment,

no correlation between the number of faecal coliforms and the prevalence of *V. vulnificus* could be observed. This finding has been confirmed in subsequent examinations by other authors (Oliver and Kaper, 1997). Given the lack of correlation, the use of faecal indicator organisms to assess health risks from *V. vulnificus* in seafoods has no value.

4.3.7. Correlation between total Vibrio *counts and* V. vulnificus

The reviewed literature gives no information on possible correlation between total *Vibrio* counts and *V. vulnificus*. Such an association is not to be expected because non-pathogenic *Vibrio* species is always present in warm sea-water.

4.4. Risk characterisation

Very few data exist on the incidence of *V. vulnificus* infections in the EU. They are not notifiable.

According to Linkous and Oliver (1999) infections with *V. vulnificus* most frequently occur among males above 50 years. Among the reviewed cases, more than 80% of the diseased persons belonged to this group.

Foodborne *V. vulnificus* disease is clearly associated with underlying medical conditions, such as chronic cirrhosis, hepatitis, thalassaemia major and haemocromatosis, and often involves individuals with a history of alcohol abuse. Less frequently, cases have occurred in people underlying malignancies or who have undergone gastrectomy (ICMSF, 1996).

The septicaemic form of *V. vulnificus* infection does occur almost exclusively among patients with underlying medical conditions, such as liver or renal failure, diabetes, immunosuppressing therapy or disorders involving the metabolism of iron (Dowdy *et al.*, 1999; Morris, 1988; Oliver and Kaper, 1997; West, 1989). Immunocompromising treatment to animals has been shown to strongly reduce the infectious dose of *V. vulnificus*. Mice with iron overload have an LD_{50} of a single cell of *V. vulnificus* compared to 10^6 cells for normal animals (Wright *et al.*, 1981). In contrast to the septicaemic form, wound infections are reported to also occur among previously healthy persons (Levine and Griffin, 1993; West, 1989).

According to Oliver and Kaper (1997), *V. vulnificus* is, together with *V. parahaemolyticus,* the etiological agents responsible for most infections involving vibrios in the United States. Infections with V. *vulnificus* alone are responsible for approximately 90% of all seafood-related deaths in this country (Evans *et al.*, 1999).

In a study conducted by Geneste *et al.* (2000) on the occurrence of non-cholera *Vibrio* infections in France during the years 1995 to 1998, five cases involving *V. vulnificus* were reported. In two of them, the main disease form were primary septicaemia, in two other cases, the main disease form were wound infection leading to secondary septicaemia while the last one case involved only wound infection. In this study *V. vulnificus* was not reported as being the etiological agent in diseases of the gastrointestinal tract.

Quantifying the risk of *V. vulnificus* infection from the consumption of seafood requires estimates of:

a) Consumption patterns of seafoods. This vary with cultural and regional preferences within the EU.

b) The prevalence and concentrations present in the implicated seafood. This vary with the source of seafood and any subsequent treatment. Imported seafood should also be considered in this respect.

c) The infectious dose of the organism. This may vary with strain.

d) The proportion of the human population susceptible to infection. This is especially relevant for V. *vulnificus* septicaemia.

Scarcity of information on seafood consumption in the EU, the widespread use of presence/absence tests with no quantitative estimates of the occurrence of the organisms in seafoods, together with the absence of a consensus with regard to infectious dose, makes it impossible to determine a numerical estimate of risk. Further research will be necessary to provide the data on which such an estimate can be based.

4.5. Methods of analysis

Two standard operating procedures have been described for the detection of *V. vulnificus*:

- FDA Bacteriological analytical manual. 8th Edition, 1995. Chapter 9. (Elliot et al.) *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and other *Vibrio* spp. (on-line, 2001: http://vm.cfsan.fda.gov/~ebam/bam-9.html).

- Nordic Committee on food analysis (NMKL) 1997 n°156 (UDC 576.851). " Pathogenic *Vibrio* species. Detection and enumeration in foods ".

Traditional phenotypic identification of *V. vulnificus* is not always straightforward. Arias *et al.* (1998) reported that confirmation of presumptive colonies could be done by PCR.

4.6. Risk management options

4.6.1. Presentation of seafood to consumers

Seafoods may be presented to retail customers or final consumers in a number of commodities:

Raw – not prepared (may or may not be chilled)

Raw – prepared (may or may not be chilled)

Raw – frozen

Raw – preserved (e.g., salted or in vinegar or brine)

Smoked – low temperature

Smoked – high temperature

Cooked – not prepared (may or may not be chilled)

Cooked - prepared (may or may not be chilled)

Cooked – frozen

Cooked – preserved

Approved heat treatment (e.g. approved methods for class C shellfish)

Canned – sterilised

Unprepared seafood will have the outer shells/skin etc. in place as well as the internal organs. In the raw, and possibly lightly cooked states, these are likely to be contaminated with the autochthonous flora and therefore with vibrios, and may present a hazard.

Removed crab and oyster/clam meats may be subsequently presented on open shells in retail outlets and restaurants; the meats may not necessarily come from the same batch as the shells and thus may not have been exposed to the same contamination sources.

4.6.2. Prevention

(1) At the harvesting step

Harvesting areas identified as at risk (see the reference below to the US transport controls) could be subject to closure during periods when the seawater temperature reaches a level that correlate with high numbers of *V. vulnificus*. However, a definite relationship between the water temperature in the harvesting area and seafood-associated illness has not been established anywhere in Europe.

(2) At the transport and storage steps

V. vulnificus is known to proliferate in temperature-abused harvested shellfish (Cook and Ruple, 1989; Cook, 1997). In the United States, control measures for *V. vulnificus* have been applied under the National Shellfish Sanitation Programme (USFDA, 1997). Additional temperature controls are imposed on the transport of shellfish harvested from state waters that have been confirmed as an original source of product associated with 2 or more cases of illness due to *V. vulnificus*. The length of time allowed from harvest to refrigeration varies with the temperature of the water in the harvesting area; e.g., when this exceeds 28.9°C the time limit is 10 hours. For areas that have not had such an association with *V. vulnificus* infection, standard controls apply, with time to refrigeration varying between 20 and 48 hours depending on the season or air-temperature.

The application of such controls require the presence of a good system of identification of the source of each harvested batch and the application and recording of temperature controls at subsequent stages up to final sale to the

consumer. However, refrigeration of harvested shellfish, especially prior to processing and packaging, is not regularly practised in Europe.

4.6.3. Decontamination

(1) Depuration technologies

Microbial depuration is a dynamic process whereby shellfish are allowed to purge themselves of contaminants either in a natural setting (relay) or in land based facilities (depuration ponds or tanks) (Richards, 1988; Council Directive 91/492/EEC[2]). According to Wood (1966), shellfish species commonly depurated in Europe were mussels (*Mytilus edulis*), European flat oysters (*Ostrea edulis*), and the Portuguese oyster (*Crassostrea angulata*). Other species depurated in France, Spain and Portugal included the palourde or carpet-shell (*Venerupis pullastra*), praire (*Venus verrucosa*), clovisse (*Venerupis geographicus*, *V. aurea*), and the avignon (*Scombricularia plana*). Richards (1988) reported that the most depurated species in the USA are clams (*Mya arenaria* and *Mercenaria mercenaria*).

Kelly and Dinuzzo (1985) have carried out a depuration study to determine the ability of Eastern oysters (*Crassostrea virginica*) to both bioaccumulate and eliminate *V. vulnificus*. According to these authors, this microorganism depurated slowly from oysters with complete elimination after 16 days. These findings may suggest that *V. vulnificus* may be present in oysters as a consequence of uptake and accumulation during normal filtration processes rather than by multiplication within the oyster (Richards, 1988). However, Barrow and Miller (1969) reported that vibrios persisted throughout commercial depuration of oysters and speculated that replication of vibrios greatly exceeded their rate of UV light inactivation. On the other hand, Steslow *et al.* (1987) concluded that oysters artificially infected with *V. vulnificus* depurated more rapidly than environmentally contaminated oysters.

a) Relay:

Relaying of oysters into waters of higher salinity than those of harvest has been shown to decrease the number of *V. vulnificus* present in the oyster: in one study the salinities were 25 ppt (Jones, 1994) and in the other averaged more than 32 ppt (Motes and DePaola, 1996). Significant decreases occurred after 7 days although some batches required 1 month or longer to reduce to <10 cells per g.

b) Depuration in tanks

It is often assumed that depurated shellfish are bacteriologically safe. However, indigenous marine bacteria (*Vibrio*) have been associated with outbreaks of gastroenteritis from the consumption of depurated oysters (Barrow and Miller, 1969). Several authors have reported that *Vibrio* does

[2] O.J. N° L 268 of 24/09/1991, pg. 1

not depurate well and may even multiply in depurating shellfish, tank water, and plumbing systems (Barrow and Miller, 1969; Eyles and Davey, 1984; Greenberg *et al.*, 1982).

Raising the temperature of depuration to a temperature in the region of 20°C tends to improve the efficiency of removal of faecal indicator bacteria and viruses pathogenic for humans (Richards, 1988; Lees, 2000). Increasing the temperature will, however, increase the likelihood that marine vibrios will multiply in the tank water and shellfish. The relative risks from such action with respect to viruses and vibrios should be assessed before such measures are propounded either in advice to industry or in the form of statutory controls.

Depuration does not seem to be an efficient method to control *V. vulnificus* in shellfish.

(2) Heat treatment

V. vulnificus is relatively sensitive to heat treatment and temperatures above 45ºC have been shown to kill the microorganism. The average reported decimal reduction time for 52 strains kept at 47ºC was 78 s, and heating to 50ºC for 10 minutes was reported to be sufficient to reduce the number of *V. vulnificus* cells to a non detectable level (Cook and Ruple, 1992). In a experiment performed by Hesselman *et al.*, (1999) oysters harvested from the Gulf Coast were subjected to meat temperatures above 50°C for one to four minutes prior to chilling, washing and packing. This treatment gave a reduction in the content of *V. vulnificus* of one to four log units in the final product.

The various heat treatment regimes allowed for class C shellfish under the Live Bivalve Molluscs Directive (91/492/EEC), e.g. 90°C for 90s, markedly exceed this level of heating and would thus yield significant inactivation of the organism. Some smoking and light cooking methods for oysters may not reach this temperature in the centre of the shellfish flesh and therefore will not provide an adequate control mechanism for this organism.

A major proportion of oysters is presented to the consumer live. Thorough, or even partial cooking, may render the shellfish unacceptable to the consumers. Combinations of heating and cooling have been investigated in order to reduce the risk of *V. vulnificus* infection while maintaining consumer acceptability. Cook and Ruple (1992) described a method which utilised heating at 50°C for 10 min followed by rapid cooling in ice-water for 5 min. This was reported to reduce naturally occurring *V. vulnificus* in oyster meats (initial concentration 59,000 per g) to undetectable levels. Previous work on the thermal inactivation of *V. parahaemolyticus* in clam homogenates (Delmore and Crisley, 1979) suggested that similar effects should be seen with that organism. The method has been used as the basis for a commercial pasteurisation procedure for oysters. A summary of their procedure follows (Andrews *et al.*, 2000):

“The basic process is to wash oysters and rubber band the shells so they stay closed. Then drop into hot water 51-53°C and hold there until the internal

temperature has stayed above 50°C for at least 5 min. When counts are high the time can be extended to as long as 20 min. Then drop into very cold or ice water to rapidly cool. Oysters can then be stored in ice for 2-3 weeks. Oysters may also be frozen for several months. The best way to thaw is in running water like thawing shrimp or fish".

The company states that establishments in Louisiana and California that serve oysters treated in this way do not have to display the usual warning signs that relate to risks of infection.

(3) Irradiation

In general, *Vibrio* species are sensitive to ionizing irradiation, and a dose of 1 kGy have been shown to be lethal to *V. cholerae, V. parahaemolyticus* and *V. vulnificus* (Hackney and Dicharry, 1988). Radish *et al.* (1992) have reported that doses of 3 kGy are required for the elimination of vibrios from frozen shrimps.

The Scientific Committee on Food (SCF) expressed its opinion on the irradiation of eight foodstuffs on 17-09-1998, shrimp among them. Most European countries have insufficient production of quick frozen, peeled and decapitated shrimps. Therefore, they satisfy their market by importation predominantly from South Asia. By the existing conditions (collection, preparation, storage and exportation) in the countries of origin, these imported products do not always meet the microbiological standards of the European countries. The Committee noted that irradiation with 5 kGy has been shown to be effective without any toxicological or nutritional relevant risks.

(4) Vacuum packing

Vacuum packing also seems to reduce the number of *V. vulnificus* found in seafood products (Parker *et al.*, 1994). Although as with freezing, vacuum packing cannot be relied upon as a sufficient measures to completely eliminate *V. vulnificus* from seafood products.

4.6.4. Existing microbiological guidelines

Present EU Directives and Decisions regarding fishery products and live shellfish do not include either standards or guidelines for vibrios. There have been a number of bans on the importation of products from third countries on the basis of their actual or suspected vibrio content.

The FDA has a zero-tolerance for *V. vulnificus* in their guidelines for microbiological contaminants in seafoods.

5. VIBRIO PARAHAEMOLYTICUS

5.1. Hazard Identification

V. parahaemolyticus is a marine organism. Disease caused by this bacterium is common in Asia and the United States. However, it is rarely reported in Europe.

Foodborne infections caused by this organism usually present as gastroenteritis. It is almost exclusively associated with the consumption of raw, improperly cooked, or cooked, recontaminated fish and shellfish, particularly oysters. Less commonly, this organism can cause an infection in the skin when an open wound is exposed to warm seawater (Daniels *et al.*, 2000).

In Asia, *V. parahaemolyticus* is a common cause of foodborne disease, up to 70% of reported foodborne infections in the 1960's. The majority of Japanese outbreaks are associated with the consumption of fish. In Japan, the annual reports from the public health institutes and health centres on isolation of *V. parahaemolyticus* from human sources have tended to increase since 1997. There were 496 registered outbreaks during the 3- year period 1996-1998 with 102 occurring in 1996, 160 in 1997, and 234 in 1998. Most outbreaks have been small, involving less than 50 people. Six percent of the outbreaks involved 50-499 cases. Large-scale outbreaks, involving more than 500 cases, were registered only twice. The first involved 691 cases and was caused by boiled crabs in Niigata prefecture in August 1996. The other involved 1167 cases and was caused by catered meals in Shiga prefecture in July 1998. In the most recent years, *V. parahaemolyticus* foodborne outbreaks in Japan have tended to be small in scale, but rather frequent (Anonymous, WER, 1999).

In Taiwan, between 1986 and 1995, 197 outbreaks of foodborne diseases were caused by *V. parahaemolyticus* (Pan *et al.*, 1997). Over 200 outbreaks were reported in 1997, including an outbreak of 146 cases acquired from boxed lunches (Anonymous, Promed, 1999).

In the United States, the first major outbreak of *V. parahaemolyticus* infection occurred in Maryland in 1971 and was related to improperly steamed crabs. Subsequent outbreaks have occurred in coastal areas of the United States. Among 42 reported outbreaks occurring between 1973 and 1987, 33 involved shellfish (Bean and Griffin, 1990). The largest United States outbreak occurred during the summer of 1978 and affected 1133 of 1700 persons attending a dinner. The food implicated was boiled shrimp. The raw shrimp had been purchased and shipped in standard wooden seafood boxes. They were boiled the morning of the dinner but returned back to the same boxes in which they had been shipped. The warm shrimp were then transported 40 miles in an unrefrigerated truck to the site of the dinner and held an additional 7 to 8 hours until served at night.

In their survey of four Gulf Coast states of the USA, Levine *et al.* (1993) found *V. parahaemolyticus* to be the most common cause of gastroenteritis (37% of 71 cases) in that area. Similarly, Désenclos *et al.* (1991) found *V.*

parahaemolyticus to be the second leading cause (over 26%) of gastroenteritis cases in those persons who had consumed raw oysters in Florida. In a 15-year survey of vibrio infections reported by a hospital adjacent to the Chesapeake Bay, Hoge *et al.* (1989) found 9 (>69%) of 13 vibrio-positive stool specimens to contain *V. parahaemolyticus* as the sole pathogen. *V. parahaemolyticus* was the most commonly isolated vibrio (35 cases) from stool samples submitted by 51 persons attending a conference in New Orleans and reporting diarrhoeal disease (Lowry *et al.*, 1989).

More recently, three outbreaks of *V. parahaemolyticus* infections linked to the consumption of raw oysters occurred in 1997 and 1998 in the USA. The first occurred in July and August 1997 and involved 209 culture-confirmed cases in persons who consumed oysters harvested from the coasts of California, Oregon, Washington, and British Columbia (CDC, 1998; Anonymous, 1997). In the summer of 1998, 416 persons developed diarrhoea after consuming oysters harvested from Galveston Bay, Texas; *V. parahaemolyticus* infection was confirmed in 110. Between July and September 1998, 23 culture-confirmed cases of *V. parahaemolyticus* infections were identified among residents of Connecticut, New Jersey and New York who had consumed oysters and clams harvested from Long Island Sound (CDC, 1999).

In France, one outbreak of *V. parahaemolyticus* has been reported (Lemoine *et al.*, 1999). In April 1997, 44 persons developed diarrhoea after consuming shrimp imported from Asia. Among 5 stools specimens studied, 3 were positive for *V. parahaemolyticus*. However, *V. parahaemolyticus* strain was not isolated from the suspected food. In a retrospective study of 29 cases of non-cholera vibrio infections which occurred in France from 1995 to 1998, 7 sporadic infections caused by *V. parahaemolyticus* were identified (Geneste *et al.*, 2000). Among these, 6 patients developed gastroenteritis and one immunocompromised patient developed septicemia. Among the 6 gastroenteritis cases, 2 were consecutive to a trip in Asia, 2 were associated with the consumption of raw oysters, and the last 2 were of unknown origin.

5.2. Hazard characterisation

5.2.1. Characteristics of human disease

The primary symptoms of *V. parahaemolyticus* gastroenteritis are watery diarrhea and abdominal cramps along with nausea, vomiting and fever. Headache and chills may also be present. The incubation period is between 4 and 96 hours (mean 15 hours) and the median duration of the illness is 2.5 days but may last for as long as 7 days. The illness is usually quite mild although some more severe cases require hospitalization. In those cases, diarrhea may be bloody with mucus and tenesmus.

5.2.2. Pathogenicity

Not much is known about the pathogenesis of the disease. While much is understood of the toxins/hemolysins of *V. parahaemolyticus,* little is known about the adherence process, which is an essential step in the pathogenesis of most enteropathogens.

At least four haemolytic components exist in *V. parahaemolyticus*: a thermostable direct hemolysin (TDH), a TDH-related hemolysin (TRH), phospholipase A, and a lysophospholipase. Of these, TDH and TRH are strongly correlated with pathogenicity. These two haemolysins are encoded by the *tdh* and *trh* genes, respectively.

TDH causes beta haemolysis of human erythrocytes in an agar medium. This reaction is known as the Kanagawa phenomenon (KP)(West, 1989) (see also section 5.5.2). The association between KP-positivity of a strain and its ability to cause gastroenteritis is well established (Sakazaki and Balows, 1981; Nishibushi and Kaper, 1995; Honda *et al.*, 1987). Recently, clinical isolates of KP-negative *V. parahaemolyticus* that produce TRH have been reported to cause gastroenteritis similar to that of TDH-producing isolates (Shirai *et al.,* 1990; Abbott *et al.*, 1989; Kaysner *et al.*, 1990).

According to the literature, virtually all strains of *V. parahaemolyticus* associated with gastrointestinal disease are either TDH- or TRH-positive. However, these TDH- and/or TRH-positive *V. parahaemolyticus* strains constitute a very small percentage of the *V. parahaemolyticus* strains found in the aquatic environments and seafoods.

Original studies in Japan showed that 96% of clinical isolates were KP-positive whereas this was the case with only 1% of the environmental isolates (Sakazaki *et al.*, 1968). A similar conclusion has been reported by Miyamoto *et al.* (1969). This observation may be explained through a natural selection of KP-positive strains in the intestinal tract and better survival of KP-negative strains in the estuarine environment (Joseph *et al.*, 1982). However, several studies have suggested that culture methods might enhance the isolation of KP-negative strains over KP-positive strains or that different physiological or nonculturable state might exist in the estuarine environment (Colwell, 1979; Colwell *et al.*, 1985; Hackney and Dicharry, 1988; Singleton *et al.*, 1982; Tamplin and Colwell, 1986).

In France, the seven *V. parahaemolyticus* strains isolated from clinical cases from 1995 to 1998 were either TDH- or TRH-positive. No TDH- or TRH-positive isolate was found among 38 *V. parahaemolyticus* strains isolated from seafood imported from Africa, Latin America and Asia, studied in the French National Reference Centre for Vibrios and Cholera in 1999. From January to October 2000, 2 strains were positive for the *trh* gene in PCR among 37 *V. parahaemolyticus* strains studied (unpublished data). Thus, the simple isolation of *V. parahaemolyticus* from water or foodstuffs does not necessarily indicate a health hazard. It has also been observed that urease production correlates with the possession of the *trh* gene in *V. parahaemolyticus* (Suthienkul *et al.*, 1995; Kaysner *et al.*, 1994).

Recently a new clone of *V. parahaemolyticus* O3:K6 emerged in India and was associated with pandemic spread in Japan, Taiwan and in the United States (Bag *et al.*, 1999; Chiou *et al.*, 2000; Okuda *et al.*, 1997). The strains of this clone are TDH positive, TRH negative and urease negative (Okuda *et al.*, 1997).

Oliver and Kaper (1997) reported a novel siderophore (vibrioferrin) for *V. parahaemolyticus* which is able to sequester iron from human transferrin. But its importance in the pathogenesis has not been established. Moreover, several adhesive factors have been proposed (outer membrane proteins, lateral flagella, pili, haemagglutinin mannose resistant), but their importance in the pathogenesis is not clear.

5.2.3. Infectious dose

Studies involving human volunteers have shown that ingestion of $2x10^5$ to $3x10^7$ CFU of KP-positive cells can lead to the rapid development of gastrointestinal illness. In contrast, volunteers receiving as many as $1.6x10^{10}$ CFU of KP-negative cells exhibited no signs of diarrhoea illness (Sanyal and Sen, 1974; Oliver and Kaper, 1997). However, in Canada and USA, oyster samples that were harvested from beds implicated in the Pacific Northwest and Oyster Bay outbreaks in 1997 and 1998, respectively yielded <200 CFU of *V. parahaemolyticus* per g of oyster meat indicating that human illness may occur at levels much lower than usually considered (CDC, 1999).

5.3. Exposure assessment

It is important to note that different methods to detect *V. parahaemolyticus* have different sensitivities. Thus, prevalence rates reported in different studies applying different detection methods may not be comparable.

5.3.1. Prevalence in the environment

V. parahaemolyticus occurs in estuarine waters throughout the world and is easily isolated from coastal waters as well as from sediment, suspended particles, plankton, but not in the open sea (Colwell, 1984). There is also evidence that *V. parahaemolyticus* is associated with the digestive tract of shellfish such as clams (Greenberg *et al.*, 1982). This supports the suggestion of Baumann and Baumann (1977) that marine vibrios may be considered to be marine enteric bacteria.

V. parahaemolyticus has occasionally been isolated from freshwater sites, but only at extremely low levels (5 CFU/liter) and only during the warmest periods of the year.

In a study conducted by Gjerde and Bøe (1981) on a total of 200 samples of mussels, seawater, bottom sediments, and fish collected in Norwegian waters, *V. parahaemolyticus* was only detected during July and August. In this survey, mussels harboured *V. parahaemolyticus* in 10 % of the examined samples. The corresponding figure for bottom sediments was 4 %. For seawater, fish gills, and intestinal content no positive samples were found. In all cases of detection, the seawater temperature was 15ºC or above. During the cold season, *V. parahaemolyticus* could not be detected in any samples.

V. parahaemolyticus was isolated from water and seafood collected from Arcachon Bay located in the Atlantic coast in the southwest of France (Urdaci *et al.*, 1988).

Macián *et al*. (2000) reported a study of the identification of *Vibrio* spp. from marine natural samples (seawater and shellfish) in the Mediterranean coast of Spain. Among 284 strains isolated, 14 (5%) were identified as *V. parahaemolyticus*.

In a two year survey carried out in Italy, 726 strains were collected from seawater samples and molluscs of the Adriatic Sea. Strains of the genus *Vibrio,* in total 340, were isolated more often than strains of other genera. The *Vibrio* strains were most prevalent during the summer months. *Vibrio parahaemolyticus* and *Vibrio vulnificus* represented each 10% of the vibrios (Croci *et al*., 2001).

5.3.2. Prevalence in food

V. parahaemolyticus occurs in a variety of fish and shellfish. The latter include at least 30 different species such as clams, oysters, lobster, scallops, shrimp and crabs. Since *V. parahaemolyticus* is more frequently found in coastal waters, it is not surprising this food from that environment is most often incriminated in food poisoning. Clams, oysters, lobsters, scallops, shrimp, and crabs have been involved in confirmed outbreaks. In addition, the microorganism has been detected in a wide variety of marine species including eels, octopus, squid, sardines, tuna, mackerel, perch, flounder, rockfish, red snapper, pompano, etc. (Beuchat, 1982; Oliver and Kaper, 1997).

In a study carried out by the FDA, 86% of the seafood samples examined were positive for *V. parahaemolyticus*. Counts have been reported as high as 1300 CFU/g of oyster tissue and 1000 CFU/g of crab meat, although levels of 10/g are more typical for seafood products (Oliver and Kaper, 1997). In a 3-year survey (Hackney *et al*., 1980), 46% of seafood samples examined were found positive for *V. parahaemolyticus* with positivity rates of 79% in unshucked oysters, 83% in unshucked clams, 60% in unpeeled shrimp, and 100% in live crabs. Oliver and Kaper (1997) reported another study in which the positive samples were between 69 to 100% of the commercially obtained or cultured oysters, clams, and shrimp studied, and 42% of the crabs.

The prevalence of *V. parahaemolyticus* in fresh seafood sold in Mexico was of 45.6%, being 71.4% in fish, 44% in oysters and 27.6% in shrimp; there was a larger number of positive samples during the warmer months (Torres-Vitela and Fernandez-Escartin, 1993).

In Italy, the concentration of *V. parahaemolyticus* in seafood is usually below 10^3 CFU/g, but can increase when the product is collected from warmer water (De Paola *et al.,* 1990). In a survey of shellfish collected from the Adriatic sea, *V. parahaemolyticus* was detected in 1.6% of the samples (Ripabelli *et al.*, 1999).

In England, Wales and Scotland, the prevalence of vibrios in seafood has been studied in harvested shellfish and cooked, ready-to-eat shellfish. In a large study of shellfish in British coastal waters during the years 1975/76, Ayres and Barrow (1978) detected *V. parahaemolyticus* in 14% of harvested shellfish with the highest proportion of isolations being made during summer

months. Conventional methods were used with enrichment in APS (or ATW) and CB and plating on TCBS. Enumeration techniques were only used by a proportion of laboratories involved in the study and counts were not included in the final report.
Over a period of 5 months in 1996, 2425 samples of a variety of cooked out-of-shell molluscs, including cockles, mussels and whelks, were sampled at retail premises and subjected to a variety of microbiological tests (Little *et al.*, 1997). Approx. one-third of the shellfish had been preserved in vinegar or brine. Presence/absence testing for pathogenic vibrios was performed by enrichment in APW (with electrolyte supplement) and CB followed by plating on TCBS. Enumeration of *Vibrio* spp was undertaken by surface plating on TCBS. The *Vibrio* Reference Laboratory of England and Wales undertook confirmation of the identification of potentially pathogenic vibrios. *V. parahaemolyticus* was detected in 3% of samples. Six samples contained *V. parahaemolyticus* at concentrations of 200 CFU/g or higher or total vibrios of $<10^3$CFU/g. Thirty-nine samples contained concentrations of total vibrios of 10^3CFU or higher.

In the Netherlands, a total of 91 samples of shellfish were examined for the presence of *Vibrio* spp. from August to October 1999 (Tilburg *et al.*, 2000). Forty samples contained *V. alginolyticus*, one sample contained *V. metschnikovii*, 25 samples contained *V. parahaemolyticus* and 6 samples contained other *Vibrio* spp. *V. cholerae* and *V. vulnificus* were not detected. None of the *V. parahaemolyticus* isolates contained the virulence genes *tdh* or *trh* as judged by PCR (see section *5.2.2.*).

In France, during 1999, a total of 193 *Vibrio* strains isolated from seafood imported from 9 countries (China, Ecuador, India, Iran, Madagascar, Senegal, Tanzania, Thailand and Vietnam) were sent to the French National Reference Centre for Vibrios and Cholera for identification. Among these strains, 94 *V. parahaemolyticus*, 53 *V. alginolyticus*, 26 *V. cholerae*, 3 *V. metschnikovii*, 2 *V. mimicus* and 15 strains of other *Vibrio* spp. were identified.

In Belgium, a total of 1299 seafood samples were tested for *V. cholerae*, 311 for *V. parahaemolyticus* and 82 for other *Vibrio* spp in 1998 (Scipioni, 2000). The samples included live and processed bivalve molluscs, crustacea and fish of various types. No vibrios were isolated from any of the samples, using selective enrichment in Alkaline Peptone Water (2% NaCl for halophilic vibrios and *V. cholerae*; 1% NaCl for only *V. cholerae*) followed by plating on TCBS and conventional biochemical identification.

In a study conducted by the Mussels Control Unit of the Acuiculture Institute, Santiago de Compostela University, 99 samples of mussels from the North-western coast of Spain were investigated. *V. parahaemolyticus* was isolated from 8% of the samples using the ISO 8914:1990 method (Martinez Urtaza, 2000).

Under the Fishery Products Directive (91/493/EEC)[3], crustaceans and bivalve molluscs that are cooked may be cooled in either drinking water or clean seawater, defined as being free from microbiological contamination. The practice of using seawater for such cooling is widespread in the EU, especially where cooking is undertaken on boats or in shore side facilities. Where the quality of such seawater is presently checked, this is usually on the basis of faecal indicator bacteria such as faecal coliforms and/or *E. coli*.

In the Rapid Alert System *V. parahaemolyticus* has since 1995 in all 61 times been notified in seafood imported to Europe from Bangladesh, Belize, Canada, China, Ecuador, India, Indonesia, Ivory Coast, Malaysia, Mozambique, Namibia, the Netherlands, Nigeria, Senegal, Spain, Sri Lanka, Tanzania, Thailand, Turkey, and Vietnam.

5.3.3. Survival an growth in the environment

Several studies concerning the interaction of bacteria and protozoa have shown that vibrios are capable of surviving and multiplying within certain protozoa, e.g. amoeba, which thus may serve as reservoir for these human pathogens (Barker and Brown, 1994).

V. parahaemolyticus has an annual cycle. The microorganisms surviving in the sediment during the winter are released into the water associated with the zooplankton when the water temperature rises to between 14-19ºC. This phenomenon is greatly affected by salinity since the adsorption of *V. parahaemolyticus* onto copepods occurs more efficiently at lower salinities (Kaneko and Colwell 1978).

A correlation exists between *V. parahaemolyticus* infection and warmer months of the year. This has been observed in Asia, United States, and France where the monthly isolation of *V. parahaemolyticus* is concentrated during the period July-October (Daniels *et al.*, 2000; Geneste *et al.*, 2000).

V. parahaemolyticus survives within the pH range 4.8- 11 and tolerates up to 10% NaCl (FDA, 1998b)

5.3.4. Survival and growth in food

V. parahaemolyticus can be reduced at low temperatures but not eliminated. Survival determined at 4, 0, -18, and -24ºC showed that the time of total decreasing number depends on the initial number of microorganisms and incubation temperature. It is possible to determine the storage time necessary to reduce *V. parahaemolyticus* hazard in fish (Mutanda Garriga *et al.*, 1995).

V. parahaemolyticus has been reported to undergo an initial rapid drop in viable counts when incubated on whole shrimp at 3, 7, 10, or -18°C, although survivors remained at the end of an 8-day study. Thus, numbers can be reduced but not eliminated by cooling. *V. parahaemolyticus* has also been observed to survive storage in shellstock oysters for at least 3 weeks at 4°C

[3] O.J. N° L 268 of 24/09/1991, pag.15

and subsequently to multiply after incubation at 35°C for 2 to 3 days. Similarly, cells of *V. parahaemolyticus* were reduced in cooked fish mince and surimi at 5°C for 48 h, but growth occurred when the product was held at 25°C. Studies involving temperature abuse of octopus, cooked shrimp, and crabmeat all have documented growth of *V. parahaemolyticus* to very large numbers when held for even short periods of time under improper refrigeration (Oliver and Kaper, 1997).

Generation times of 13 to 18 minutes and 12 minutes at 30°C were observed in raw horse mackerel and boiled octopus, respectively. Growth rates at lower temperatures are lower, but counts increased from 10^2 to 10^8 CFU/g after 24 h storage at 25°C in homogenised shrimp, and from $5x10^3$ to $5x10^8$ CFU/g after 7 days storage at 12°C in homogenised oysters (Twedt, 1989).

Vibrios, including the enteropathogenic species, are readily destroyed by cooking. However, heat resistance depends on several factors, including heating menstruum and physiological conditions. There is also considerable variation between the different species, and *V. cholerae* seems to have a higher level of heat resistance than *V. parahaemolyticus* (Delmore and Crysley, 1979). The commercial practice of heat shocking oysters in boiling water to facilitate opening reduces counts of *V. parahaemolyticus* and other non *V. cholerae* vibrios to "undetectable" levels (Hackney *et al.*, 1980).

5.3.5. Estimation of growth, predictive model

At temperatures in the 15-40ºC range, growth of *V. parahaemolyticus* can be very rapid, with a generation time as short as 8-9 minutes in broth culture under optimal conditions (37ºC).

A mathematical model to describe the effect of temperature and a_W on the growth rate of *V. parahaemolyticus* in food has been developed. Using accuracy and bias factors, comparisons between predicted generation time and those reported in the literature has been made. Results showed that the model predictions agreed well with published data (Miles *et al.*, 1997).The growth rate was modeled as a function of temperature and water activity.

$$\sqrt{K} = b(T - T_{min})\{1 - \exp[c(T - T_{max})]\} \cdot \sqrt{(a_w - a_{w,min})\{1 - \exp[d(a_w - a_{w,max})]\}}$$

Where:

K = growth rate

$a_{w,min}$, $a_{w,max}$ = theoretical lower and upper water activity limits

T = temperature

T_{min} , T_{max} = the lower and upper temperature at which the filled equation equals zero

b, c, d = coefficients fitted by non-linear regression

The observed minimum temperature for growth of *V. parahaemolyticus* was 8.3ºC while the maximum temperature for growth was 45.3ºC. The optimum was between 37 and 39ºC.

V. parahaemolyticus strains grew over a water activity range of 0.936 to 0.995 (9.6-0.4% NaCl) with an optimum between 0.982-0.987.

The formula is claimed to hold within the pH range of 6.5-8.9.

5.3.6. Correlation between indicator organisms and V. parahaemolyticus

No correlation have been observed between the occurrence of *V. parahaemolyticus* and faecal coliforms or other indicators (Hackney *et al.*, 1980; Martinez-Manzares *et al.,* 1992).

5.3.7. Correlation between total Vibrio *counts and* V. parahaemolyticus

No correlation can be expected between total *Vibrio* counts and *V. parahaemolyticus* because non-pathogenic *Vibrio* species will always be present in warm seawater.

5.4. Risk characterisation

Very few data exist on the incidence of *V. parahaemolyticus* infections in the EU. These infections are not notifiable.

Table 2 summarises the current knowledge about the incidence of *V. parahaemolyticus* infections in Europe.

Table 2 Available data on the incidence of human infections in Europe

Country	Period considered	Cases number	Symptoms	Origin of data
Denmark	1987-1992	13 10	wound infection ear infection	Hornstrup and Gahrn-Hansen, 1993
	1980-2000	2	gastroenteritis	Statens Serum Institut, Copenhagen
England and Wales	1995-1999	115		PHLS, Colindale
France	1995-1998	6 1	gastroenteritis septicemia	Geneste *et al.*, 2000
	1997	44	gastroenteritis[1]	Lemoine *et al.*, 1999
Northern Ireland	1990-99	0		CDSC (Communicable Disease Surveillance Centre, NI)
Scotland	1994-1999	6		
Spain	1995-98	19	gastroenteritis	Anonymous, 1996 Anonymous, 1998
Sweden	1992-97	350	gastroenteritis[2]	Lindquist *et al.*, 2000
Norway	1999	4		Unpublished data – personal communication

Notes: 1. One outbreak associated with seafood imported from Asia.
2. One outbreak associated with consumption of crayfish imported from China.

Based on the available information on the incidence of *V. parahaemolyticus* infection, the risk of attracting such an infection seems to be low in the EU.

Risk of infection with pathogenic *V. parahaemolyticus* are most strongly associated with:

(i) Consumption of raw, undercooked or recontaminated seafood;

(ii) Occupational or recreational use of natural aquatic environments.

Since these factors are clearly increasing in general, the risk of *V. parahaemolyticus* infections will probably increase in the future.

A strict risk quantification of *V. parahaemolyticus* infection from the consumption of seafood would require information on:

a) Seafood consumption patterns in the EU including an estimate on how much has been harvested locally and how much have been imported from high-endemic regions, and the relative amounts consumed raw and cooked.

b) Quantitative data on the occurrence of TDH- and TRH-positive *V. parahaemolyticus* in seafood harvested inside and imported into the EU.

c) The infectious dose of the organism.

Scarcity of data on the seafood consumption in the EU, the widespread use of presence/absence tests without enumeration or virulence characterisation in studies on the occurrence of the organisms in seafoods, together with the absence of a consensus with regard to infectious dose, makes it impossible to determine a numerical estimate of risk. Further research will be necessary to provide the data on which such an estimate can be based.

5.5. Methods of analysis

5.5.1. Detection of V. parahaemolyticus

Three standard operating procedures have been described by different authorities:

- ISO method 8914 (1990) “Microbiology - General guidance for the detection of *Vibrio parahaemolyticus*”. This method is actually under revision and probably will be substituted by a common method for the detection of *V. cholerae* and *V. parahaemolyticus*. During the 20th meeting of ISO held in Vienna on June 1999, it was established to carry out additional trials until the end of December 2000.

- FDA Bacteriological Analytical Manual. 8th edition, 1995. Chapter 9. (Elliot *et al.*) *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and other *Vibrio* spp. (on-line, 2001: http://vm.cfsan.fda.gov/~ebam/bam-9.html)

- Nordic Committee on Food Analysis (NMKL) 1997 n°156 (UDC 576.851). " Pathogenic *Vibrio* species. Detection and enumeration in foods ". This method is applicable to *V. cholerae, V. parahaemolyticus, V. vulnificus,* and *V. alginolyticus*.

These three procedures describe the detection and identification of *V. parahaemolyticus*. The FDA and NMKL procedures include a quantitative determination of *V. parahaemolyticus*. The FDA method describes a Most Probable Number (MPN) format enumeration of the vibrios in the specimen whereas the NMKL procedure quantifies by direct inoculation of a fixed amount of the specimen to a solid medium.

5.5.2. Determination of pathogenicity

Determination of pathogenicity based on detection of the Kanagawa phenomenon is described in the FDA procedure. As noted in 5.2.2., this determination will not detect all pathogenic isolates of *V. parahaemolyticus*.

In 1968, Wagatsuma formulated a special high-salt blood agar to detect the haemolytic activity of *V. parahaemolyticus*. The study was developed in the Kanagawa Prefectural Public Health Laboratory, and the haemolysis of human red blood cells was interpreted as a positive or Kanagawa phenomenon-positive (KP-positive). The reaction was caused by a heat-stable direct haemolysin with molecular weight of approximately 42,000 (Honda *et al.*, 1976), and it was named as TDH (thermostable direct haemolysin) or Kanagawa haemolysin. This protein is encoded by the *tdh* gene and is only partially inactivated at 100ºC for 30 min at pH 6.0. It produces oedema, erythema, and induration in skin and has capillary permeability activity. The protein is lethal for mice, lyses erythrocytes from a large variety of animals (but not from horse), and its action is temperature dependent (Oliver and Kaper, 1997).

Moreover, Nishibuchi *et al.* (1989) found that during an outbreak of gastroenteritis KP-negative strains produced a TDH related haemolysin (TRH) encoded by the *trh* gene. This gene shares significant nucleotide homology with *tdh*. Its biological, immunological, and physicochemical properties are similar to those of TDH, but not identical. The study showed that strains with the *trh* gene were also associated with gastroenteritis. Thus, TRH could be an important virulence factor and probably the origin of diarrhoea in patients from whom only KP-negative *V. parahaemolyticus* are isolated.

Sensitive and rapid molecular methods, using PCR, have been applied to identify the presence of *tdh* and *trh* genes from *V. parahaemolyticus* (Lee and Pan, 1993; Suthienkul *et al.*, 1995).

A sandwich Enzyme-Linked Immunosorbent Assays (ELISA) has been developed for the detection of TDH with a sensitivity of <10 ng/ml (Honda

et al., 1985). In Japan, reverse passive latex agglutination (RPLA) and ELISA kits are commercially available and have been shown to cross-react with TRH with a sensitivity of approximately 10 ng/ml (Yoh *et al.*, 1995). However, discrepancies have been noted between the Kanagawa reaction including recognition of the *tdh* gene by molecular probe technique and the reaction in a commercial RPLA test, with the latter yielding positive results in only 62.1% of the KP-positive isolates (Wong *et al.*, 2000).

It seems that the determination of the *tdh* and/or *trh* genes in *V. parahaemolyticus* by molecular methods correlates well with virulence.

5.6. Risk management options

5.6.1. Prevention

(1) At the harvesting step

Both the occurrence of *V. parahaemolyticus* in the estuarine/marine environment, and the occurrence of illness, is associated with summer months (Kaneko and Colwell, 1978; Hackney *et al.*, 1980). Control by prohibiting harvest of shellfish from areas that have been associated with outbreaks of the illness if the seawater temperature exceeds a certain temperature in an analogous manner to US controls for *V. vulnificus* (see section *4.6.2*) could be considered. The widespread occurrence of KP-negative *V. parahaemolyticus* strains in tropical, sub-tropical and temperate coastal and estuarine waters would represent difficulties with any controls based solely on the demonstration of the presence, even in high concentrations, of the microorganism in the environment. This situation may change with further development of methods for the direct detection and enumeration of TDH- and/or TRH-positive *V. parahaemolyticus* in seafood and environmental samples.

(2) At the transport or storage steps

Levels of pathogenic vibrio were shown to increase in oysters after harvest with the increases being temperature dependent: *V. parahaemolyticus* and *V. vulnificus* did not multiply in oysters held at 10ºC. Levels of *V. parahaemolyticus* in oysters stored above 10ºC often approached the infectious dosage level for KP-positive strains of 10^5 to 10^7 viable cells (Cook and Ruple, 1989). However, since most environmental strains of *V. parahaemolyticus* are KP-, multiplication in oysters does not necessarily indicate a human health risk.

Refrigeration of harvested shellfish, especially prior to depuration, is currently not widely practised in Europe. Transport of harvested shellfish and fishery products should be undertaken under refrigerated conditions. Since the concentration of *V. parahaemolyticus* in freshly harvested shellfish and other seafoods is generally low and multiplication does not occur under 10ºC, rapid cooling after harvest and effective subsequent temperature control is likely to reduce the incidence of human disease at least in high-endemic regions.

5.6.2. Decontamination

(1) Depuration technologies

a) Relay:

Less information is available on the effects of relay on concentrations of *V. parahaemolyticus* in bivalve shellfish than for *V. vulnificus* (see Section 4.6.3.(1)). The concentration of *V. parahaemolyticus* in *Crassostrea commercialis* was shown to reduce from 18 per g to 5 per g after being relayed from a harvesting area to a pollution free waterway (Son and Fleet, 1980). No information was given on the temperature or salinity of the relay area. Further studies need to be undertaken if relay is to be considered as a control measure for *V. parahaemolyticus*.

b) Depuration in tanks:

See also section 4.6.3.(1)b)

Son and Fleet (1980) found that *V. parahaemolyticus* counts decreased from 18 CFU/g to 5 CFU/g in 2 days and remained at 5 CFU/g after 6 days in Sydney rock oysters (*Crassostrea commercialis*) during depuration, relaying, and storage.

The influence of temperature on depuration of hard shell clams (*Mercenaria mercenaria*) was evaluated by Greenberg *et al.* (1982) for *Escherichia coli* and *V. parahaemolyticus*. The study concluded that *Escherichia coli* was rapidly eliminated at 8, 15 and 25ºC in a recirculating depuration system, but that *V. parahaemolyticus* was not. The most rapid depuration of *Vibrio* was at 15ºC, and at 8 or 25ºC was approximately 1 log after 72 h. The persistence of *Vibrio* in hard clams may have been the result of a closer association between the bacteria and the host's intestinal cells, which resulted in the apparent growth of *Vibrio* in clams detected in the studies (Richards, 1988).

Son and Fleet (1980) found that *V. parahaemolyticus* depurated from 10^7 CFU to 10^2 CFU/100 g after 72 h in Sydney rock oysters (*Crassostrea commercialis*). However, *Escherichia coli* was reduced to non-detectable levels and *Salmonella* spp. decreased dramatically by about 4 log cycles both within 48 h.

In contrast, Eyles and Davey (1984) found that counts of *V. parahaemolyticus* in individual samples of *C. commercialis* of 48 CFU/g persisted after depuration. Moreover, *V. parahaemolyticus* was detected in 39% of 54 depurated oyster samples obtained from 13 out of 25 plants studied. Three fourths of the contaminated oysters were detected during the warmest months of the year.

The present state of knowledge is such that depuration on its own cannot be considered to be a satisfactory control measure for pathogenic vibrios.

(2) Heat treatment

Heat treatment conditions specified for *V. vulnificus* (see section *4.6.3.*) will also reduce the concentration of *V. parahaemolyticus* in raw shellfish. However, *V. parahaemolyticus* is also associated with the consumption of cooked seafood. Normal cooking conditions for many seafoods may not kill all vibrio cells and these may subsequently regrow if the cooked food is not eaten directly or kept at a temperature of less than 4°C or greater than 60°C. Processing, handling and subsequent transport should be undertaken under conditions which prevent recontamination and growth of vibrios (Blake, 1984 and Paparella, 1984).

Heating of *V. parahaemolyticus* cells at 60°, 80°, or 100ºC for 1 min is lethal to a small population ($5x10^2$ CFU/ml), although some cells survive heating at 60ºC and even 80ºC for 15 min when populations of $2x10^5$ are used. Cells do not survive boiling for 1 min (Vanderzant and Nickelson, 1972).

The use of seawater containing pathogenic vibrios in direct contact with cooked seafood will present a hazard of recontamination of the product.

(3) Irradiation.

See section 4.6.3.(3)

5.6.3. Existing microbiological guidelines

See Section 4.6.4.

A number of governmental and non-governmental bodies have produced guidelines for vibrios in post-processing or ready-to-eat seafoods. Some of these are presented in Annex 2. In general, there seems to be an agreement of a limit of acceptability of 10^2-10^3 CFU per g for *V. parahaemolyticus*, with the FDA limits being 10-fold higher.

6. CONCLUSIONS

(1) As there are insufficient data on the incidence of infections caused by *V. vulnificus* and *V. parahaemolyticus* in Europe, at present their incidence cannot be quantified. Yet, in the EU, the risk of infections caused by these pathogens seems to be low. Increasing international trade and consumption of raw seafood together with an increasing number of susceptible persons is causing concern that the incidence of infections due to *V. vulnificus* and *V. parahaemolyticus* in Europe may increase. The incidence of *V. parahaemolyticus* infections seems to be rather high in Japan, South East Asia and USA, especially along the Gulf of Mexico. *V. vulnificus* infections occur in the same regions, but at a much lower incidence than *V. parahaemolyticus* infections.

(2) The major clinical presentation of foodborne infections caused by *V. vulnificus* is primary septicaemia and, to a lesser extent, gastroenteritis. Almost all recorded *V. vulnificus* infections in Europe are not foodborne in

the strict sense of the word, but occur after contact with contaminated seawater or after handling contaminated seafood, usually shellfish. Most foodborne infections due to *V. vulnificus* are reported in the USA, primarily associated with consumption of raw oysters.
For *V. parahaemolyticus* the major clinical presentation is gastroenteritis. This disease is almost exclusively associated with the consumption of raw, improperly cooked, or cooked recontaminated fish and shellfish, particularly oysters. Most human infections of *V. parahaemolyticus* in Europe are associated with consumption of imported seafood, especially from Asia.

(3) Data on the infectious dose are insufficient although it appears that the dose for pathogenic *V. parahaemolyticus* is high, while that for *V. vulnificus* seems to be lower. Conditions causing immunosuppression and iron accumulation are risk factors for *V. vulnificus* infections. For *V. parahaemolyticus* such an association has not been described.

(4) *V. parahaemolyticus* and *V. vulnificus* occur in estuarine or marine waters throughout the world and are part of the natural flora of zoo-plankton and coastal fish and shellfish. Their numbers are dependent on the salinity and temperature of the water. *V. parahaemolyticus* has not been detected in water with a temperature below 15°C, while *V. vulnificus* has been detected in water with a temperature of 8°C.

(5) *V. parahaemolyticus* and *V. vulnificus* may be present in seafood produced or harvested in Europe and in imported seafood harvested from warmer waters. Their prevalence seems to be low in products from Europe. However, detailed quantitative data on *V. vulnificus* and *V. parahaemolyticus* in different seafoods and their patterns of consumption are lacking.

(6) The pathogenicity of *V. parahaemolyticus* is correlated to the production of either of two haemolysins, TDH and TRH. These haemolysins, or the genes encoding them, may be detected by a range of traditional, and molecular methods. The vast majority of strains isolated from the environment or seafood do not produce these haemolysins. For *V. vulnificus*, no virulence characteristics have been described that could be used to distinguish pathogenic from non-pathogenic strains.

(7) There is no observed correlation between either *V. vulnificus* or *V. parahaemolyticus* and faecal coliforms counts or other indicators.

(8) Because non-pathogenic vibrios can be present in all seafood from coastal waters, a positive correlation between total vibrio counts and potentially pathogenic vibrios, such as *V. vulnificus* and *V. parahaemolyticus*, cannot be expected. Thus, total *Vibrio* counts are not indicative for the presence of pathogenic vibrios.

(9) The sensitivity of different methods for detection and enumeration of *V. vulnificus* and *V. parahaemolyticus* in seafood products varies, making it difficult to compare results from different studies.

(10) *V. vulnificus* and *V. parahaemolyticus* grow readily in seafood at temperatures between 10°C and 45°C. Their growth is inhibited by chilling

and freezing, but they are not eliminated. Neither can the current depuration technologies for oysters and other shellfish by relay or in tanks or in ponds be relied upon to eliminate *V. vulnificus* and *V. parahaemolyticus*.

(11) *V. vulnificus* and *V. parahaemolyticus* are eliminated by properly designed heat treatment or ionising irradiation.

7. RECOMMENDATIONS

(1) Methods for detection, enumeration and virulence characterisation should be standardised and harmonised to facilitate comparison of data on pathogenic vibrios in seafood.

(2) Further basic research is needed on the characterization of virulence determinants of *V. vulnificus* and *V. parahaemolyticus*.

(3) To monitor infections caused by *V. vulnificus* and *V. parahaemolyticus* in order to assess their risk to human health, these pathogens could be included in the "European Network for Epidemiologic Surveillance and Control of Communicable Diseases" (9118/98/CE). *V. vulnificus* and *V. parahaemolyticus* should be included in all microbiological sentinel surveillance systems for infectious gastroenteritis. Clinicians and microbiologists should be made aware of the illnesses caused by these organisms.

(4) Because infections caused by *V. vulnificus* are invasive and thus usually diagnosed, they should be made notifiable.

(5) In any clinical case of infection due to *V. vulnificus* or *V. parahaemolyticus*, investigations should attempt to identify the source of infection, the species, markers of pathogenicity, and in case of a food source, the amount of food consumed and the numbers of microorganisms involved.

(6) Further information regarding cultural and regional seafood consumption patterns and seafood preparation practices within the EU Member States should be gathered.

(7) Data on pathogenic *V. vulnificus* and *V. parahaemolyticus* in seafood and infections caused by them should be considered for inclusion in the Zoonoses Report under the Zoonosis Directive.

(8) Good hygienic practices (GHP), including maintenance of the cold chain, should be enforced at all stages from harvest to the consumer. The time from harvest to landing should be kept as short as possible. To avoid cross contamination, processed seafood should not be cooled in untreated sea-water nor stored in containers used before processing, unless cleaned and disinfected.

(9) Depuration or relay technologies should not be relied upon for elimination of vibrios.

(10) The practice of judging seafood exclusively based on total *Vibrio* counts as indicative for the presence of pathogenic vibrios is not appropriate and should be discontinued.

(11) The practice of judging seafood exclusively based on total *V. parahaemolyticus* counts without consideration of the virulence factors TDH/TRH (or *tdh/trh*) is not appropriate and should be discontinued.

(12) Currently available scientific data do not support setting specific standards or microbiological criteria for pathogenic *V. vulnificus* and *V. parahaemolyticus* in seafood. Codes of practice should be established to ensure that GHP has been applied.

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9. ACKNOWLEDGEMENTS

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10. GLOSSARY.

APW: Alkaline peptone water (1% NaCl)

ATW: Alkaline salt tryptone water

APS: Alkaline-peptone-salt broth (3% NaCl)

CB: Salt colistin broth

CDC: Centers for Disease Control and Prevention, USA.

CFU: Colony Forming Units

CPC: Cellobiose.polymixin B-colistin agar.

HACCP: Hazard Analysis and Critical Control Points.

HAE: Horie arabinose-ethyl violet broth.

HB: HAE.

FDA: Food and Drug Administration, USA.

GMPs: Good Manufacturing Practices.

GST: Glucose salt teepol broth.

KP: Kanagawa phenomenon.

MNS: Monsur broth.

MRN: Marine broth.

PCR: Polymerase Chain Reaction.

SDS: Sodium dodecyl sulfate-polimixin B-sucrose agar.

TCBS: Thiosulfate-citrate-bile salts-sucrose agar.

TDH: Thermostable direct haemolysin or Kanagawa haemolysin.

TRH: TDH related haemolysin.

WB: Water blue-alizarin-yellow agar.

11. ANNEXES

11.1. ANNEX 1 Summary of characteristics of *Vibrio vulnificus* and *Vibrio parahaemolyticus* and preventive measures to control them

Characteristics to be used as a general guide into a HACCP system (from : Mortimore and Wallace, 1994 unless otherwise stated)

	V. vulnificus	***V. parahaemolyticus***
Naturally found	Salt water, molluscs, seafood.	Seafood, coastal marine environments, intestines of marine animals
Associated foods	Seafood, especially shellfish.	Seafood
Why important	Invasive and rapidly lethal pathogen. 56% fatal.	Particularly important in raw seafood. Responsible for 50-70% of enteritis cases in Japan.
Infective dose	Not known.	Not fully established. Probably high ($>10^6$)
Incubation period	Short, 7 h to several days (median 16-38 h).	4-96 h
Symptoms	Fever, chills, hypotension, nausea. Less frequently vomiting, diarhoea, abdominal pains.	Acute gastroenteritis: nausea, vomiting, abdominal cramps, fever chills, diarrhoea. Can be fatal.
Morphology	Gram-negative straight or curved rods. 0.5-0.8 x 1.4-2.6 um	Gram-negative curved or straight rods with flagellum. 0.5-0.8 x 1.4-2.6 um
Oxygen requirements	Facultative anaerobe	Facultative anaerobe
Growth temperatures (ºC)	40 37 5	Maximum: 43 Optimum: 37 Minimum: 12.8
pH range	Maximum:10 Minimum: 5 (FDA, 1998b)	Maximun: 11 Optimum: 7.5-8.5 Minimum: 4.5-5
Minimum a_W for growth	Not known.	0.94
Salt tolerance (%)	Maximum: 6 Optimum: 1-2 Minimum: 0.1	Maximum: 10 (FDA, 1998b) Halophile (minimum salt 0.5%)

Preventative measures to biological hazards such as *V. vulnificus* and *V. parahaemolyticus* (Mortimore and Wallace, 1994):

Raw materials
Lethal heat treatment during process
Specification for organism(a)
Testing(a)
Evidence of control during supplier process(a)
Certificate of analysis(a)
Temperature control to prevent growth to hazardous levels
Intrinsic factors such as pH and acidity; a_W – salt, sugar, drying; organic acids; chemical preservatives
Processes such as irradiation, electrostatic field sterilization, etc.
Cross contamination at the facility (from the environment and raw materials)
Intact packaging
Pest control
Secure building (roof leaks, ground water, etc.)
Logical process flow, including where necessary:
(i) segregation of people, clothing, equipment, air, process areas
(ii) direction of drains and waste disposal

(a) = critical when the process has no lethal heat treatment

11.2. ANNEX 2 Existing microbiological limits and guidelines for vibrios in seafood

Table 1. ICMSF Recommended Microbiological Limits for *Vibrio parahaemolyticus* in Seafoods (ICMSF, 1986).

Product	n	c	Count per gram or cm^2 m	M
Fresh and frozen fish and cold-smoked fish	5	2	10^2	10^3
Frozen raw crustaceans	5	1	10^2	10^3
Frozen cooked crustaceans	5	1	10^2	10^3
Cooked, chilled and frozen crabmeat	10	1	10^2	10^3
Fresh and frozen bivalve molluscs	10	1	10^2	10^3

Table 2. FDA Guidelines for Microbiological Contaminants in Seafoods (FDA, 1998a).

Seafood type	Organism	Action level
Ready to eat fishery products	*V. cholerae*	Presence of toxigenic 01 or Non-01
	V. vulnificus	Presence of pathogenic organism showing mouse lethality
	V. parahaemolyticus	Levels equal to or greater than $1x10^4$ /g and Kanagawa positive or negative

Table 3. Guidelines from the Guides to Good Practice of the Dutch Commodity Board for Fish - Preparation of molluscs and crustacea (Produktschap voor Vis en Visproducten, The Nederlands, 1994).

Microbiological parameter	Raw/cooked, fresh or frozen (imported) lobster/crab/prawn (CFU per gram)	
	At import	At time of sale
V. parahaemolyticus	Not specified	<100

Table 4. PHLS (UK) Guidelines for vibrios in Ready to Eat Foods at sale level.

Organism	Microbiological quality (CFU per gram unless stated)			
	Satisfactory	Acceptable	Unsatisfactory	Unacceptable/ potentially hazardous*
Vibrio cholerae	Not detected in 25g	-	-	Detected in 25 g
V. parahaemolyticus	< 20	20 - <100	100 - $<10^3$	Greater or equal than 10^3

*Prosecution based solely on high colony counts and/or indicator organisms in the absence of other criteria of unacceptability is unlikely to be successful.

11.3. ANNEX 3 Methods of analysis for *V. vulnificus*

11.3.1. Introduction

In the course of several studies (Aono *et al.*, 1997; Kaysner *et al.*, 1987; Oliver *et al.*, 1983) it has been found that numerous vibrios similar to *V. vulnificus* in biochemical characteristics occurred in the marine environment, suggesting that with the conventional technique based on identification of phenotypic characteristics it is difficult to correctly identify this species.

11.3.2. Isolation

Of all the pathogenic vibrios, this species is the most dangerous in the USA, and responsible of the 90% of all deaths related with seafood. So, the first detailed clinical epidemiologic and taxonomic study was carried out by CDC (Centers for Disease Control and Prevention, USA) in 1979 (Blake *et al.,* 1979). The FDA (1995) has established the protocol (similar to those described below for *V. parahaemolyticus* but with some differences): Enrichment in alkaline-peptone-salt broth (APS) + 3% NaCl (35-37ºC, 16 h) and streaking onto cellobiose-polymyxin B-colistin (CPC) and TCBS agar plates to obtain isolated colonies. Typically, *V. parahaemolyticus* does not grow on CPC, but grows on TCBS, while *V. vulnificus* grows in both of them (Sloan *et al.*, 1992).

CPC agar was originally described by Massad and Oliver (1987). This medium take advantage to the resistance of *V. vulnificus* and *V. cholerae* to colistin and polymyxin B, and of cellobiose fermentation. Moreover, the recommended incubation temperature is 40ºC, because eliminates other marine bacteria. However, Kaysner *et al.* (1989) reported that even at 35ºC allowed the growth of pure cultures of *V. vulnificus* on CPC agar.

See Annex 1

11.3.3. Identification

There are three biotypes, 1, 2, and 3. Biotype 1 is a estuarine microorganism highly invasive and pathogen to human being. Biotype 2 is pathogen for eels, although Amaro and Biosca (1996) reported a *V. vulnificus* biotype 2 strain that was isolated from a human leg wound suggesting that is also an opportunistic pathogen for humans. Biotype 3 is highly invasive and has so far only been described in Israel (Bisharat *et al.*, 1999)

Arias *et al.* (1995, 1998) have reported that the best approach consisted of a combination of culture methods starting with a short enrichment and subsequent inoculation on a selective medium, followed by confirmation of the presumptive colonies by PCR.

The gene region encoding the cytolysin elaborated by *V. vulnificus* was sequenced by Yamamoto et al. (1990). Hill *et al.* (1991) used a 519-bp portion of the cytolysin-haemolysin gene, and when 10^2 CFU/g were

inoculated into oyster homogenates, the microorganism was recovered after 24 h in alkaline peptone water.

The study of Arias *et al.* (1999) about the incidence of *Vibrio vulnificus* from sea water and shellfish of western Mediterranean coast from Spain stressed the importance of designing an appropriate detection strategy, especially when this is a microorganisms present in low numbers.

V. vulnificus can adopt a viable but nonculturable state in the environment. This metabolic state may make its presence difficult to demonstrate by traditional culture methods and by antibody-based assay. But the DNA of the cells may be detectable by PCR (Hill, 1996). However, *V. vulnificus* cells treated in the laboratory to achieve a viable but nonculturable state were more difficult to detect by PCR than was expected (Brauns *et al.*, 1991). The reason for this is not presently understood (Hill, 1996).

11.3.4. Limitation of methods.

In 1992, Sloan et al. compared five enrichment broths and two isolation agar media for their ability to detect and enumerate *V. vulnificus* in oysters. The enrichment broth tested were alkaline peptone water (APW), glucose salt teepol (GST) broth, Marine (MRN) broth (ZoBell, 1941), Horie arabinose-ethyl violet (HAE/HB) broth, and Monsur' (MNS) broth (Monsur, 1963). The two isolation media studied were CPC agar and sodium dodecyl sulfate-polymixin B-sucrose (SDS) agar (Kitaura *et al.*, 1987).

The log MPN/g of *V. vulnificus* detected in oysters decreased with enrichment broth in the sequence of APW (average of 4.2/g) > MRN > HAE > MNS > GST (average of 1.3/g). Statistically, MRN broth gave equivalent counts, but lower, than APW.

With respect to the isolation agar media, atypical growth was less on CPC agar than on SDS agar. Moreover, *V. vulnificus* strains were isolated more frequently from CPC agar (81%, 580/468) than from SDS agar (61%, 342/207).

Based on Sloan *et al.* (1992) results, APW enrichment broth with CPC isolation agar was more efficient than other media. These results were consistent with FDA' rules.

The study of Arias *et al.* (1999) about the incidence of *Vibrio vulnificus* from sea water and shellfish of western Mediterranean coast from Spain stressed the importance of designing an appropriate detection strategy, especially when this is a microorganisms present in low numbers. In Japan, Aono *et al.* (1997) reported similar results also using PCR for the identification of presumptive isolates. Both research groups found a low percentage of confirmed isolates: 6.6% (2/30) for shellfish and 4.7% (1/21) for sea water (Arias *et al.*, 1999); 2.1% for oyster samples and 0.2% for sea water or sediments (Aono *et al.*, 1997). This means that a large proportion of the environmental strains able to grow on selective media giving *V. vulnificus*-like colonies belong to other *Vibrio* species that are not successfully inhibited (Arias *et al.*, 1999).

11.4. ANNEX 4 Methods of analysis for *V. parahaemolyticus*

11.4.1. Introduction

The reported studies use different techniques of analysis. This fact make it very difficult or even impossible to compare the results. Therefore, if standards are going to be established, the most appropriate methods must be selected.

11.4.2. Isolation.

Reviews of the taxonomy and the variety of media for isolating pathogenic *Vibrio* species have been provided by Sakazaki and Balows (1981), Joseph *et al.* (1982), Twedt (1989), West (1989), and Oliver and Kaper (1997). McLaughlin (1995) provided tests which are of special value in differentiating the 12 human pathogenic vibrios species into six groups.

As is the case with many of the vibrios, strain variation is common, and phenotypic testing is often insufficient for identification to species (Oliver and Kaper, 1997). As an example can be considered the case of the percentage of isolates of *V. parahaemolyticus* that has been found sucrose positive (1%), the species generally being sucrose negative (McLaughlin, 1995). Moreover, some probes as H_2S production are dependent on the medium or assay method employed (Oliver and Kaper, 1997). Additionally, although Kaysner *et al.* (1994) have reported a correlation between urea hydrolysis and the production of the Kanagawa haemolysin (see section 5.5.2.), other authors have found that urea hydrolysis is not a good marker for the production of Kanagawa haemolysin (Osawa *et al.* 1996).

In 1963, Sakazaki described a teepol-containing medium, water blue-alizarin-yellow agar (WB). Horie et al. (1964) devised a broth used successfully for isolation of *Vibrio parahaemolyticus* from plankton and fish, the arabinose-ethyl violet (HAE or HB) broth.

The Food and Drug Administration (FDA, 1995) specifies, as pre-enrichment, alkaline-peptone-salt broth (APS) containing 3% NaCl to be subsequently streaked on thisulfate-citrate-bile salts-sucrose agar (TCBS) in a protocol designed to isolate the microorganisms from contaminated seafood.

Studies carried out by the FDA (data unpublished) and Beuchat (1976, 1977b) indicate that HB and WB may be superior to GST and TCBS, particularly in recovery of cold-stressed microorganisms (Peterson, 1979).

The main difference in the composition of these media is the inhibitors employed. HB is a non-selective broth and WG and GST contain teepol. The selectivity of TCBS depends on oxgall and sodium cholate.

In order to study the efficacy of that media, Peterson (1979) carried out a multicenter comparative study (universities, food industries and FDA laboratories studied shrimps inoculated with *V. parahaemolyticus* and *V. alginolyticus* mixtures) contrasting GST with HB and TCBS with WB. The

following combinations were made: HB-WB, HB-TCBS, GST-WB, and GST-TCBS. The HB combinations seemed to be the best choice.

A previous study (Beuchat, 1977a) demonstrated that HB was superior to GST in the recovery of *V. parahaemolyticus* from chilled and frozen crab meat. Ray *et al.* (1978) showed the need to consider providing a non-selective medium for injury-repair microorganisms before more selective media, and this may be the clue to the superiority of HB (Peterson, 1979).

Additionally, most participants in Peterson's study found it difficult to differentiate *V. alginolyticus* from *V. parahaemolyticus* colonies on WB agar. Although it was not indicated in the procedure, participants found that identifiable colonies would develop on WB agar with an additional 20 h of aging at room temperature.

V. parahaemolyticus is susceptible to sublethal injury when exposed to environmental stress. Several authors have studied injury resulting from heat (Beuchat 1976; Heinis *et al.*, 1977, 1978) and cold (Beuchat 1977a, 1977b; Ma-Lin and Beuchat, 1980) treatments. They recommended the use of HB as an additional enrichment broth, together with GST, in order to enhance the probability of isolating all viable cells from seafoods. A similar recommendation was reported by Peterson (1979) from the FDA collaborative study cited above.

The ISO method 8914 "General guidance of the detection of *Vibrio parahaemolyticus*" (see section 5.5.1) is actually under revision and probably it will be substituted by a common method for the detection of all pathogenic vibrios. The standard 8914 will be maintained until the standard on the pathogenic vibrios is available. During the 20th meeting of ISO held in Vienna on June 1999 it was established to carry out additional trials till the end of December 2000.

11.4.3. Identification.

(a) Conventional biochemical identification

For the detection of *V. parahaemolyticus*, known amounts of the food are inoculated into a liquid selective medium (usually APW with 2% salt, pH 8.6). After incubation, samples are plated on a solid selective medium (usually TCBS agar). Suspect colonies are from isolation of the media subjected to further cultural and biochemical examinations (Gram, motility, oxidase, arginine dihydrolase, lysine decarboxylase, ONPG, acid-gas from glucose, saccharose, cellobiose, and halophilic characteristics).

Table 3 summarises the biochemical tests used for identification of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*.

Table 3. Biochemical tests for the identification of 3 species of vibrios

Test	Reaction		
	V. cholerae	*V. parahaemolyticus*	*V. vulnificus*
Oxidase	+	+	+
ONPG	+	–	+
Arginine dihydrolase	–	–	–
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	+	+	V
Fermentation:			
Sucrose	+	–	–
Lactose	–	–	+
Arabinose	–	+	–
Growth in:			
0% NaCl	+	–	–
3% NaCl	+	+	+
8% NaCl	–	+	–
Voges-Proskauer	V	–	–
Lipase	+	+	+
Reaction to O/129*:			
10 µg	S	R	R
50 µg	S	S	S

* At present, *cholera vibrio* strains resistant to O/129 as well as to various antibiotics are being increasingly isolated. This test therefore no longer possesses the same validity as an indicator of *V. cholerae* species.

S= sensitive

R= resistant

V= variable

(b) Serotyping

V. parahaemolyticus is serotyped according to both somatic O and capsular polysaccharide K antigens. The serotyping scheme was developed by Sakazaki *et al.* (1968) who studied 2720 strains. The current serotyping scheme contains 12 O antigens and 59 K antigens. The H or flagellar antigen is common to all strains of *V. parahaemolyticus*, rendering this component of little value for serotyping. The majority of the clinical strains can be classified by their O antigen (Oliver and Kaper, 1997).

Serotyping maybe of value in investigating the source of outbreaks but be not useful for isolates from foodstuffs in the absence of human disease.

(c) Molecular methods

Molecular techniques, particularly specific oligonucleotide probes, constitute a very sensitive and specific tool for detecting very low numbers of bacteria, or even viable but nonculturable forms including *Vibrio* species (Koch *et al.*, 1993; Arias *et al.*, 1995).

A cloned DNA fragment from *V. parahaemolyticus*, designated pr72H has been used as species specific DNA probe for *V. parahaemolyticus*. A polymerase chain reaction (PCR) assay, using this DNA fragment, was found to be highly specific for *V. parahaemolyticus* (Lee *et al.*, 1995). The specificity of this fragment for *V. parahaemolyticus* was confirmed by the French National Reference Center for Vibrios and Cholera (unpublished data).

Molecular methods have been used for the detection of *V. parahaemolyticus* as well as for its identification after isolation by conventional means. These increasingly involve the use of the Polymerase Chain reaction (PCR) for amplifying target nucleic acid rather than relying on direct probing. Detection of all *V. parahaemolyticus* types in shrimp samples was achieved by applying PCR to the part of the gyrase B gene, following an 18 hour pre-enrichment (Venkateswaran *et al.*, 1998).

Enzyme-Linked Immunosorbent Assays (ELISAs) have been developed for the detection of TDH with a sensitivity of <10 ng/ml when sandwich methods were used (Honda *et al.*, 1985). In Japan, reverse passive latex agglutination (RPLA) and ELISA kits are commercially available and have been shown to cross-react with TRH with a sensitivity of approximately 10 ng/ml (Yoh *et al.* 1995). However, discrepancies have been noted between both the Kanagawa reaction and recognition of the *tdh* gene by molecular probe technique with the reaction in a commercial RPLA test, with the latter yielding positive results in only 62.1% of the K+ isolates (Wong *et al.*, 2000).

11.4.4. Limitation of methods.

According to Pace and Chai (1989), a number of entities may be involved in the pathogenicity of *V. parahaemolyticus*, and the mechanism may vary from strain to strain. In this study they found that KP-positive and KP-negative strains differ somewhat in their cell envelope protein profiles, lipopolysaccharide levels under certain growth conditions, and alkaline phosphatase activities. These results indicate that pathogenicity may be related to the cell envelope composition and that may be related to strain variation and cultural conditions. Thus, *V. parahaemolyticus* may be able to enter in the viable but non-culturable state, which might be responsible for the difficulty in isolating KP-positive strains from the environment.

The viability of microorganisms has routinely been estimated by plating techniques using solid media and enumeration of the resulting number of organisms. Such plating methods may be misleading and underestimate the number of viable cells, and that could be due to the presence of stressed cells or cells in a dormant state (McFeters *et al.*, 1986; Stevenson, 1978).

Many aquatic bacteria appear to enter a natural dormant state when subjected to certain environmental stresses, such as low temperatures or nutrient deprivation. This response appears to be a strategy for survival and that cells are named as nonrecoverable or “viable but nonculturable” (Roszak and Colwell, 1987).