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BACTERIAL ASSOCIATIONS WITH COMMERCIALLY IMPORTANT MARINE BIVALVES

by

Eileen Lane

Thesis presented for the Degree of Doctor of Philosophy

in the Faculty of Science, University of Glasgow

Department of Microbiology, May 1997

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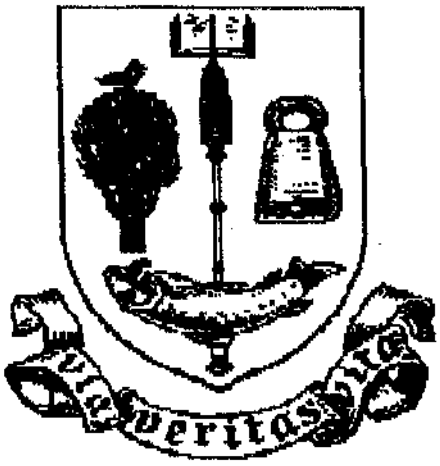

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Dedicated to my mother and late father, Angela and Morgan Lane. To my family and friends, thank you for all the kindness that you have shown to me.

Many a mickle maks a muckle

(Author unknown)

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LIST OF ABBREVIATIONS

A	Absorbance
BRD	Brown Ring Disease
C	Control
c.f.u.	Colony forming units
CAM	Calcein-AM
CLED	Cysteine Lactose Electrolyte Deficient Medium
C.P.D.	Critical point dryer
CT	Ciliostatic toxin
Da	Dalton
DMSO	Dimethyl sulphoxide
D-PBS	Dulbecco's phosphate buffered saline
ETHD	Ethidium Homodimer
FH	Filtered haemolymph
FSW	Filtered sea water
h	Hour
g	Gram
l	Litre
LD_{50}	Lethal dose 50
LPS	Lipopolysaccharide
M.W.	Molecular weight
MA	Marine agar
MB	Marine broth
min	Minute
ml	Millilitre
mM	Millimolar
MOF	Marine Oxidative/Fermentative Medium

MPS	*Mytilus edulis* physiological saline.
NCIMB	National Collection of Industrial and Marine Bacteria
NMWL	Nominal molecular weight limit
OD	Optical density
ONPG	*0*-Nitrophenyl-*β*-D-galactopyranoside
OVVD	Oyster Velar Virus Disease
PEG	Polyethylene glycol
PMS	Phenazine methosulphate
PT	Pertussis toxin
SDS	Sodium dodecyl sulphate
SEM	Scanning Electron Microscopy
S_J	Jaccard Similarity Coefficient
Sp.	Species
S_{SM}	Simple Matching Similarity Coefficient
TCBS	Thiosulphate Citrate Bile Salts Sucrose
U	Units
µg	Microgram
µl	Microlitre
µm	Micrometre
UPGMA	Unweighted pair group method with averages
VP	Voges-Proskauer
v/v	Volume per volume
w/v	Weight per volume
XTT	2, 3 -bis [2-methoxy-4-nitro-5-sulfophenyl]-2-H-tetrazolium-5 carboxanilide inner salt

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SUMMARY

This study is concerned with the interactions between bacteria and commercially important bivalves. It is divided into two sections, the first part consists of the isolation and identification of bacteria collected from hatcheries at Reculver, Guernsey and Conwy in the U.K. Identification of the bacteria was carried out using phenotypic analysis combined with numerical taxonomy. Results showed that diverse microbial flora was detected in these shellfish farms, and that the principal groups consisted of *Vibrio, Enterobacter, Pseudomonas, Cytophaga, Flavobacterium, Moraxella, Micrococcus* and *Aeromonas* species. The distribution of these bacteria was as follows; *Vibrio* were predominant at Guernsey whereas *Enterobacter* and *Cytophaga* were the most common isolates at Reculver and Conwy. Antibiotic resistance patterns were performed inorder to establish the profile of bacterial resistance to commonly used aquaculture and medical important antibiotics. The outcome was a heterogenous resistance profile.

Part two includes the analysis and screening of the tentatively identified bacterial isolates for their toxicity to bivalve haemocytes and known pathogens. In parallel other isolates from France, Spain, Norway and Scotland were included in the study for comparative analysis. Isolates were subjected to cytotoxicity tests inorder to detect potential bivalve pathogens. A cytotoxocity test was developed and optimised for this purpose using natural conditions where possible. Alternative methods such as neutral red dye and molecular probes were used inorder to obtain better results but were not successful. Thus visual observation of haemocyte-bacteria interactions proved to be more reliable. Principally bacteria from *Vibrio* and *Cytophaga /Flavobacterium* produced marked effects on haemocytes. Further tests revealed that this effect was more prominent in filtered haemolymph when compared to filtered sterile sea water. This was probably due to the presence of factor(s) that may have played an important role in this reaction, which merit further investigation to elucidate their exact role. Species specificity of several bacterial pathogens of bivalves is reflected in their interaction with bivalve haemocytes.

1.1 HISTORY OF EUROPEAN BIVALVE AQUACULTURE.

Culture of fish and molluscs has a long history in Europe stretching back several centuries before the birth of Christ (cited by Kirk, 1990). Rearing techniques changed very little between the time of the Roman empire and the 18th century, when the possibilities for fish culture were greatly increased by the application of artificial fertilisation to the rearing of trout (Jacobi, 1765). The decline in natural populations of both shellfish and fish, along with the ever-increasing world population, has encouraged interest in aquaculture (Trust, 1986). It is clear that fishing cannot supply the increasing demand for luxury products as the natural stocks are overexploited and the hunt for these species has had a detrimental effect on the species that are caught at the same time and rejected (Barnabé, 1994).

Bivalves such as mussels, oysters and clams have been subjected to varying degrees of cultivation in different parts of the world, with high density commercial production being made possible by the development of intensive husbandry systems for the early life stage of the molluscs. In spite of the threat of pollution, uncertain profitability and various disasters which have affected aquaculture, the industry continues to develop. World production from aquaculture has more than doubled in 15 years from 6 million tons in 1976 (Nash, 1987) to over 16 million tons in 1992 (Barnabé, 1994). European aquaculture produces 1.3 million tons per year and is the second highest producing area in the world; also, France is one of the largest producers of molluscs in the world (Barnabé, 1994).

From the introduction of mussel farming in Galicia in the North-West of Spain in 1946, mussel production in Spain grew to 130,000 tons yr^{-1} within twenty years (Andreu, 1968). This area is now the main mussel producer in Europe, with an annual output of approximately 200 - 250,000 tonnes yr^{-1}.

Oyster culture dates back from the latter half of the 19th Century (Roche, 1898) and approximately 95 % of the present output is from France alone (Héral,

1990). The species most commonly reared in France up to 1969 was the Portuguese oyster, *Crassostrea angulata,* which had almost taken over from the native oyster, *Ostrea edulis,* until it too was virtually wiped out by disease in the late 1960s. The Japanese oyster, *C. gigas,* was then imported in large quantities to revive the French oyster industry (Héral, 1990). Since the introduction of *C. gigas,* around 1970, oyster growers suffered from a succession of problems such as the devastating effects described by Comps (1970, 1980), of the protozoan parasites, *Marteilia refringens* and *Bonamia ostreae.* Numerous oil tanker groundings polluted oyster beds in Brittany and the use of tributyl tin (TBT)-based paints on pleasure crafts also had an effect on the marked decline in the quality of oysters being produced (Anon, 1980).

Increased European production of clams has resulted from the increasing demand for market-sized *Tapes semidecussatus* and *T. decussatus* (Le Borgne, 1990). Worldwide demand for scallops has promoted research on *Pecten maximus* because natural settlement is poor, but much progress remains to be made on the conditioning of broodstock and on scallop larval diseases too (Franklin *et al.,* 1980; Dao, 1990; Thouzeau, 1991).

Annual importation of oysters into the United Kingdom from the U.S.A. ceased in 1939 and from France around 1970, following massive mortalities of the Portuguese oyster by viral gill disease (Spencer, 1990). The lack of supplies and the decline of natural oyster beds, together with a failure of natural spatfalls to replenish stock, stimulated the search for improved hatchery techniques for the production of oyster seed under a fully controlled environment at the Fisheries Laboratory, Conwy, U.K. (Spencer, 1990). In Scotland, six species of shellfish are cultured with reported annual production of 898 tonnes of mussels, 2.6 million Pacific oysters, 194,000 native oysters, 489,000 scallops and 1.5 million queen scallops (Fraser, 1993). Production of Pacific oysters, native oyster, scallops, and queens in Scotland rose by 10, 59, 55 and 1 % on previous years output respectively, whereas mussel production

fell by 12 %. There has been some experimental growth of *T. semidecussatus* in Scotland (Fraser, 1993).

1.2 HATCHERY CULTURE OF BIVALVE LARVAE AND JUVENILES.

Hatcheries are designed to control reproduction throughout the year (Loosanoff & Davis, 1963; Walne, 1966, 1970, 1974; Elston *et al.*, 1981; Drinkwater, 1987; Castagna & Manzi, 1989; Barnabé, 1990, 1994; Utting & Spencer, 1991). The various stages of culture require separate areas, to prevent the mixing of sea water and the potential for spreading disease or problems due to hygiene (Elston *et al.*, 1981). Figure 1 outlines the general aspects of bivalve hatchery culture. Bivalve species most commonly reared in the United Kingdom and Northern Europe are shown in Table 1 (Utting & Spencer, 1991).

1.2.1 Conditioning and Spawning of Broodstock.

Adult bivalves require a rich supply of food during conditioning. Unfiltered sea water offers a diverse and abundant supply of natural food, but additional cultured species of marine algae such as *Tetraselmis suecica, Thalassiosira pseudonana* and *Skeletonema costatum* have been used as food (Whyte *et al.*, 1990; Utting & Spencer, 1991).

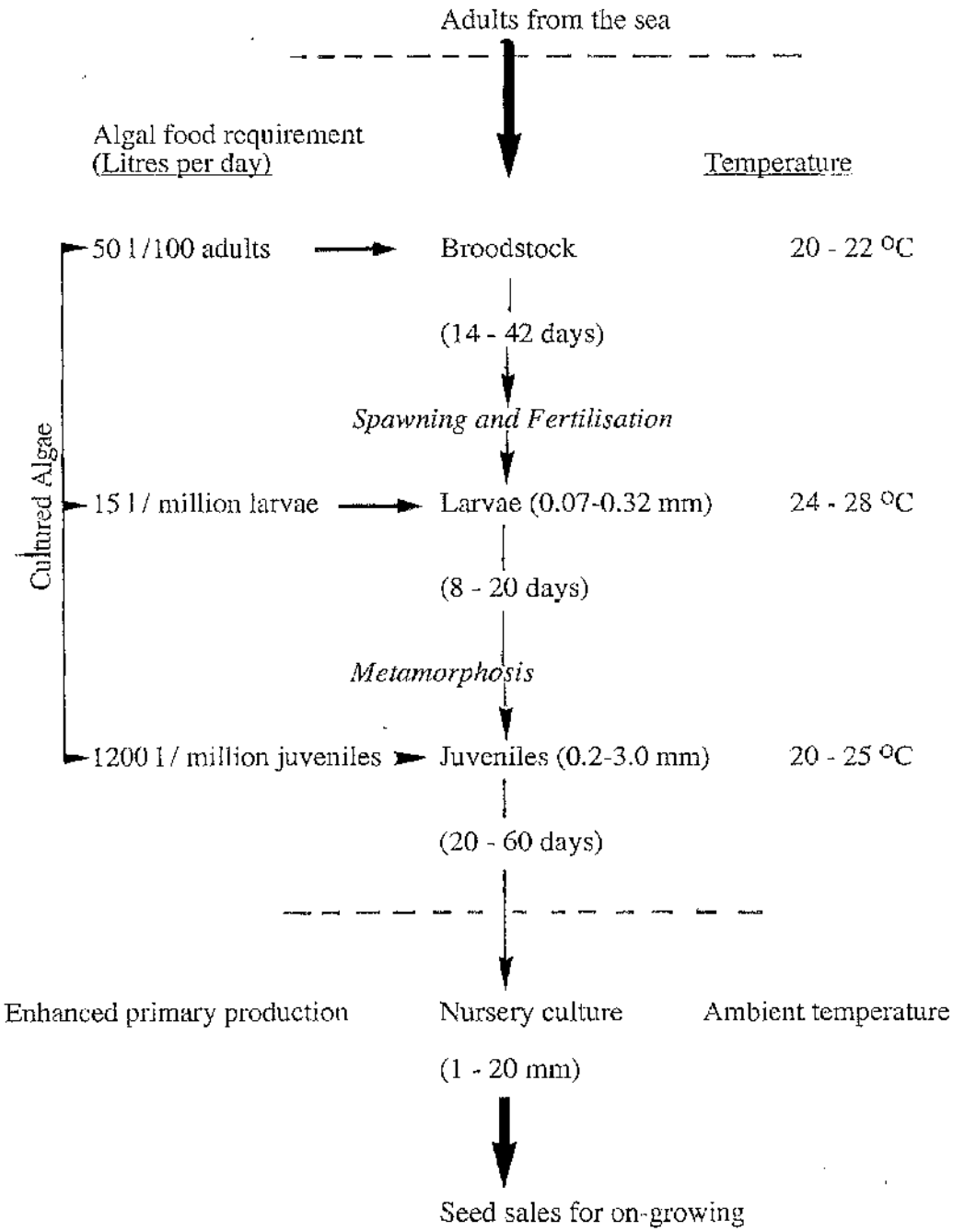
Female oysters (30 gram live weight) are capable of releasing about 1 million larvae (*O. edulis*) or 50 million eggs (*C. gigas*) per individual (Utting & Spencer, 1991). Small female clams (10 - 20 g live weight) will spawn 5 - 8 million eggs on average depending on their condition and the time of year (Spencer, 1990). Thermal (temperatures raised from 20°C to 28°C), chemical or physical shock and the addition of sexual products or ultraviolet radiation are used to induce spawning (Loosanoff & Davis, 1963; Walne, 1974; Morse, 1978; Utting & Spencer, 1991). Another spawning technique involves the removal of gametes and artificial fertilisation (Helm & Millican, 1977) with the exception of clams (gametes mature

TABLE 1 Commercial bivalve species reared in the U.K. hatcheries.

Species	Status	Year of introduction (source)
Crassostrea gigas Thunberg	Introduced	1965 (Canada)
(Pacific oyster)		1972 (Canada)
		1978 (U.S.A.)
Mercenaria mercenaria (quahog)	Introduced (Accidently ?)	1910-1920 (U.S.A.)
Ostrea edulis (European native flat oyster)	Native	____
Tapes decussatus (palourde clam)	" "	____
Tapes semidecussatus (Manila clam)	Introduced	1980 (U.S.A.)

Reference: Utting & Spencer, 1991.

FIGURE 1 Outline of hatchery culture.



Reference: Utting & Spencer, 1991.

during passage down the oviduct) and the larviparous species, *O. edulis* (Utting & Spencer, 1991).

1.2.2 Fertilisation and Embryonic Development.

Fertilised eggs develop into the fully shelled D-veliger stage within 24 hours (Utting & Spencer, 1991). Normal D-larvae should have a mean shell length of approximately 90 - 95 µm (small clams), 70 - 75 µm (*C. gigas*), 170 µm (*O. edulis*) and 100 - 250 µm (scallop), respectively (Dao, 1990; Utting & Spencer, 1991). Approximately 80 - 85 % of oyster eggs should develop successfully to the "D" shaped stage, although sometimes this can fall to 50 % (Loosanoff & Davis, 1963).

D-larvae are reared in heated filtered sea water (23 - 25 ^{o}C) and fed on mixed algal diets composed of *Pavlova lutheri*, (*Monochrysis lutheri*), *Isochrysis galbana*, *Chaetoceros calcitrans*, *Isochrysis* species and *Chaetoceros gracilies* (Helm *et al.*, 1979; Laing, 1979). Healthy larvae have a yellow-brown colouration with a dark digestive gland (Brown, 1973).

1.2.3 Growth and Metamorphosis of Larvae.

Rapid growth is observed in actively swimming larvae of clams, *C. gigas* and *O. edulis*, for up to days 8, 12 and 7 respectively (Utting & Spencer, 1991). Growth is reduced in clam larvae as they reach the pediveliger stage (approx. 200 µm shell length) when they gradually change to a more sedentary habit, crawl by means of a foot and undergo metamorphosis (approx. 230 µm shell length) to the juvenile or spat stage. Young spat crawl up vertical surfaces and cluster together using their byssal threads (Spencer, 1990).

Scallops spend 3 weeks as swimming larvae before they undergo metamorphosis and attachment to a support by the byssus. Juvenile scallop then spend up to 6 months growing from 250 µm to 25 - 30 mm in size (Dao, 1990). Afterwards they become free living for up to 1 year while growing from 25 mm to approximately

70 mm. The following year is spent sedentary, until maturation at 2 years old (Dao, 1990).

Before metamorphosis (at 280 - 300 μm), oyster larvae cement themselves permanently to a suitable substratum ('settlement' phase). When the spat reach 2 mm or more they can be transferred from the hatchery to the nursery, which is a transition point between the controlled environment (warm water enriched with food) and the wild conditions where they will finally mature to adults (Utting & Spencer, 1991).

1.3 MICROBIAL PROBLEMS IN AQUACULTURE.

1.3.1 Diseases in General.

All organisms including humans and animals, among which are fish and shellfish, are subjected to diseases. Shellfish are vulnerable to algal, fungal, viral, protozoan and bacterial disease. In this review, emphasis will be on viral, protozoan and bacterial diseases, in particular "vibriosis" in economically important shellfish.

1.3.1.1 Viruses in commercial shellfish.

Viral presence in commercial bivalves was unknown up until the early 1970s, when Farley *et al.* (1972) detected a herpes-like infection in the cell cytoplasm of oysters (*C. virginica*) subjected to elevated water temperatures. Approximately 20 presumptive viral infections have been described in marine molluscs (Table 2). Due to the lack of a stable molluscan cell culture system, none of the shellfish viruses have been adequately characterised beyond histopathology and ultrastructural morphology and in most instances the viruses have not satisfied Rivers' postulate (Sindermann, 1990).

Some viruses have been detected in shellfish affected by other diseases or under some form of environmental stress, so it is uncertain whether the viruses are primary pathogens or secondary invaders. An example of this was observed when an icosahedral virus appeared to be associated with velar erosion in *C. gigas* larvae, but

TABLE 2: Viruses affecting bivalves important in European aquaculture.

Host	Virus Type	Effect on Host	References
Ostrea edulis	IPN-like reovirus	Necrosis (digestive gland).	Hill (1976a, b), Underwood *et al.*, (1977), Hill & Alderman (1979).
Crassostrea gigas	Herpes-like (presumptive)	Associated with enzootic "summer disease" of *C. gigas*.	Alderman (1980), Buchanan and Richards (1982).
	Icosahedral virus	Oyster velar virus disease (OVVD).	Leibovitz *et al.*, (1978), Elston (1979, 1980), Elston & Wilkinson (1985).
	Iridovirus	Gill and palp disease.	Marteil (1968, 1969), Comps (1970, 1972).
	Icosahedral virus	Causes grayish discolouration of the visceral mass.	Comps & Bonami (1977), Comps (1978, 1980, 1983).
Crassostrea angulata	Iridovirus	*Maladie des branchies*.	Comps (1969, 1970, 1972, 1978), Comps & Duthoit (1976), Marteil (1968, 1989).
	Irido-like virus	Destruction of connective tissue.	Comps *et al.*, (1976), Marteil (1976), Bonami (1977).
Mercenaria mercenaria	Herpes-like	Role in neoplasia not determined.	Barry & Yevich (1972), Farley (1978).

the etiology was confused by the effects of fungus-like organisms which seemed to be responsible for gastrointestinal impactions in the same shellfish (Leibovitz *et al.*, 1978; Elston, 1979, 1980). Later, Elston & Wilkinson (1985) reported specific viral effects on the velum. Recently, Nicolas *et al.* (1992a) found a herpes-like virus infecting hatchery-reared Pacific oyster larvae, after abnormal mortality and morbidity were noted. A similar virus was found associated with further outbreaks in 1992 and 1993 in *C. gigas* (Le Deuff *et al.*, 1994) and in *O. edulis* (Comps & Cochennec, 1993) in French hatcheries. The herpes-like virus found in *O. edulis* 5-month-old spat was suspected to be responsible for the 90 % mortality observed in these animals (Comps & Cochennec, 1993). When *O. edulis* was inoculated with the herpes-like virus it produced 80 % mortalities in young spat, whereas larval stages appeared to be more resistant (Comps & Cochennec, 1993).

Studies of oyster viral diseases which affect survival have been given priority and epizootics caused by several iridoviruses are probably the most significant. The first outbreak of viral disease occurred in *C. angulata*, on the French coast during 1966 - 67 and was initially attributed to several protistan pathogens. Later a viral (iridovirus) etiology was proposed by Comps & Duthoit (1976). When *C. angulata* stocks were seriously reduced in 1970, iridovirus, gill necrosis virus and haemocytic virus were implicated (Comps, 1988). A more resistant species, *C. gigas* was introduced in large quantities to ensure survival of oyster culture in France (Comps *et al.*, 1976; Marteil, 1976; Bonami, 1977; Comps, 1970, 1978). An apparent decrease in the resistance of *C. gigas* to the iridovirus was later described by Comps (1980).

A B-type retrovirus has been isolated from the soft-shell clams, *Mya arenaria* and the neoplastic disease has been reproduced experimentally in clams (Oprandy *et al.*, 1981, Oprandy & Chang, 1983). Molluscan viruses other than the iridovirus and the retrovirus previously mentioned can cause pathology but lethality has usually been demonstrated only under abnormal conditions. Other viruses isolated from *C.*

virginica, include a herpes-type (Farley, 1973), papovavirus (Farley, 1973, 1975, 1976, 1978) and a reo-like virus (Meyers, 1979; Meyers & Hirai, 1980).

1.3.1.2 Protozoan diseases in shellfish.

According to Sparks (1985), protozoan parasites are responsible for significant occurrence of disease in invertebrates. Extensive scientific research has been carried out with specific emphasis on *O. edulis* and *C. virginica*. Protozoans such as *Haplosporidium nelsoni* (Haskin *et al.*, 1966; Couch *et al.*, 1966; Farley, 1968; Andrews & Frierman, 1974; Haskin & Andrews, 1988), *Haplosporidium costale* (Andrews & Castagna, 1978) and *Perkinus marinus* (Mackin *et al.*, 1950; Ray, 1952; Mackin & Boswell, 1956; Perkins, 1976), parasitize the oyster *C. virginica*. Delaware Bay, Seaside and Dermo diseases, were associated with the parasites *H. nelsonii*, *H. costale* and *P. marinus* respectively (Sindermann, 1990).

Infected oysters suffer weight loss, and in the case of *H. nelsoni* the initial infections occur in the gill and palp epithelia, spreading later to adjacent connective tissue, provoking infiltration of hyaline haemocytes (Farley, 1968) and in affected areas mortalities exceed 95 % (Andrews, 1964). *H. costale* infects the digestive gland tubule epithelium and, unlike *H. nelsoni*, this parasite sporulates synchronously in the host connective tissues; thus, mortalities (20 - 50 %) may occur 10 months after exposure to *H. costale* (Andrews & Castagna, 1978). Finally, *P. marinus* infections occurs in high salinity waters and provokes intensive leukocyte invasion of tissues, necrosis of the digestive tract epithelium and terminal systemic infections (Mackin, 1951; Perkins, 1976; Andrews, 1988).

Several haplosporidian spp. have been reported as rare parasites of other species of oysters, but were not recognised as severe pathogens. They were first identified in Dutch flat oysters by van Banning (1979) and later in French oysters by Cahour *et al.* (1980). Further reports of this type of parasite in *O. edulis* were made by Bachére & Grizel (1982) and in *C. gigas* (Kerns, 1976).

European oysters have had recent problems with *M. refringens* and *B. ostreae*. Comps first reported *M. refringens* in 1970. It is associated with 'digestive gland disease' (Aber disease) and exerted serious pathological affects on the intestine and digestive tubule (Grizel & Tige, 1979; Aufret & Poder, 1985; Figueras & Montes, 1988). This parasite has been observed without severe side-effects in *C. gigas* (Cahour, 1979) and *M. edulis* (Comps *et al.*, 1975).

Bonamia was first identified in France in 1979 (Comps, 1980). It produces the disease 'bonamiasis' in *O. edulis* which is characterised by yellow discolouration of the oysters, extensive gill lesions, the presence of 'microcells' and a massive host cellular response (Comps *et al.*, 1980; Andrews, 1988; Grizel *et al.*, 1988). Bonamiasis, spread rapidly within dense oyster populations (Elston, 1986). Associated mortalities have been reported from Spain, Denmark, the Netherlands, the United Kingdom and the United States (van Banning, 1979, 1985; Bucke & Feist, 1985; Elston *et al.*, 1986; Auffret & Poder, 1987; Montes, 1991). Exposure of several genera of molluscs (*C. gigas, M. edulis, T. decussatus* and *T. semidecussatus*) to *Bonamia* did not result in infections. *Ostrea* species appear to have very poor resistance.

Comps *et al.* (1987) reported that the oocytes of *C. gigas* were invaded by the parasite *Marteilioides chungmuensis,* and Elston (1986) observed rickettsia-like infections of bivalves, but no severe host response or pathological effect were found in either case. Haplosporidian and *Perkinus*-like parasites have been observed in *T. decussatus* (Chagot *et al.*, 1987a, 1987b). Characteristics of the latter were uncommon in comparison with the similar protozoosis known in marine bivalves. A *Perkinus* sp. similar to *P. marinus* was reported to be widespread in bivalve molluscs from Italian coasts and was associated with heavy infections and mortalities in *T. decussatus* (Daro & Canzonier, 1985).

1.3.1.3 Shellfish associated bacteria.

Bacteria are ubiquitous in the marine environment; some are opportunistic pathogens for weakened animals and a few may be severe primary pathogens (Austin, 1988). Originally, bacteria may enter the semi-closed system of mollusc hatcheries or nurseries and expand in numbers either in algal cultures or be present elsewhere in the system. A wide variety of bacteria may exist in the husbandry systems (Leibovitz, 1978). Few bacterial groups (Table 3) have been identified as the causative agents of disease in shellfish. Bacterial pathogens, such as *Vibrio anguillarum* (Hoff, 1989), the causative agent of vibriosis which affects both fish and shellfish, are not restricted to marine environments. They are found in both marine or freshwater (Hoff, 1989), whereas *Aeromonas hydrophila*, which produces septicaemia, has only been isolated from freshwater (Allen *et al.*, 1983). Bacterial pathogens have been observed in the wild as well as in farmed fish and shellfish, causing huge economical losses in the latter (Paillard *et al.*, 1989).

Marine animals are subject to a wide spectrum of diseases of infectious and non-infectious etiology. Some highly virulent bacteria may be the primary cause of diseases; these should be distinguished from other species which cause bacterial infections secondary to the initial lesions caused by mechanical damage or parasitic infestation. It has been reported that bacteria are rarely the primary cause of disease in invertebrates (Paillard *et al.*, 1989).

Extensive bacterial growth in confined habitats, such as aquaculture tanks and ponds, can lead to massive populations of opportunistic pathogens such as vibrios (Sindermann, 1990). This does not exclude the potential role for virulent primary pathogens which are capable of sweeping through captive or wild shellfish stocks.

The taxonomic composition of the microflora appears to be quite varied. Austin *et al.* (1987) identified the bacteria from bivalve larvae as *Caulobacter, Flavobacterium, Hyphomicrobium, Prosthecomicrobium* and *Vibrio*. Colwell and

Liston (1960) determined that the natural microflora of *Crassostrea gigas* comprised mostly *Achromobacter, Flavobacterium, Pseudomonas* and *Vibrio*. Also, Lovelace *et al.* (1968) reported a diversity of bacterial taxa recovered from *C. virginica* which include *Achromobacter, Bacillus,* Coryneforms, *Cytophaga-Flavobacterium,* Enterobacteriaceae, *Micrococcus, Pseudomonas* and *Vibrio*. Among the vibrios associated with the mortalities of larvae and cultivated bivalves, *Vibrio anguillarum, V. parahaemolyticus* and *V. alginolyticus,* were commonly found during outbreaks of disease. However, there is controversy over the role of *V. parahaemolyticus* as a fish pathogen. Sindermann (1990) reported that the characteristics of the most bivalve pathogenic *Vibrio* isolates corresponded very closely to those of *V. alginolyticus. V. tubiashii* was also shown to be pathogenic to shellfish (Hada, *et al.*, 1984). Numachi *et al.* (1965) reported that uncharacterised Gram-positive bacteria were implicated in bivalve diseases. Sindermann (1990) reported that many vibrios are strongly proteolytic, chitinolytic and halophilic, which probably accounts in part for their success as opportunistic pathogens of marine species in culture.

Vibriosis ('bacillary necrosis') is recognised as the most important disease of bivalve larvae in aquaculture. It was first described in *C. virginica* and four other bivalves by Tubiash *et al.* (1965). Adult bivalves exposed to high densities of *Aeromonas* and *Vibrio* were unaffected, while lower concentrations produced disease in larvae (Tubiash *et al.*, 1965). Martin (1976) reported that adult mussels survived exposure to *V. anguillarum,* while larvae died even at low concentration levels.

Many aspects of vibriosis have been studied (Garland, 1959; Tubiash *et al.*, 1965; Murchelano & Bishop, 1969; Tubiash *et al.*, 1970; Helm & Smith, 1971; Brown, 1973; Tubiash, 1975; Brown & Losee, 1978; Di Salvo *et al.*, 1978; Elston & Leibovitz, 1980; Elston *et al.*, 1981; Jeffries, 1982; Garland *et al.*, 1983; Brown & Roland, 1984; Nottage & Birkbeck, 1986, 1987a, 1987b, 1990; Nottage *et al.*, 1989; Birkbeck & Gallacher, 1993) including an outbreak of the disease in a New York oyster hatchery, described by Leibovitz (1979), Elston & Leibovitz (1980), and

TABLE 3 Shellfish pathogens.

Pathogen	Disease	Host range	Ecology
Vibrio sp.	Larval infections	Molluscs	Ubiquitous marine
Vibrio anguillarum	Vibriosis	Fish & shellfish	Ubiquitous marine
Aeromonas hydrophila	Septicaemia	Fish & shellfish	Freshwater
*F. columnaris**	Columnaris disease	Fish & shellfish	Freshwater
F. branchiophilum	Gill disease	Fish & shellfish	Freshwater
Mycobacterium sp.	Tuberculosis	Fish & shellfish	Marine
Streptococcus sp.	Septicaemia	Fish & shellfish	Marine

* F:- *Flexibacter*

After Sindermann (1990)

Leibovitz & Elston (1980), where larval mortalities were extensive. Di Salvo *et al.* (1978) reported that infections in *C. gigas* larvae which resulted in persistent mortalities were attributed to *V. anguillarum*. Bacillary necrosis has been identified by Castagna (1975) in the bay scallop (*Argopecten irradians*).

The effects of bacteria may vary with the species of bivalve larvae being tested or with the stage of larval development. Loosanoff (1974) found that different species of bivalve larvae varied in susceptibility to infection by defined bacterial isolates. Brown (1973) studied the effects of *Vibrio* and *Pseudomonas*, and the influence of the larval developmental stage and/or bivalve larval species in relation to susceptibility to disease. Some of the test isolates were found to be pathogenic for *C. virginica* embryos, but had less effect on presetting larvae. Abnormal embryonic development, decreased growth, or larval death before reaching the veliger stage, were additional effects after exposure to particular strains of *Vibrio* and *Pseudomonas* (Brown, 1973, 1981). A prodiginine pigment produced by a red pigmented marine *Pseudomonad* and *V. psychroerythrus,* was toxic to embryos and larvae of *M. mercenaria* and *C. virginica* (Brown, 1974, 1981). Helm and Smith (1971) described an outbreak of disease that resembled bacillary necrosis in *O. edulis* at a hatchery at Conwy, Wales, where a highly pathogenic strain of *Pseudomonas* was identified as the causative organism.

Brown (1981) reported an outbreak of disease in *C. virginica* larval populations in which the disease could be initiated experimentally by adding very low numbers of the highly pathogenic *Vibrio* sp. Further studies of infected oyster larvae at another hatchery identified a *Vibrio* sp. as the causative organism which was present in low numbers in the larvae of *C. virginica* (Brown & Losee, 1978). Leibovitz (1978) reported that outbreaks of vibriosis were associated with peaks in abundance of *Vibrio*.

Three types of pathogenic effect associated with vibriosis were described by Elston & Leibovitz (1980); these involved progressive mantle disruption, severe velar deformation and damage, and progressive visceral lesions and atrophy. Very limited information is available on the effects of *Vibrio* and other bacteria on wild populations of shellfish.

Tubiash *et al.* (1973) reported that *Vibrio* infections in adult oysters were rare and showed that *V. anguillarum,* isolated from pericardial fluids in high densities, proved to be pathogenic to oyster larvae but not to adults. *V. parahaemolyticus* was a consistent isolate obtained from oysters which died in laboratory tanks after being removed from commercial beds (Lipovsky & Chew, 1971), and these infections were thought to be temperature related. *Vibrio* infections have been provoked by environmental stressors such as high temperatures (Lipovsky & Chew, 1971, 1972; Grischkowsky & Liston, 1974), oxygen saturation (Elston & Lockwood, 1983) and high larval population densities (Brown, 1973).

Complete elucidation of the effects of bacteria on bivalve molluscs has been complicated due to uncertainties in the taxonomy of important facultatively pathogenic bacterial groups, especially *Vibrio, Pseudomonas* and *Aeromonas* (Sindermann, 1990). Pathogens may be part of the normal marine flora and may be present in the tissues of presumably healthy animals. Vibrios and other bacterial groups consist of numerous strains, biovars and biotypes, and the numbers recognised is continuously expanding (Regal, 1986, Ortigosa *et al.*, 1989; Pujalte *et al.,* 1993; Onarheim *et al.,* 1994; Ortigosa *et al.*, 1994; Ishimaru *et al.*, 1995). The genus *Vibrio* has currently more than 35 species, most of which are of marine origin (Holt *et al.*, 1994). In addition to the 22 *Vibrio* species described in *Bergey's Manual of Systematic Bacteriology* (Kreig & Holt, 1984), 14 new species have been recognized.

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Opportunistic bacterial diseases, particularly vibriosis, are presently the most significant diseases. Bacteria flourish in culture systems and subsequently cause disease, both by direct bacterial infection and by toxin-mediated processes (Nottage &

Birkbeck, 1986). Primary disease, in metamorphosing and juvenile animals, involves direct infection and growth of bacteria in ligament and soft tissues (Elston, 1984).

Bacterial diseases of larval and juvenile molluscs are the most important diseases caused by opportunistic bacterial pathogens and can severely depress bivalve mollusc production (Elston, 1984). Vibriosis is the most significant of these bacterial diseases (Tubiash *et al.,* 1965, 1970; Brown, 1973; Brown & Losee, 1978; Elston & Leibovitz, 1980; Brown & Roland, 1984; Nottage & Birkbeck, 1986). Experimental studies of vibriosis have been carried out in larval and juvenile bivalves (Leibovitz, 1978; Elston & Leibovitz, 1980; Elston *et al.,* 1981; Brown, 1981; Elston *et al.,* 1982; Jeffries, 1982; Garland *et al.,* 1983; Brown & Roland, 1984; Nottage & Birkbeck, 1986, Nottage *et al.,* 1989) and the disease in juvenile molluscs is more significant and costly in intensive culture. It has been suggested that bacteria pathogenic to bivalve larvae in culture may not be pathogenic in natural populations (Brown, 1973).

The first description of bacterial disease in larval molluscs was in the United Kingdom by Walne (1958). Later, it was described as occurring in continental Europe (Le Pennec & Pricur, 1977), North America (Elston *et al.,* 1982) and Australia (Garland *et al.,* 1983). In outbreaks of vibriosis in British hatcheries the causative organisms were identified as *V. anguillarum* (Austin *et al.,* 1988) and *V. tubiashii* (Jeffries, 1983). Other factors such as temperature, salinity and depletion of food may debilitate oyster larvae and make them more susceptible to vibriosis (Nottage *et al.,* 1989). In contrast, debilitated larvae and juveniles not exposed to vibrios survived (Nottage *et al.,* 1989).

Although bacterial diseases are not frequent in adult bivalves, Paillard *et al.* (1989) described one particular disease, Brown Ring Disease (BRD) in the Manila clam, *Tapes phil ippinarum. Vibrio* P1 was identified as the pathogenic bacterium associated with this disease by reproduction of the disease in healthy individuals (Paillard & Maes, 1990). BRD symptoms are localised at the edge of the shell and bacteria have been observed by electron microscopy in the digestive gland of *Vibrio*

P1 infected clams (Plana & Le Pennec, 1991). Maes and Paillard (1992) have demonstrated that this pathogen is not specific since *T. decussatus* and other Veneridae can also present the symptoms of BRD, with less mass mortalities. The symptoms of the disease have been observed in several European aquaculture sites since 1987 and the disease has caused reduced performance in aquaculture of clams (Oubella, *et al.,* 1993).

Other bacterial genera have also been implicated as possible pathogenic agents in larval diseases including *Pseudomonas* (Brown, 1973) and those included in the genus *Alteromonas* (Garland *et al.,* 1983). Vibrios are considered the most important group but further work is required to determine the significance of these other bacteria. There are still some poorly described vibrios which may be important disease causing agents (Elston, 1984).

1.4 BIVALVE DEFENCES AGAINST BACTERIA.

Sparks (1981) observed that to induce disease bacterial pathogens needed to develop mechanisms to escape phagocytosis, survive phagocytosis or overwhelm stressed hosts. The immune systems of larval and juvenile bivalves are underdeveloped and because it is very difficult to obtain samples in large quantities, most research has been carried out on the defense mechanisms in adult bivalves. Bivalve embryos are extremely vulnerable to attack, and this vulnerability is marginally reduced as D-larvae undergo shell formation and maturity. D-larvae may have limited protection because of their constant filtration of particles and swimming activity. Veliger larvae are partially protected by their possible inability to ingest most naturally occurring bacteria (>1 μm) (Bayne, 1983). Douillet (1993a) reported a bacterium which enhanced survival and growth of the larvae of *C. gigas.* He also noted that bacterial carbon contribution decreased with increasing size of larvae (Douillet, 1993b). Therefore, he concluded that during a particular stage of larval growth, the larvae are more vulnerable to potentially pathogenic bacteria if the larval diet has a bacterial dependency phase.

Mature shellfish possess external (shell) as well as internal (ciliary activity of the gill) defences against invasion by infectious agents of all types. Extensive literature has been published on the internal defence mechanisms of invertebrates. The internal defence network, contained within an open vascular system which includes a heart, is composed of both cellular (Stauber, 1961; Cheng, 1981; Feng, 1988) and humoral (Tripp, 1966; Feng, 1967; Renwrantz *et al.*, 1981; Nakamura *et al.*, 1985; Leippe & Renwrantz, 1988) components. They act together to diminish the effects of, and eliminate, invading agents. Haemocytes not only function in cellular defense but also in wound healing, collagen deposition, aggregation and shell repair (Cheng, 1981).

According to Read (1958), susceptibility (degree of vulnerability of animal to penetration and establishment of the pathogen) and resistance (responses of animals to invasion by infectious agent) are two factors a pathogen must be able to overcome to infect and cause disease. Mechanisms of resistance may include cellular or humoral responses.

Phagocytosis and haemocyte infiltration are the principal cellular defences of shellfish (Cheng, 1981), with lysosomal enzyme digestion within phagocytic vacuoles (Cheng & Rodrick, 1974; McHenery *et al.*, 1979; Cheng, 1981; Birkbeck & McHenery, 1982; McHenery & Birkbeck, 1985) and in the haemolymph assuming a critical role in killing microorganisms. Accessory cellular responses in shellfish (Cheng & Rifkin, 1970) include haemocytosis (mobilization, infiltration and aggregation of haemocytes), thrombosis (cellular and extracellular clot formation), encapsulation (of invaders too large to be phagocytosed) and nacrezation (secretion by the mantle of nacre around foreign bodies). Indigestible particles are transported across the epithelial surface by phagocytes (Tripp, 1960; Cheng, 1967; Feng, 1967; Cheng, 1981). Foley & Cheng (1975) noted that all types of haemocytes could be phagocytic but that granular cells played the most active role. Haemocyte cell cytoplasmic granules are possible lysosomes that store acid hydrolases (Yoshino &

Cheng, 1976). Cheng (1981) reviewed the literature on the functional morphology of bivalve haemocytes, and proposed a scheme that placed haemocytes into three categories, namely granulocyte, halinocytes and serous cells.

Phagocytosis involves the recognition, adherence, uptake, destruction, and dispersal of foreign particle. Several phagocytosed parasites such as *P. marinus* (Mackin, 1951) and *H. nelsoni* (Farley, 1968) have been observed. Bang (1961) studied bacterial phagocytosis in molluscs and found that bacteria adhered to haemocytes before phagocytosis. He also reported that certain bacteria were not phagocytosed (Bang, 1961). After phagocytosis, the bacteria were either digested within the phagosomes or they remained within the cell cytoplasm, where they multiplied and eventually killed the host cell (Sindermann, 1990).

Humoral defences of molluscan shellfish consist of pre-existing, and in some instances induced, lysins and agglutinins, augumented by an array of lysosomal enzymes in haemocytes and haemolymph (Ratcliffe & Rowley, 1981; Auffret, 1988; Feng, 1988; Fu-Lin, 1988; Olafsen, 1988; Sindermann, 1990; Olafsen *et al.,* 1992). Cellular responses are involved indirectly in humoral responses because cellular secretion, fragmentation or biochemical alteration confer bacteriostatic, lytic or other properties on the haemolymph (Stauber, 1961). Feng (1967) summarised evidence for a wide range of natural lectin-like agglutinins in molluscs. Tripp (1966) reported oyster haemagglutinins to be heat labile, with an opsonic effect on the phagocytosis of rabbit erythrocytes by oyster haemocytes *in vitro*. Immunoglobulins are not produced in mollusca or any invertebrates, but other humoral (cell products) factors that are protective against certain diseases have been identified (Anderson, 1981).

Lysins, and agglutinins (multivalent, non-immunoglobulin proteins) which bind carbohydrate (Olafsen, 1988), are common components of molluscan haemolymph. They appear to be involved in the response to invasion by microorganisms by acting as opsonins and enhancing phagocytic activity (Arimoto & Tripp, 1977: Leippe & Renwarntz, 1988). According to van der Knaap *et al.* (1981)

haemocytes synthesize the agglutinins or opsonins which are expressed on the cell surface as receptors for foreign material. Another non-specific defense factor, hydrogen peroxide was shown to be produced *in vitro* by bivalve haemocytes in both resting and stimulated states (Nakamura *et al.*, 1985; Pipe, 1992). Cytotoxicity of molluscan haemocytes has been reported in mussels and clams (Wittke & Renwrantz, 1984; Leippe & Renwrantz, 1988).

MacDade & Tripp (1967) identified lysozyme in the haemolymph of oysters which lysed certain Gram-positive bacteria (*Bacillus megaterium* and *Micrococcus lysodeikticus*). Lysozyme-like activity has been identified in many invertebrate species as a basic protein with a molecular weight of approximately 15, 000 daltons (MacDade & Tripp, 1967a, 1967b; Cheng *et al.*, 1975). Other serum enzymes (acid and alkaline phosphatases, aminopeptidase, β-glucuronidase, hexosaminadase, lipase and non-specific esterases) may act on their respective substrates, which may include pathogens (Cheng & Rodrick, 1975; Cheng & Yoshino, 1975). Antimicrobial substances have been identified in haemolymph and tissue extracts of oysters and clams (Fu-Lin, 1988). They were protein or glycoprotein and were found in serum, haemocytes or both (Fu-Lin, 1988).

Although these lysosomal enzymes may be an integral part of the defence system in bivalves, Cheng (1981) noted that low levels have been detected in unchallenged bivalves. Hardy *et al.* (1977) demonstrated a primary response in oysters consisting of an increase in antibacterial agglutinins in the haemolymph after initial exposure, but not in several gastropods. Acton & Evans (1968) reported secondary responses in oysters in that a faster clearance of bacteriophage was detected. This was not observed in gastropods tested (Nelstrup *et al.*, 1968). The oysters were later exposed to, and rapidly cleared, an antigenically unrelated bacteriophage without primary stimulation; this implied that the response was non-specific. Evidence for inducible humoral protective factors in molluscs is very limited. Weinheimer *et al.* (1969) could not demonstrate an inducible bactericidin in

oysters. Exposure of oyster and soft-shelled clam haemocytes to *B. megaterium, in vitro,* induced elevated intracellular aminopeptidase and lysozyme activity (Yoshino & Cheng, 1976). Feng and Canzonier (1970) pointed out that the failure to demonstrate humoral responses may have been because the experimental animals were injected with a variety of fluids or particles whereas humoral responses to natural infections were not observed to the same extent.

Boman (1995) decribed broad spectrum innate immunity in insects, for example *Drosophila* and the pupae of two giant silk moths (*Bombyx* and *Cecropia*). Associated antibacterial factors called cecropins were first isolated in 1980 in insects after bacterial infections. Steiner *et al.* (1981) observed the primary structure of these insect cecropins and reported that they were specifically antibacterial and unable to attack insect or mammalian cells; they can function without either high specificity or memory. Host destruction by cecropins is also avoided by cellular compartmentalisation or by specificity of the cecropins for a microbial target that is absent in the host. These peptide antibiotics curb, delay, and avoid microbial growth shortly after infection.

Two insect cecropins A and B, were highly active against different strains of *Escherichia coli* and other Gram-negative bacteria like *Salmonella typhimurium, Acinetobacter calcoaceticus, Pseudomonas aeruginosa* and some Gram-positive organisms like *B. megaterium* (Boman, 1995). Cecropins are not contained in phagocytes but induced as a response to a bacterial infection and secreted into the haemolymph; cytotoxicity of these anti-bacterial factors is greater than tetracycline (Boman, 1995).

1.5 PATHOGENIC MECHANISMS.

Both direct infection of tissues by bacterial cells (Elston & Leibovitz, 1980; Elston *et al.*, 1982) and bacterial toxin-mediated disease (Brown, 1973; Brown & Losee, 1978; Elston & Leibovitz, 1980; Jeffries, 1982; Brown & Roland, 1984; Nottage &

Nottage & Birkbeck, 1987a, 1987b; Nottage *et al.*, 1989) have been reported. The two modes of pathogenesis involved are neither distinct nor always clear cut. Experimental evidence (Elston & Leibovitz, 1980) as well as trials in production hatcheries (Jeffries, 1982) have supported the theory that the disease is complex, requiring both bacterial cell infection and toxic metabolites to cause the disease. As larval molluscs increase in size and age they become less susceptible to the effects of bacterial toxins (Tubiash *et al.*, 1965; Helm & Smith, 1971; Elston & Leibovitz, 1980; Jeffries, 1983; Nottage & Birkbeck, 1986).

Pathogenesis of vibriosis in both larval and juvenile bivalves has been studied by Elston & Leibovitz (1980), Elston *et al.* (1982), Jeffries, (1983), Elston, (1984), Brown & Roland, (1984), and Nottage & Birkbeck (1986, 1987a, 1987b), Nottage *et al.*, (1989). Elston & Leibovitz (1980) and Elston *et al.* (1981) summarised the mechanisms of infection of larvae and juveniles as follows. In larvae, the bacteria attach to and grow along the periostracum. Pallial infections begin at the shell periphery and ligaments, with eventual progressive infection of the soft tissues. Water-soluble toxins mediate the disease and resistance increases with age. Foci of bacteria are located in the digestive gland. In juvenile bivalves, the course of infection involves the attachment of the bacteria to the periostracum followed by liquefaction and erosion of the ligament. Pallial infection progresses from ligament and peripheral shell borders with eventual branchial and systemic infection (Elston, 1984). Elston (1984) suggested that the infection proceeded from the shell surface to the ligament, to the mantle and the gill tissues. The disease appears to be irreversible once it has reached the mantle tissue. Bacterial presence on the shell surface is not unusual and therefore is not indicative of disease although it may be a sign of potential disease (Elston, 1984).

Bivalve larval and juvenile diseases are very significant depressants of production in large scale rearing facilities. Some success has been achieved in limiting the disease by chemotherapy (Kraeuter & Castagna, 1984) and this suggests

that the bivalve mortalities are related to the disease mechanisms described. Metamorphosis and the early post-metamorphic life stages are critical phases in the mollusc culture. Surface associated bacteria may be responsible for retardation of normal shell growth and calcium deposition (Elston *et al.*, 1982). Microbial flora control of the surfaces of the culture systems during metamorphosis and early post-metamorphosis is essential for successful mollusc culture.

Nottage & Birkbeck, (1986) observed vibriosis in larvae and noted specific characteristics of the disease that involved bacterial attachment, and invasion resulted in rapid large scale tissue damage which lead to larval mortalities in excess of 90 % within 24 hours of exposure. In order to prevent problems which limit hatchery production of bivalve larvae and conquer vibriosis, the mechanisms of the disease must be deciphered. These mechanisms have not been defined although it has been suggested that toxins play a major role.

The mode of infection and the pathogenic mechanisms of these bacteria are still obscure, it has been reported that the pathogenicity of *V. anguillarum* involves adhesion of the organism to the animal (Kanno *et al.*, 1990) and the mechanism of its virulence has prompted extensive work. Crosa *et al.* (1977) reported that virulent *V. anguillarum* contains a plasmid which encodes for siderophores, thus, invading bacteria may multiply in the host by scavenging successfully for the iron which is bound by high affinity iron-binding proteins. Also, possible interactions of exotoxins and/or endotoxins of *V. anguillarum* have been implicated in virulence (Bullock and Conroy, 1971). Inamura *et al.* (1984), De La Cruze and Muroga (1989), Munn, (1978, 1980) demonstrated that haemolysins were involved in the virulence of *V. anguillarum*. These were described as thermolabile enzymes with an estimated molecular weight of 191 kDa, also a 36 kDa zinc metallo protease was found to play a role in the virulence and has been associated with invasion processes (Inamura *et al.*, 1984; Kanemori *et al.*, 1987; Norquist *et al.*, 1990). Several toxins and products expressed by other bacteria were described to cause fish mortalities in laboratory

trials and the mechanisms of action of these toxins are still unclear. *Cytophaga* representatives were reported to cause disease in fish via extracellular products (ECPs), haemolysin, proteases, neuromuscular toxins of unknown nature and possibly collagenases (Pacha, 1968; Meyer *et al.*, 1969; Mudarris and Austin, 1989). Also proteases and lipases produced by *Pseudomonas* were reported to be involved in the virulence of these organisms in fish (Li & Traxler, 1971). Another vibrio isolate, *V. vulnificus* expressed and excreted several virulence factors which include haemolysins, lipases, phospholipases and proteases (Amaro *et al.*, 1992). *Aeromonas* representatives (*A. salmonicida, A. hydrophila*) produced many virulence factors such as A-layer, haemolysins, glycerolphospholipid:cholesterolacyl transferase and acetylcholinesterase (Belland & Trust, 1985; Titball & Munn, 1981; Buckley *et al.*, 1982; Massad *et al.*, 1991; Nieto *et al.*, 1991). Few workers reported the involvement of bacterial toxins in bivalve disease. Production of a water soluble, heat-stable toxin by *V. anguillarum* inhibited larval *O. edulis* and contributed to mortality was demonstrated by Di Salvo *et al.* (1978). A heat-labile extracellular product produced by *V. anguillarum* and lethal to oyster larvae was identified by Jeffries (1983), Brown & Roland (1984) and Nottage & Birkbeck (1987). Brown & Roland (1984) suggested that this specific toxic factor had a molecular weight of 68 kDa.

Mortalities of shellfish were more prevalent at higher temperatures. High density rearing conditions may have been conducive to localised areas of low oxygen tension, which is advantageous for growth of facultative anaerobic vibrios and may be associated with their pathogenicity (Elston *et al.*, 1982)

According to Elston (1984) no biochemical characteristics of *Vibrio* have been linked with their pathogenicity. However, Jeffries (1982) correlated the ability of *V. tubiashii* to ferment sucrose and degrade xanthine with pathogenicity (Hada *et al.*, 1984).

Species of vibrio such as *V. anguillarum* and *V. alginolyticus* have been implicated in molluscan pathology. *V. alginolyticus* has been associated with the

typical shell surface attachment phenomenon and it is an extremely common coastal marine bacterium (Elston, 1984).

Nottage & Birkbeck (1987a, 1987b) reported that virulent strains of vibrio produced heat-labile proteinases (M. W. 39 and 41 kDa) which were lethal to spat and capable of rapid degredation of *Mytilus* gill tissue; the same authors identified a second factor which consisted of a heat-stable ciliostatic toxin of low molecular weight (< 5 kDa) (Nottage & Birkbeck, 1986, 1987a, 1987b). Among these proteinases, a gelatinase and a caseinase were identified by Simidu & Tsukamato (1985). Moreover *V. alginolyticus* has been shown to produce 5 serine proteinases, a collagenase (Kothary & Keil, 1978; Hare *et al.*, 1983) and heat-labile toxin (Di Salvo, 1978; Jeffries, 1983; Brown & Roland 1984). Inamura *et al.* (1985) suggested that proteinases produced by *Vibrio* strains were implicated in causing vibriosis in fish.

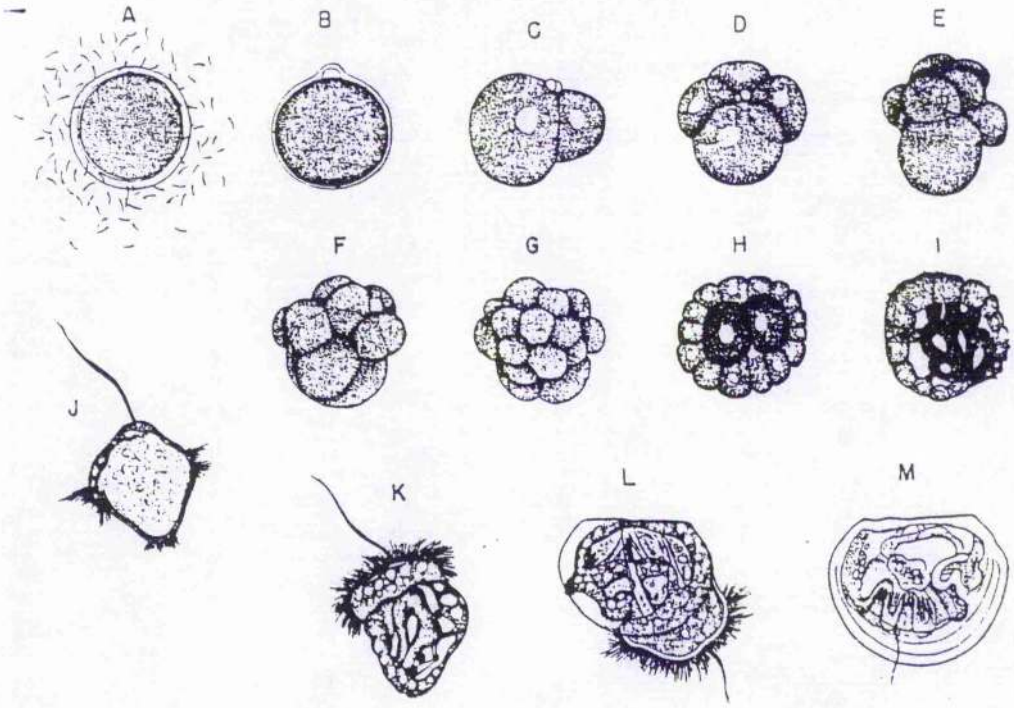
1.6 SHELLFISH DISEASES AND PUBLIC HEALTH CONCERN.

Several studies have shown that direct transmission of shellfish pathogens to humans can occur, causing enteric disorders if infected animals are eaten raw or undercooked, as in the case of *V. parahaemolyticus* causing summer bacterial enteritis outbreaks in Japan (Aiso & Matsuno, 1961; Sakazaki *et al.*, 1963). This organism, together with *Bacillus cereus, Clostridium perfringens* and occasionally *Salmonella* may cause food poisoning (Van den Broek *et al.*, 1979; Thi-Son & Fleet, 1980). However, it should be noted that most pathogens and parasites of shellfish are not directly infective to humans. Passive transmission of diseases from shellfish to humans was described as a process that results from contact with, or ingestion of, marine animals which carry, but are usually not themselves severely affected by, the microorganisms or the toxins involved. The role of the marine animal is usually that of concentrating or passively transporting the etiological agent (Sindermann, 1990). Also, uptake and retention of microorganisms by shellfish and ingestion of such passive carriers, uncooked and undercooked, can unite to produce human infections; examples of typhoid fever, cholera and infectious hepatitis outbreaks traceable to the ingestion of raw shellfish

are widely documented (Hart, 1945; Shewan & Liston, 1954: Mason & McLean, 1962; Klein *et al.*, 1974). Therefore, it seems that much of the public health significance of shellfish disease resides not in the disease itself, but rather in the passive transmission by the shellfish of human pathogens and toxic chemicals.

Additional note

The development of the Manila clam embryo to the fully shelled D-larva (this early veliger stage (M) is known as the D-larva stage because of the characteristic "capital D" shape of the shell).



OBJECT OF RESEARCH

The main objectives of this study concern the isolation and the identification of bacteria associated with commercially-produced bivalves in 3 U.K. hatcheries. The study formed part of a wider survey and also involved hatcheries in France and Spain. Larval toxicity or cytotoxicity tests will be developed to screen the isolates to determine which are potential pathogens. The eventual aim is to identify bacterial isolates capable of causing diseases in bivalve larvae.

Materials & Methods

2.1 BACTERIA.

Bacteria (Appendices I.1 and I.2) were isolated from material received from the Reculver hatchery (Mr. J. Bayes, Seasalter Shellfish Ltd.) and Guernsey Sea Farms (Mr. M. Dravers), during outbreaks of disease at both shellfish hatcheries. Other bacterial isolates (Appendix I.3) were obtained from larval material received from an experimental hatchery at Conwy (Dr. S. Utting, MAFF Experimental Shellfish Cultivation Unit, Conwy). Presumptive vibrio isolates were received from Dr. J-L Nicolas (France) and Dr. A-E. Toranzo and Dr. J-L. Barja, (Spain). Turbot bacterial isolates were obtained from the Bacterial Culture Collection, Division of Infection and Immunity, University of Glasgow. Standard vibrios were obtained from the national collection of industrial and marine Bacteria (NCIMB).

2.1.1 Isolation of Bacteria.

Bacteria were isolated by direct plating of hatchery sea water and oyster larvae homogenates diluted in filtered sterile sea water (FSW), on marine agar 2216 (MA) (Difco) and thiosulphate citrate bile salt sucrose (TCBS) agar (Oxoid). The MA and TCBS agar plates were incubated at 20 ^{o}C for 5 days and 48 hours, respectively.

2.1.2 Maintenance of Bacterial Cultures.

Bacteria were subcultured two or three times to obtain pure cultures and were maintained as stab cultures in MA, covered with liquid paraffin in screw-capped tubes and stored at room temperature in the dark, at 4 ^{o}C and as suspensions of marine broth 2216 (MB) (Difco) containing 25 % (v/v) glycerol at -70 ^{o}C. Organisms were routinely checked for purity by gram staining.

2.1.3 Bacterial Isolate Culture.

Overnight cultures of bacteria grown at 25 ^{o}C with shaking (100 rpm), in MB, were adjusted with MB to A600 = 1 (approximately 1 x 10^{9} cells ml^{-1}). The bacterial cells were collected by centrifugation, washed twice with FSW and resuspended to the original volume in MB, FSW or filtered haemolymph (FH).

2.2 IDENTIFICATION.

2.2.1 Biochemical Tests.

A total of 42 physiological and biochemical properties were recorded for each bacterial isolate. These tests are listed in Table 4. The incubation temperature was 20 °C unless indicated otherwise. A description of some of the methods used is given in Appendix II.

The mode of metabolism of glucose was tested by the method of Hugh and Leifson (1953) using MOF (marine oxidative/fermentative) medium (Difco). A Gram stain was performed and the colonies were examined for luminescence in the dark after incubation for 24 h on MA plates. In addition, pigmentation, swarming and growth at 4 °C and 37 °C were determined after 5 days incubation on MA plates. Motility was determined by examination of an inverted drop of culture using a light microscope (Vickers Instruments) after 24 h incubation in 1 % tryptone with 2 % NaCl. Growth on CLED (cysteine lactose electrolyte deficient medium) (Oxoid) was determined after 24 h incubation at 20 °C.

The following tests were carried out as decribed by Cowan (1965) with the addition of 1 % NaCl to the media: acid production from arbutin, salicin and sucrose with phenol red as the indicator, amylase, gelatinase (plate method), indole, ONPG (O-nitrophenyl-β-D-galactopyranoside) and oxidase. The ability to utilise compounds as sole carbon source was determined by the replica plating method of Baumann *et al.* (1971) and aesculin hydrolysis was tested as described by the Lee and Donovan (1985). Nitrate reduction was tested by the method of Lee *et al.* (1979). The following tests were performed as described by Furniss *et al.* (1979): arginine dihydrolase, lysine and ornithine decarboxylases, lysis of sheep red blood cells, lecithinase, and the Voges-Proskauer (V-P) test.

To test for resistance, or sensitivity, bacteria were grown overnight in MB at 20 °C, 200 µl of each bacterial suspension was spread on to MA plates which were

TABLE 4 : List of identification tests

Tests
Growth on TCBS medium
Gram Stain
Oxidation / Fermentation of Glucose
Pigment
Motility
Swarming
Growth on CLED medium
Arginine Dihydrolase
Lysine Decarboxylases
Ornithine "
Nitrate Reduction
Oxidase Test
Indole Production
ONPG Hydrolysis
Voges Proskauer Test
Resistance to: O/129 10 µg
O/129 150 µg
Ampicillin 10 µg
Aesculin Hydrolysis
Production of enzymes: Gelatinase
Lecithinase
Amylase
Haemolysis of sheep red blood cells
Acid from carbohydrates: Arbutin
Salicin
Sucrose
Utilization of single carbon sources:
L-arabinose
D-cellobiose
D-galactose
D-melibiose
D-gluconate
D-glucuronate
L-citrulline
D-glucosamine
DL-3-hydroxybutyrate
Succinate

incubated at 20 °C for up to 1 week and examined daily. Resistance to 0/129 (10 and 150 μg) (2, 4-diamino-6, 7-diisopropyl-pteridine) was determined after 48 h incubation using discs (Oxoid). Sensitivity to 10 μg ampicillin and 50 U polymyxin B was determined using filter paper discs impregnated with 10 μl antibiotic solution.

Decomposition of elastin and xanthine was determined by spot inoculation of bacteria on to MA plates containing 2 % elastin or xanthine which were incubated for 28 days at 20 °C. A clear zone around the colony indicated a positive result.

2.2.2 Antibiotic Resistance Tests.

Antibiotic resistance tests were carried out using 19 different antibiotics (chloramphenicol (25 μg), erythromycin (5 μg), fusidic acid (10 μg), methicillin (10 μg), novobiocin (5 μg) Penicillin G (1 unit), streptomycin (10 μg), tetracycline (25 & 100 μg), ampicillin (10 & 25 μg), cephaloridine (5 μg), colistin sulphate (25 μg), gentamicin (10 μg), sulphatriad (200 μg), cotrimoxazole (50 μg), nitrofurantoin (50 μg), ticarcillin (75 μg), nalidixic acid (30μg), trimethoprim (2.5 μg) and sulphamethoxazole (50 μg)), prepared in discs (Mastring-S M11, M14 & M46, Mast Diagnostics) and were used to identify the antibiotic profile of the bacterial isolates. Bacteria were grown overnight in MB at 20 °C, 200 μl of each bacterial suspension was spread on to MA plates which were incubated for up to 1 week at 20 °C and examined daily.

2.2.3 Identification of Bacteria.

Identification of the bacterial isolates to genus level was based on the taxonomic schemes of Oliver (1982) and Muroga *et al.* (1987). *Vibrio* and related genera were further identified by the computer program Bacterial Identifier, the revised edition of Bactid (Bryant *et al.*, 1986), run on an Amstrad pc 1512. Reference bacteria identification characteristics are listed in Appendix III (Kreig & Holt, 1984; Bryant *et al.*, 1986; Holt *et al.*, 1994).

2.2.4 Numerical Taxonomy.

Bivalve strains were examined for 50 characteristics (Appendix III) using two coefficients (Sneath and Sokal, 1973), Jaccard (similarity, S_J) and Simple Matching (similarity and dissimilarity, S_{SM}) in connection with the UPGMA cluster method (Sneath and Sokal, 1973). Analysis of data was conducted on an IBM-AT microcomputer using the programmes Simil and Agrusim (Dr. M. Serra, Dept. Microbiologia, Unidad de Ecologia, Universidad de Valencia, Spain).

Cluster analysis of the turbot isolates was performed using BMDP P2M (BMDP Statistical Software Inc. 1964 Westwood Blvd., Suite 202, Los Angeles, California 90025) run on an ICL 3890 computer.

2.3 MICROSCOPY.

2.3.1 Still Photography.

Larvae were fixed in 2.0 % formalin before photography using phase contrast microscopy. Live and dead haemocytes were photographed using a camera mounted on an inverted microscope (Olympus CK2) and using black and white film (Ilford).

2.3.2 Time-Lapse Photography.

Time-lapse video recording was carried out using phase-contrast optics and x24 time compression, with a Panasonic S-VHS time-lapse video cassette recorder. Haemocytes were added to wells in a Nunc, 24 multi-well plate and allowed to adhere for 1 h at room temperature. Cells were washed twice carefully and then FH (see 2.5.1), with or without added bacteria, was added.

2.3.3 Scanning Electron Microscopy (SEM).

Infected larvae were rinsed carefully in FSW, treated with 0.1 % (w/v) benzalkonium chloride for 30 seconds or FSW (untreated) and then rinsed in FSW. The following steps involving highly toxic chemicals were performed in a fume cupboard and plastic gloves were worn. Larvae were suspended in fixative (Appendix II) for 1 h in

a plastic universal bottle. The fixative was then removed using a Pasteur pipette and replaced with buffer (Appendix II). Larvae were then stored at 4 °C until required for further processing. This involved washing with fresh buffer, then resuspending in a few drops of buffer and an equal volume 0.2 % osmium tetroxide. The mixture was left at room temperature for 1 h, then the larvae were rinsed twice, for 10 min each time, with distilled water. This solution is toxic and slightly radioactive. The specimens were left at room temperature in the dark for 1 h, then rinsed twice with distilled water.

A dense suspension of larvae, treated as described above was prepared in distilled water and one drop was transferred to the drying apparatus. The larval suspension was held in a teflon ring sandwiched between two Nuclepore polycarbonate filters of 0.5 µm pore size and 13 mm diameter supported by two metal mesh discs. The 'sandwich' was held in a brass filter holder.

The specimens were dehydrated with a series of acetone solutions (30, 50, 70 and 90 %, absolute and dried absolute acetone). Each acetone solution was applied for 10 min. The brass filter holder was quickly transferred to the critical point dryer (C.P.D.) and the door of the C.P.D. was filled with liquid carbon dioxide, flushed twice then refilled. This step was repeated every 15 min for 1 h. Approximately one third of the volume of liquid carbon dioxide was drained then the C.P.D. was heated using two hairdryers until the pressure reached 96 bar. The pressure was reduced slowly over a period of approximately 15 min while continuing to heat the C.P.D. with one hairdryer.

The polycarbonate filters were removed and mounted on double-sided sticky tape on a metal stub. The edges were carefully cut away and silver paint applied to improve conductivity between the filter and the stub. The specimens were gold coated in a sputter coater (Polaron Equipment Ltd.) at 15 mA for 8 min then examined with a Philips 500 scanning electron microscope.

2.4 VIABILITY TESTS.

2.4.1 Molecular Probes Tests.

Neutral red (Sigma), XTT (2, 3 -bis [2-methoxy-4-nitro-5-sulfophenyl]-2-H-tetrazolium-5 carboxanilide inner salt, Sigma), Calcein AM (CAM) and Ethidium homodimer (ETHD) which were purchased from Molecular Probes, Inc., were prepared according to the manufacturer's recommendation.

Neutral red experiments were carried out using Nunc 96 well plates containing *Mytilus edulis* haemocyte monolayers; bacteria were washed and resuspended in FSW. Co-cultures were incubated for 3 h at 20 ^{o}C. Following incubation, sterile neutral red dye was added to each test at a final concentration of 0.05 % (w/v) for 30 min. at 20 ^{o}C. Viability of haemocytes was determined microscopically. Controls were prepared by adding FSW without bacteria to the haemocyte monolayers.

Spectrophotometric analysis of neutral red uptake by haemocytes after exposure to bacteria was carried out using similar preparations as above except that the wells were washed with FSW twice to remove bacteria, excess neutral red and dead haemocytes. Supernatants were removed and replaced with the same amount of citrate-buffered ethanol for 1 h to produce a pink colour, the absorbance of which could be measured at 540 nm with a microplate reader (Anthos). Increasing optical density (O. D.) units correlated with increasing cell viability.

Experiments using XTT were carried out using Nunc 96-well plates containing *Mytilus* haemocyte monolayers. Test bacteria were washed and re-suspended in FSW or FH. Co-cultures were incubated for 3 h at 20 ^{o}C. Following incubation 60 µl of sterile XTT working solution (stock solution: 1 mg/ ml in FSW) was added to each test (final XTT concentration of 200µg/ml in the culture medium) and incubated for 45 min at 20 ^{o}C. Gentle mixing was used to enhance the dissolution and dispersion of the XTT formazan. Viability of the haemocytes was determined by measuring the absorbance of the resulting orange solution at a wavelength of 450 nm.

Background absorbance of the multiwell plates was recorded at 690 nm and subtracted from the 450 nm measurement. Appropriate controls (haemocytes with FSW and no bacteria) were also incorporated into the assay. Test antibiotics concentrations ranging between 10 and 100 µg/ml were prepared in FSW or FH.

Calcein-AM experiments were carried out using Nunc 96-well plates containing *Mytilus* haemocyte monolayers; bacteria were washed and resuspended in FSW or FH. Co-cultures were incubated for 3 h at 20 °C. Following incubation, 100 µl sterile Calcein-AM (6 µl of stock solution of Calcein-AM in DMSO was added to 10 ml FSW to give an approximate 3 µM solution), was added to each test to give a final concentration of 1 µM and was incubated for 45 min at 20 °C. Haemocyte viability was determined by measuring the calcein fluorescence (excitation at 485 ± 20 nm and emission 530 ± 30 nm; sensitivity 5) using a multi-well plate scanner (Millipore Cytofluor™ 2300). Controls were prepared by adding FSW without bacteria to the haemocyte monolayers.

Analysis of ethidium homodimer uptake by haemocytes after exposure to test bacteria was carried out using similar preparations as before. After incubation, 100 µl of ethidium homodimer solution (initial 12 mM stock solution diluted to 150 µM in 2 ml of tissue grade Dulbecco's phosphate buffered saline (D-PBS); 400 µl of this aqueous stock solution was then added to 10 ml FSW, yielding a final solution of approximately 6 µM) was added at 3x concentrations to allow for three-fold dilutions upon addition to wells (final concentration range was between 0 and 6 µM) and incubated for 45 min at 20 °C. Increasing fluorescence units (excitation at 485 ± 20 nm and emission 645 ± 45 nm; sensitivity 6) correlated with increasing levels of cytotoxicity and were measured using a Millipore Cytofluor™ 2300. Appropriate controls were incorporated into the assay to determine the maximum signal when all the haemocytes were dead and to account for possible background fluorescence.

2.4.2 Preparation and Experimental Procedure for Bivalve Larvae.

To obtain axenic larvae the gametes were stripped aseptically from conditioned adults. Both the eggs and sperm were pooled in separate containers and fertilisation of the eggs was initiated by adding 2 ml of a dense sperm suspension to 1 litre of slightly opaque egg suspension in filtered sea water (Utting & Spencer 1991; Redshaw, 1991). The fertilised eggs divide within 60 - 90 min at 25 °C and within 24 h fully shelled D-veliger larvae are formed when incubated at 20 °C.

Culture of axenic algae (*Pavlova lutheri*) was initiated using a bacteria-free inoculum, kindly provided by Mr. J. M. Scott, Dunstaffnage Marine Laboratory, Oban. The algae were grown in aerated spherical glass flasks of 500 ml volume. Glass tubing was used inside the flasks and as connectors at the end of the input and output tubes. The ends of the tubes were clamped and stored in small bottles of iodine solution (Appendix II). Silicon bungs and tubing (Altec, Mill Lane Estate, Alton, Hampshire) were used throughout. Fresh growth medium for the algae (Appendix II) was sterilised by autoclaving at 121 °C for 15 min and this along with a filtered vitamin supplement was added to the algae cultures regularly.

Bivalve larvae, obtained from hatcheries, were counted and resuspended in 2 ml FSW. To prepare samples of the oyster larvae for homogenisation they were washed with 0.1 % (w/v) benzalkonium chloride for 30 seconds to remove surface bacteria or washed carefully with FSW only. The larvae were collected on separate sterile nylon mesh discs, transferred to a glass homogeniser with a close fitting teflon plunger and homogenised in 5 ml FSW. Dilutions were prepared in FSW and 0.1 ml volumes were spread onto MA and TCBS agar and incubated at 20 °C for 5 days and 48 h respectively.

Using a system similar to the algal culture apparatus, experiments were carried out with co-cultures of bacteria and axenic larvae (10 larvae/ml) in aerated 1 litre spherical bottom flasks containing FSW over 72 h at 15 °C. Samples were collected

at time 0; every 24 h the viability of larvae was determined using neutral red, as described for the haemocyte neutral red assay. Also, the bacterial count and larval size were estimated. The larvae were provided with a diet of axenic algae (50 cells /µl/ day) every 24 h.

2.4.3 Ciliostatic Toxin Assay (Gill Assay).

Mytilus edulis (50-70 mm shell length) were maintained in aquaria containing sea water at 10 °C. Prior to their use in experiments, the mussels were acclimated for 1 h to 20 °C. Gill tissues were removed from the mussels and segmented. These segments were then placed in square well tissue culture plates (Flow Laboratories Ltd) containing 1 ml of 25 ‰ artificial sea water (a.s.w.) (with penicillin 100 iu / ml and streptomycin (100 µg/ml). Then, 1 ml of heat-treated (100 °C /10 min) or untreated 72 h bacterial culture supernatant was added to each well in triplicate except for the controls where 1 ml of the medium used to culture the bacterial strains was added. The plates were incubated at 20 °C for 24 h, after which the ciliary activity of the epithelial cells and integrity of the gill section was examined microscopically. The viability of the cells was determined using neutral red dye (0.01 % w/v).

2.5 HAEMOCYTE ASSAY PROTOCOL.

2.5.1 Diluents.

For the haemocyte assay, FSW was obtained by filtration through a 0.45 µm filter (Whatman), autoclaved and left for 2 days before use to restore the normal pH to 8-8.2 and the air content to normal sea water level. This was used for washing and diluting the bacteria. Bivalve haemolymph, extracted aseptically from the posterior adductor muscle sinus using a sterile syringe, was centrifuged (10, 000 g for 10 min), filtered through 0.2 µm filter (Whatman) for use as a diluent (FH). Mixtures of bacteria and haemocytes were prepared in the diluents at different ratios (10, 50, 100, 500, 1000 c.f.u. per haemocyte). Toxicity of bacteria was recorded using

still photography and direct microscopic observation and was determined using the following formula:-

$$\% \text{ Normalised total of rounded cells} = \frac{(\text{Test} - \text{Control})}{(100 - \text{Control})} \times 100$$

2.5.2 Haemocyte Preparation.

Haemolymph was collected from the posterior adductor muscle sinus of each bivalve. Haemocytes were counted using a Neubauer haemocytometer and the cell density was expressed as the number of haemocytes per ml of haemolymph (approximately 10^6 per ml).

Extracted haemolymph was loaded into 24 well microtitre plates and allowed to stand for 1 h at room temperature, to allow the haemocytes to adhere to the plastic surface. The haemocytes were washed carefully, twice in FSW. Finally, FSW or FH was added to the attached haemocytes. *Pecten* haemocytes required longer incubation of up to 24 h for adherence to the plastic surface.

2.5.3 Trypsin-Treated FH.

Mytilus FH was incubated with trypsin (Sigma) at 50 μg ml^{-1} for 1 h at 25 °C. Following incubation, *V. anguillarum* 2981 was suspended in the trypsin-treated FH and tested for toxicity using the *Mytilus* haemocyte assay.

2.5.4 Effect of Nutrient on Bacterial Toxicity.

Overnight cultures of the test bacteria were grown in different concentrations of peptone (ranging from 0 to 5 %) and FSW, at 20 °C. The bacteria were collected and tested for toxicity using the *Mytilus* haemocytes assay as above.

2.5.5 Effect of Temperature.

Mytilus haemocytes were incubated with 10 or 50 c.f.u. of V2981 per haemocyte for 2 h 30 min at temperatures, 4, 10, 20 or 37 °C. Control haemocytes were prepared without bacteria and these were also incubated at the test temperatures.

Results were recorded using still photography and the numbers of live or dead haemocytes were then quantified.

2.5.6 Toxic Effect of Lipopolysaccharide (LPS).

LPS of *V. anguillarum* 2981 was extracted using the method of Westphal and Jann (1965). Overnight bacterial cell cultures grown in MB (100 ml) at 20 °C with shaking, were harvested by centrifugation (10, 000 g for 15 min at 4 °C), and pellets were resuspended in 10 ml of hot (68 °C) sterile distilled water and 10 ml of 90 % (w/v) hot phenol (68 °C). Mixtures were incubated at 68 °C for 30 min in a water bath with occasional shaking, then cooled to 10 °C and centrifuged at 3, 000 g for 45 min at 4 °C. After further centrifugation the phases were separated, the phenol layer twice mixed with 10 ml of distilled water and the aqueous layers collected; the combined water extracts were dialyzed against cold tap water for 2 days and against distilled water for 1 day. Samples were concentrated to a volume of 30 - 40 ml with PEG and centrifuged at 15, 000 g for 15 min in a Sorval centrifuge to remove traces of insoluble material. The supernatants were ultracentrifuged at 100, 000 g for 3 h at 4 °C and both the supernate and pellet were retained. The cell pellet was resuspended in distilled water and spun together with the supernate at 100, 000 g for 3 h. This step was repeated with the cell pellet only. The final cell pellet was weighed, freeze-dried and stored at 4 °C. Purified LPS was mixed with FH (2 mg ml^{-1}) and tested for toxicity to *Mytilus* haemocytes after incubation for 3 h at 20 °C. Results were recorded as for trypsin-treated FH.

2.6 GEL ELECTROPHORESIS.

2.6.1 SDS-Polyacrylamide Gel Electrophoresis of Proteins.

The method was based on that described by Laemmli (1970) using a vertical slab gel tank. The stock solutions and recipes for gel and buffer preparation are shown in Appendix II.

Separating (lower) and stacking (upper) gels contained 12.5 % (w/v) and 4.5 % (w/v) acrylamide respectively. The gel was formed between two glass plates of 17 cm x 19 cm x 0.3 cm with spacers of 1.5 mm thick. Gel and electrophoresis (running) buffers contained 0.1 % (w/v) SDS.

2.6.2 Preparation of Sample for SDS-PAGE.

Test samples were collected in sterile microfuge tubes and equal volumes of appropriate solubilizing buffer (see Appendix II) were added to each sample and heated at 100 ^{o}C for 5 min in a boiling water bath.

To estimate the molecular weight of separated polypeptides, a mixture of polypeptides of known molecular weight was used (SDS-7, Sigma); this contained a mixture of the following seven proteins: α-lactalbumin (14, 200 Da), trypsin inhibitor (20,100 Da), trypsinogen (24,000 Da), carbonic anhydrase (29,000 Da), glyceraldehyde-3-phosphate dehydrogenase (36,000 Da), egg albumin (45,000 Da) and bovine albumin (66,000 Da). Protein markers were also incubated at 100 ^{o}C for 3 min prior to electrophoresis.

2.6.3 Electrophoresis of Protein Samples.

Samples of 30 µl were loaded onto the gel and electrophoresis was performed at room temperature at a constant current of 30 mA, using a Shandon VoKam SAE 2761 power unit, until the tracking dye had reached the bottom of the gel. Following electrophoresis the gel was silver stained.

2.6.4 Silver Staining of Gels.

This method was adapted and modified from the procedure of Oakley *et al.* (1980). Gels were prefixed in a solution containing 50 % (v/v) absolute alcohol and 10 % (v/v) acetic acid in distilled water, overnight at room temperature; the fixing solution was removed and replaced by a solution containing 10 % (v/v) glutaraldehyde (BDH) in distilled water. After gentle shaking for 30 min gels were then rinsed in a large volume of distilled water overnight, then in fresh distilled water for 30 min. Gels

were soaked in a freshly prepared solution containing 5 µg/ml of dithiothreitol (Sigma) in distilled water for 30 min, the solution discarded and replaced by 0.1 % (w/v) silver nitrate solution (BDH) for 30 min. Gels were rinsed once in distilled water and then twice rapidly in developer (50 µl of 37 % formaldehyde in 100 ml of 3 % (w/v) sodium carbonate solution) until the desired level of staining was reached, then 5 ml of 2.3 M citric acid solution was added to developer and mixed for 10 min. Finally, gels were soaked in 0.03 % (w/v) sodium carbonate solution for 10 min and stored at 4 ^{o}C in heat-sealed cellophane bags.

Results

3.1 MICROSCOPIC ANALYSIS OF OYSTER LARVAE, ISOLATION, CHARACTERISATION AND IDENTIFICATION OF BACTERIAL ISOLATES.

One of the objectives of this study was to identify the Gram-negative bacterial communities associated with shellfish fisheries to characterise the potentially pathogenic species. A number of bacterial isolates were obtained from turbot hatcheries for comparison. This section describes the microscopic analysis of the oyster larvae, the isolation and overall characterisation (biochemical, taxonomic and antibiotic sensitivities) of the bacterial isolates, and determination of the main bacterial species associated with oysters under different conditions.

3.1.1 Microscopic Analysis of Oyster Larvae.

Benzalkonium chloride-treated oyster larvae (*Ostrea edulis*) and untreated larvae, both from Reculver, were examined by phase contrast microscopy and scanning electron microscopy (SEM), to determine whether the bacterial isolates were associated with the internal or external body of the larvae.

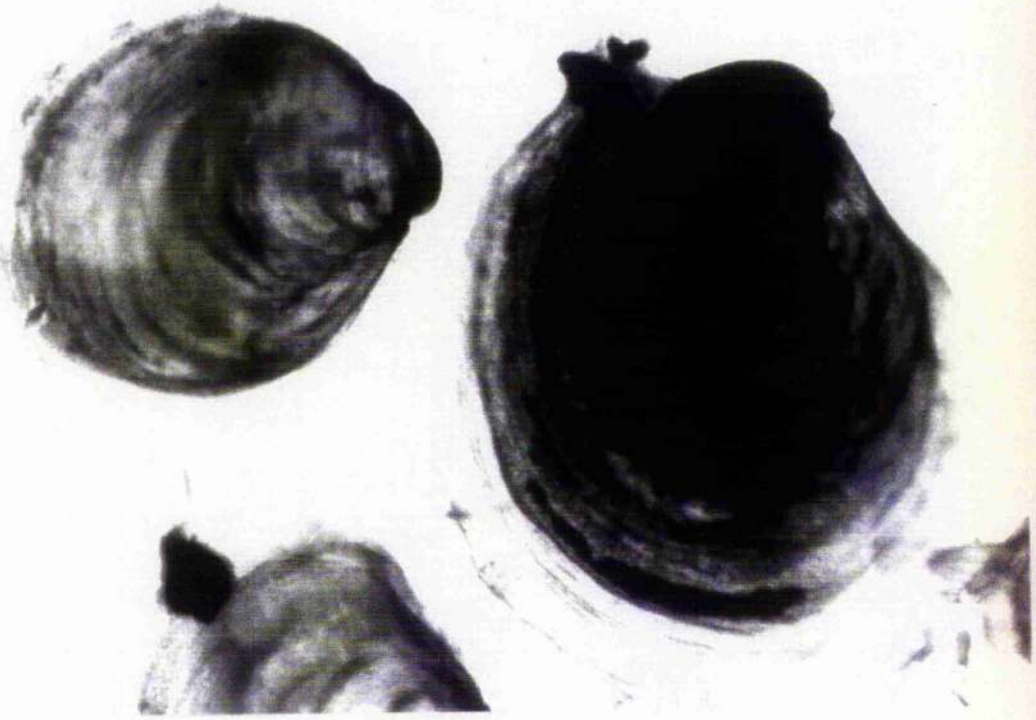
Figure 2 shows phase contrast photographs and SEM micrographs which show the absence of bacteria on the surface of the treated and untreated oyster larvae. This indicated that the bacterial isolates were present internally in the oyster larvae. Shell-bound bacteria were only weakly bound as they were lost during the initial washing step.

3.1.2 Isolation of Bacteria.

Marine micro-organisms require salt for the stability of the cell membrane and some of their enzymes, and *Vibrio* are no exception. Bacterial isolates were selected on the basis of their ability to grow on marine agar and thiosulphate-citrate-bile-sucrose-salts (TCBS) medium. All isolates that were Gram-negative rods, were tested for their ability to ferment carbohydrate.

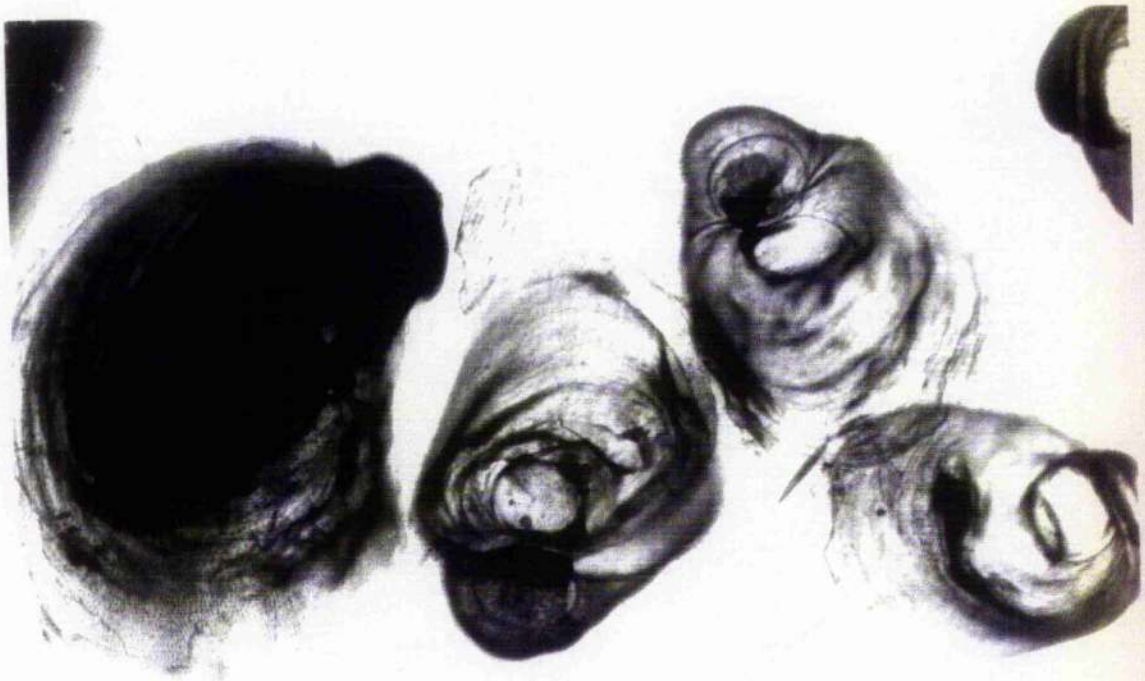
FIGURE 2 Phase contrast photographs (Ai and Bi, x 40) and scanning electron micrographs (Aii, x 200), (Bii, x 100) of oyster larvae (*Ostrea edulis*), (A) treated or (B) not treated with benzalkonium chloride.

A (i)



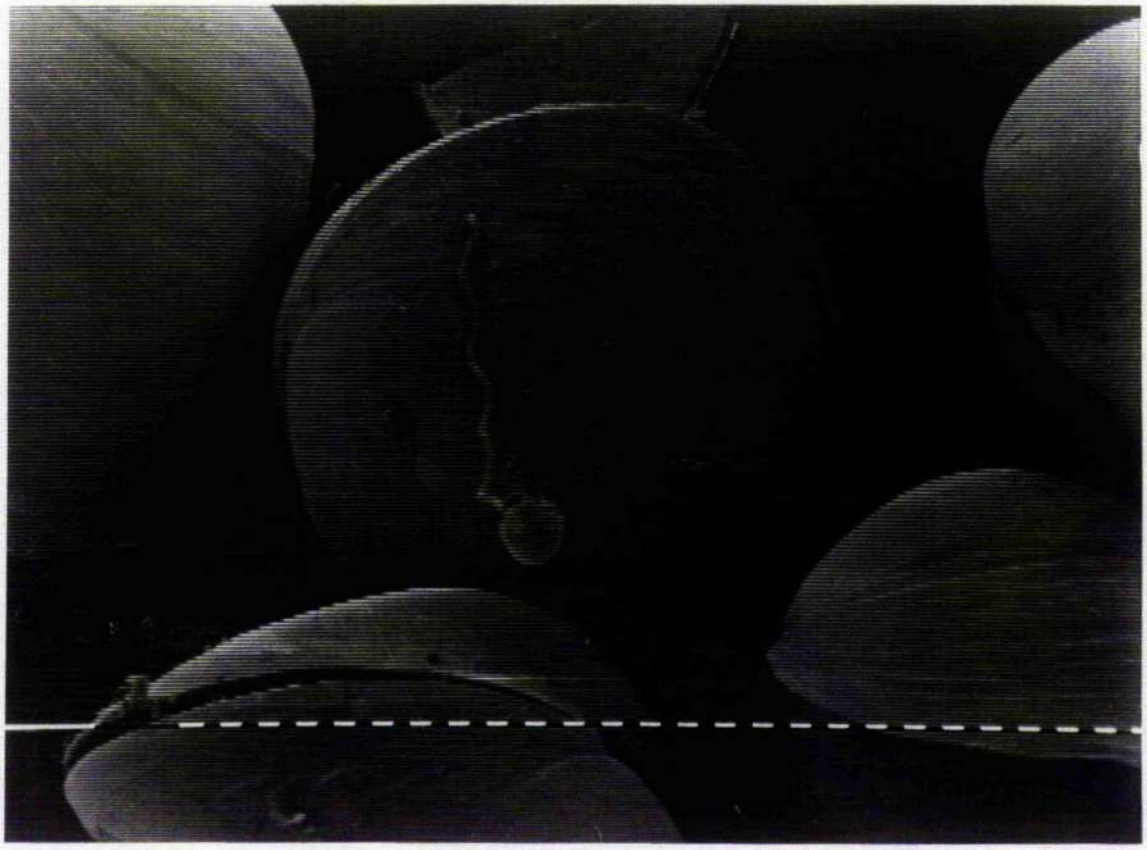
x40

B (i)

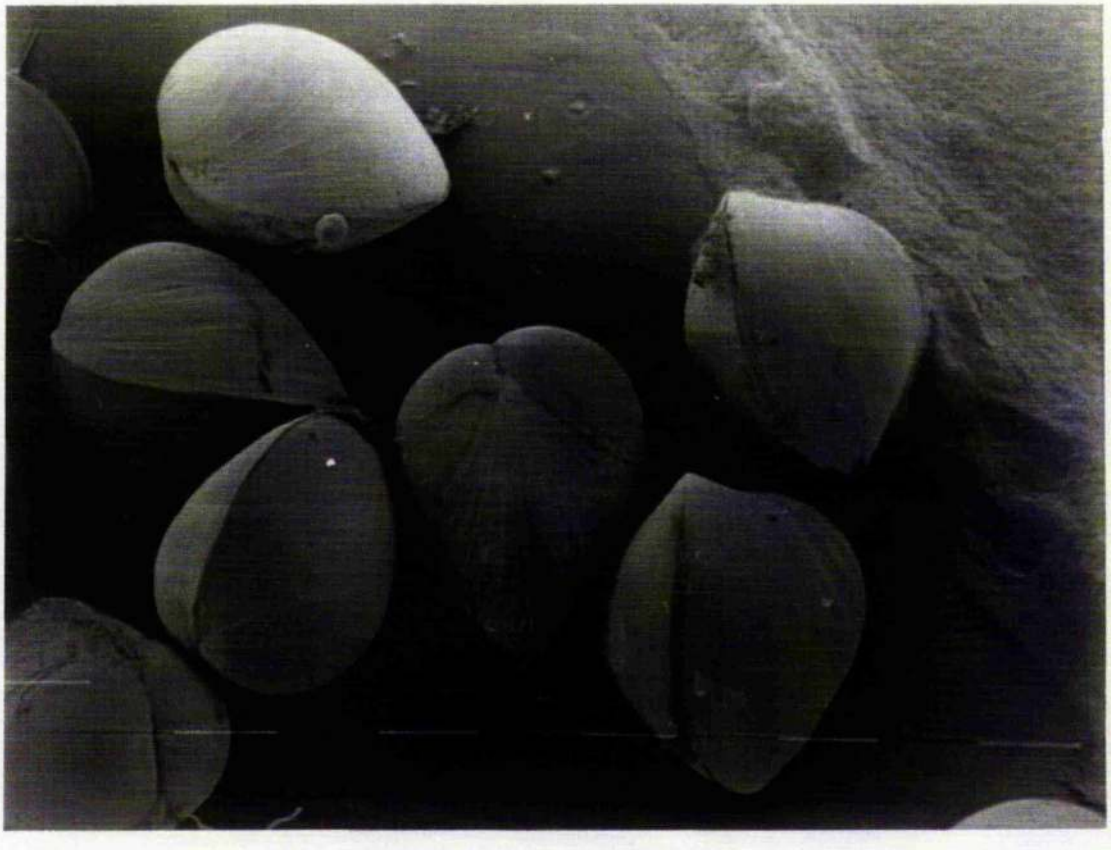


x40

A (ii)



B (ii)



The bacterial strains were isolated from material obtained from shellfish hatcheries experiencing outbreaks of disease at Reculver (group A I) and Guernsey (group G) and from an experimental shellfish hatchery at Conwy (group A II), where there was no evidence of disease at the time of sampling. Also, bacterial isolates obtained from turbot hatcheries in Scotland, Norway and Spain, which were previously characterised by Dr. P. Munro, were analysed, together with French and Spanish isolates which originated from bivalve hatcheries during infections.

3.1.3 Phenotypic and Biochemical Characteristics of the Bacterial Isolates.

The phenotypic characteristics of the isolates were determined on TCBS and marine agar plates. Most of the isolated Guernsey strains grew readily on TCBS (80 %) in comparison to the isolates from Conwy (55 %) and Reculver (32 %) (Table 5). All isolates of group G were non-pigmented and 46 % of these strains swarmed on marine agar. In contrast, the Reculver and Conwy isolates were pigmented (35 and 45 % respectively) and swarmed (5 and 9 % repectively) on marine agar. Appendix III (3.1 to 3.4) shows the most important physiological characteristics of the bacterial isolates.

A series of biochemical characteristics was used to identify the isolates from Conwy, Reculver and Guernsey; these characteristics are shown on Appendix III (3.1 to 3.4) and a summary of the experimental results is shown in Table 5.

These results show that approximately 5 % of the bacterial isolates from Guernsey and Reculver were Gram-positive, the remaining isolates and the Conwy isolates were Gram-negative rods. All the Conwy isolates and approximately 90 % of the Guernsey and Reculver strains were unable to grow on electrolyte deficient medium (CLED). Almost half of each group of isolates lysed sheep red blood cells, utilised L-arabinose and produced arginine dihydrolases. Most of the bacterial strains were indole positive, were resistant to the vibriostatic agent 0/129 (10 μg) and produced gelatinase (an average of 95, 88 & 81 % respectively). On average, less

TABLE 5 : Overall percentage of bacterial isolates from Conwy, Guernsey and Reculver showing positive tests.

Isolate origin	Conwy (11)*	Guernsey (24)	Reculver (67)
Tests			
Growth on TCBS medium	55	80	32
Gram Stain	0	4	5
Fermentation of Glucose	27	75	37
Pigment	45	0	37
Motility	55	96	74
Swarming	9	46	5
Luminescence	9	ND	16
Growth on CLED medium	0	8	11
Arginine Dihydrolase	55	46	42
Lysine Decarboxylase	36	75	37
Ornithine "	9	77	5
Nitrate Reduction	45	96	68
Oxidase Test	64	100	68
Indole Production	91	96	100
ONPG Hydrolysis	36	29	37
Voges Proskauer Test	9	42	5
Resistance to:			
O/129 (10 µg)	100	71	95
O/129 (150 µg)	73	29	79
Ampicillin	27	25	63
Polymyxin B	55	ND	47
Hydrolysis of: Aesculin	9	0	11
Elastin	45	ND	37
Gelatin	73	96	74
Lecithin	0	92	0
Starch	55	87	32
Xanthine Decomposition	9	ND	16
Haemolysis (sheep rbcs)	45	46	42
Acid from: Arbutin	9	87	5
Salicin	9	0	5
Sucrose	55	96	42
Utilisation of:			
L-arabinose	64	42	58
D-cellobiose	64	84	59
D-galactose	73	92	63
D-melibiose	64	24	68
D-gluconate	64	79	84
D-glucuronate	82	29	63
L-citrulline	27	21	37
L-leucine	36	ND	53
D-glucosamine	73	67	74
DL-3-hydroxybutyrate	73	46	58
Succinate	55	91	47

ND: Not determined

*Number of isolates per group

than 10 % of all the bacterial isolates produced acid from salicin or hydrolysed aesculin. An average of 70 and 30 % of all the bacterial isolates grew in the presence of the single carbon source D-glucosamine, and hydrolysed ONPG (O-nitrophenyl-β -D-galactopyranoside), respectively.

The characteristics of the Group G isolates were significantly different from those of the isolates in group A (I & II). Group G was composed predominantly of presumptive vibrios compared to the miscellaneous marine bacteria in group A (I & II). Differences in biochemical characteristics were observed in the following groups; the G isolates were fermentative (75 %) and grew on TCBS medium (80 %). All of the Guernsey isolates were oxidase positive and did not produce acid from salicin. They produced lecithinase, acid from arbutin, and were ornithine decarboxylase positive (greater than 90, 79 & 68 % respectively), to a greater extent than the isolates in group A (I & II). A higher percentage of the group G isolates utilised succinate, produced lysine decarboxylase and were V-P positive, than that observed in group A (I & II) isolates. The group G had up to 50 % more isolates that reduced nitrates, produced acid from sucrose and were susceptible to 0/129 (150 µg) in comparison with the results of the bacteria in group A (I & II). More of the Conwy and Guernsey isolates were susceptible to ampicillin, utilised D-galactose, hydrolysed starch and did not use L-citrulline, compared to the Reculver isolates. Reculver strains shared similarity with the Guernsey isolates in that the majority of the isolates were motile and utilised D-gluconate.

Conwy isolates shared the following characteristics with the strains from Reculver: they were resistant to 0/129 (10 µg), utilised D-melibiose and D-glucuronate. Few of the isolates produced acid from arbutin, showed lecithinase activity, swarming or luminescence, and most of the isolates were V-P positive. The majority of these isolates were non-fermentative. Also, approximately 50 % of the isolates from Conwy were pigmented, motile and grew on TCBS medium. Around 50 % of the Conwy isolates produced amylase and were positive for nitrate production.

Most of the Conwy isolates (73 %) grew in the presence of the single carbon source DL-3-hydroxybutyrate compared to the isolates from Guernsey and Reculver (50 %).

Further tests were carried out on the bacterial isolates in group A (I & II) which disclosed that few of these isolates were luminescent and/or decomposed xanthine. These tests also showed that approximately half of the isolates from Conwy and Reculver were susceptible to Polymyxin B and did not produce elastase or utilise L-leucine. The biochemical characteristics of the isolates are given further consideration in the cluster analysis of the bacterial isolates from Conwy, Guernsey and Reculver.

3.1.4 Antibiogram Analysis of Bacterial Isolates.

Bacterial isolates from the three locations, Conwy, Guernsey and Reculver, were characterised according to their sensitivity or resistance to 19 broad activity spectrum antibiotics. Results are shown in Appendix III (3.5 to 3.9) and are summarised in Tables 6, 7, 8 and 9.

All of the bacterial isolates tested were resistant to one or more of the β-lactams and streptomycin. With the exception of the tentative *Pseudomonaceae* (Conwy), all of the remaining bacterial groups showed resistance to gentamicin (Table 6). Variable bacterial resistance to the sulphonamides, sulpha drugs, pyrimidines and the miscellaneous antibiotics was prevalent within all 3 locations. Tetracycline and the miscellaneous antibiotics were effective against presumptive *Vibrio* spp. and many other bacterial groups isolated from Conwy. Greater than 60 % resistance to erythromycin was evident in the bacterial groups *Cytophaga/Flavobacterium, Enterobacteriaceae* (Conwy), *Vibrio* spp. (Conwy and Reculver) and *Moraxella* (Reculver).

Chloramphenicol and tetracycline (100 µg) were the most effective antibiotics against all the bacteria tested. Some bacteria showed low levels of resistance to nalidixic acid. Specificity of antibiotics to the bacterial isolates from 2 out of 3

TABLE 6 : Summary of percentage of bacteria from Conwy, Guernsey and Reculver resistant to selected antibiotics.

Antibiotic group	Origin of bacterial isolates		
	Conwy (11)*	Guernsey (23)	Reculver (68)
ß-Lactam group			
Penicillin G	73	75	84
Methicillin	55	83	84
Ampicillin (25 µg)	36	25	23
Cephaloridine	73	96	84
Ticarcillin	0	46	24
Aminoglycoside group			
Streptomycin	91	96	92
Gentamicin	64	33	49
Quinolones & Tetracyclines			
Tetracycline (100 µg)	0	0	4
Nalidixic acid	18	0	13
Macrolide group			
Erythromycin	64	75	45
Aromatic antibiotic			
Chloramphenicol	0	0	4
Sulphonamides /Sulpha drugs / Pyrimidines			
Sulphamethoxazole	64	12	58
Trimethoprim	82	33	69
Cotrimoxazole	18	0	42
Sulphatriad	18	17	49
Derivatives of Polymyxin, Fusidane, Coumarin and Nitrofuran			
Colistin Sulphate	45	4	25
Fusidic acid	36	79	55
Novobiocin	36	12	51
Nitrofurantoin	0	8	12

*Number of isolates per group.

TABLE 7 : Frequency of bacterial resistance to antibiotics of isolates from Guernsey.

Frequency (%) of bacterial resistance to antibiotics of isolates from Guernsey

Antibiotic group	*Vibrio*	*Aeromonas*
Penicillin G	72	100
Methicillin	86	50
Ampicillin (25 µg)	50	50
Cephaloridine	95	100
Ticarcillin	45	50
Streptomycin	72	100
Gentamicin	95	100
Tetracycline (100 µg)	0	0
Nalidixic acid	5	0
Erythromycin	81	0
Chloramphenicol	0	0
Sulphamethoxazole	9	50
Trimethoprim	36	50
Cotrimoxazole	0	100
Sulphatriad	18	100
Colistin Sulphate	0	50
Fusidic acid	23	100
Novobiocin	5	100
Nitrofurantoin	5	50

TABLE 8 : Frequency of bacterial resistance to antibiotics of isolates from Reculver

Antibiotic group	Frequency (%) of bacterial resistance to antibiotics of isolates from Reculver					
	Vibrio	*Enterobacteriaceae*	*Moraxellaceae*	*Pseudomonas*	*Micrococcus*	*Cytophaga/Flavobacterium*
Penicillin G	88	75	100	79	50	90
Methicillin	88	80	100	71	75	90
Ampicillin (25 µg)	13	15	33	29	50	10
Cephaloridine	75	85	66	50	75	90
Ticarcillin	38	10	0	21	75	30
Streptomycin	100	85	100	93	75	100
Gentamicin	50	25	33	36	25	100
Tetracycline (100 µg)	4	5	33	7	25	0
Nalidixic acid	50	5	0	14	50	0
Erythromycin	100	40	66	43	50	20
Chloramphenicol	0	5	0	0	25	0
Sulphamethoxazole	50	5	33	59	50	86
Trimethoprim	75	90	100	79	75	0
Cotrimoxazole	13	25	0	43	25	96
Sulphatriad	13	35	33	57	50	82
Colistin Sulphate	0	5	0	7	25	70
Fusidic acid	100	70	66	50	75	20
Novobiocin	0	25	33	79	75	60
Nitrofurantoin	0	25	0	14	25	0

TABLE 9 : Frequency of bacterial resistance to antibiotics of isolates from Conwy.

	Frequency (%) of bacterial resistance to antibiotics of isolates from Conwy				
Antibiotic group	*Vibrio*	*Enterobacteriaceae*	*Moraxellaceae*	*Pseudomonas*	*Cytophaga/Flavobacterium*
Penicillin G	33	100	0	100	100
Methicillin	66	100	0	100	25
Ampicillin (25 µg)	33	0	0	0	43
Cephaloridine	66	100	0	100	88
Ticarcillin	0	0	0	0	0
Streptomycin	66	100	100	100	100
Gentamicin	66	0	100	0	100
Tetracycline (100 µg)	0	0	0	0	0
Nalidixic acid	33	0	100	0	0
Erythromycin	66	100	0	0	87
Chloramphenicol	0	0	0	0	0
Sulphamethoxazole	66	0	0	100	87
Trimethoprim	66	100	0	100	100
Cotrimoxazole	33	0	0	0	0
Sulphatriad	66	0	0	0	0
Colistin Sulphate	33	0	100	0	75
Fusidic acid	100	0	0	100	0
Novobiocin	0	0	0	0	0
Nitrofurantoin	33	100	0	100	50

locations was observed. Ticarcillin showed 100 % inhibition against the bacterial isolates from Conwy compared to the bacterial isolates from Reculver and Guernsey. Tetracycline (100 µg) and chloramphenicol were 100 % competent in killing all the *Vibrio* isolates from Guernsey only and there was no specific antibiotic against the Reculver isolates.

Low frequency of resistance was not evident in the Conwy bacterial isolates. Both the Reculver and Guernsey bacterial isolates showed low frequency of resistance to nalidixic (13 & 0 %) and nitrofurantoin (12 & 8 %) respectively. Low levels of resistance were expressed in the bacterial isolates from Guernsey to novobiocin (12 %) and sulphamethoxazole (12 %), and from Reculver to chloramphenicol (4 %). Detailed analysis of antibiotic resistance for each bacterial group (Tables 7, 8 and 9) will be reviewed in section 4.

3.2 CLUSTER ANALYSIS OF BACTERIAL ISOLATES.

Cluster analysis of the data from biochemical, physiological (Appendix III, 3.1 to 3.4) and antibiotic sensitivity (Appendix III, 3.5 to 3.9) testing of the bacterial isolates from Conwy, Guernsey and Reculver was done. The strains were examined for 50 characteristics using the two coefficients (Sneath & Sokal, 1973), Jaccard (similarity, S_J) and Simple Matching (similarity and dissimilarity, S_{SM}) methods, both analysed with the UPGMA method (Sneath & Sokal, 1973) and the programme, Bacterial Identifier (Bryant *et al.*, 1986). Two dendrograms were produced for each group of isolates but as these were very similar only the S_{SM} dendrograms were analysed further.

The vast majority of the isolates showed similarities in the range of 50 to 100 % but only the isolates which showed between 85 and 100 % similarity were defined as phenons and were the subject of further consideration.

3.2.1 Cluster Analysis of Group G Isolates.

The bacterial isolates in this group were obtained from sea water, quarry water (sea water from an artificial lake in an old quarry) and oyster larvae from the shellfish hatchery. The phenons (see 3.2) produced by both S_{SM} and S_J were the same with the exception of one phenon that contained negative matches and was absent from the Jaccard analysis (Fig. 3). Identification of the bacteria to the genus or species level was attempted using the Bactid programme for identification of Gram-negative fermentative bacteria and by comparison with bibliographic reference on bacterial systematics (Krcig & Holt, 1984; Holt *et al.*, 1994). The detailed results are shown in Appendix III.

Three major clusters were formed, A, B and C, and 5 phenons could be distinguished on the basis of 85 % similarity with the S_{SM} coefficient (Fig. 4). The percentages of positive responses to the biochemical tests of these phenons are shown in Table 10. Detailed analysis of these results is presented in section 4.

3.2.2 Cluster Analysis of Group AI and AII Isolates.

Reculver (68 isolates, group AI) and Conwy (11 isolates, group AII) bacterial strains were isolated from *Ostrea edulis* or *Crassostrea gigas* larvae, hatchery water or algae (Appendix I) and were identified using the computational identification programme. The clustering techniques used gave almost identical results when resemblance between strains from Conwy and Reculver was estimated using coefficients, S_{SM} and S_J, with the exception of a few individual isolates (A24, A81, A95 and P1-1-1) and a large cluster broken down into two neighbouring clusters when S_J was employed (Fig. 5). Fig. 6 shows the S_{SM} cluster analysis of the group A (I & II) the resulting dendrogram contained three large clusters A, B and C, that were further sub-divided into 16 phenons which were analysed separately. Group A (I & II) biochemical, physiological and antibiotic characteristics are shown in Appendix III and were summarised to show the percentage of positive responses (Tables 11 and 12) to the set of tests by the different phenons defined at 85 % similarity by the S_{SM} coefficient

FIGURE 3 Cluster analysis of bacterial isolates from Guernsey, analysed by Jaccard Similarity Coefficient (SJ) linked with UPGMA method and the programme, Bacterial Identifier (Bryant *et al.*, 1986). A reference strain V006 (*V. anguillarum* 6) was included in *Vibrio anguillarum.*

Legend:

Bacterial tentative identification

□ *Aeromonas.*

○ *Pseudomonas* 1-1-1.

● *V. alginolyticus.*

■ *V. anguillarum.*

✱ *V. furnissii.*

▲ *V. tubiashii.*

Percentage similarity
50
85
100
Cluster
A
B
C
G018
G017
G019
G016
G015
G024
G021
G013
G012
G009
P111
V006
G004
G022
G023
G026
G025
G008
G007
G002
G022
G020
G005
G003
G010
G001
Presumptive identification

FIGURE 4 Cluster analysis of bacterial isolates from Guernsey, analysed by Simple Matching, Similarity Coefficient (S_{SM}) linked with UPGMA method and the program Bacterial Identifier (Bryant *et al.*, 1986). A reference strain V006 (*V. anguillarum* 6) was included in *V. anguillarum.*

Legend:

Bacterial tentative identification

□ *Aeromonas.*

○ *Pseudomonas* 1-1-1.

● *V. alginolyticus.*

■ *V. anguillarum.*

✱ *V. furnissii.*

▲ *V. tubiashii.*

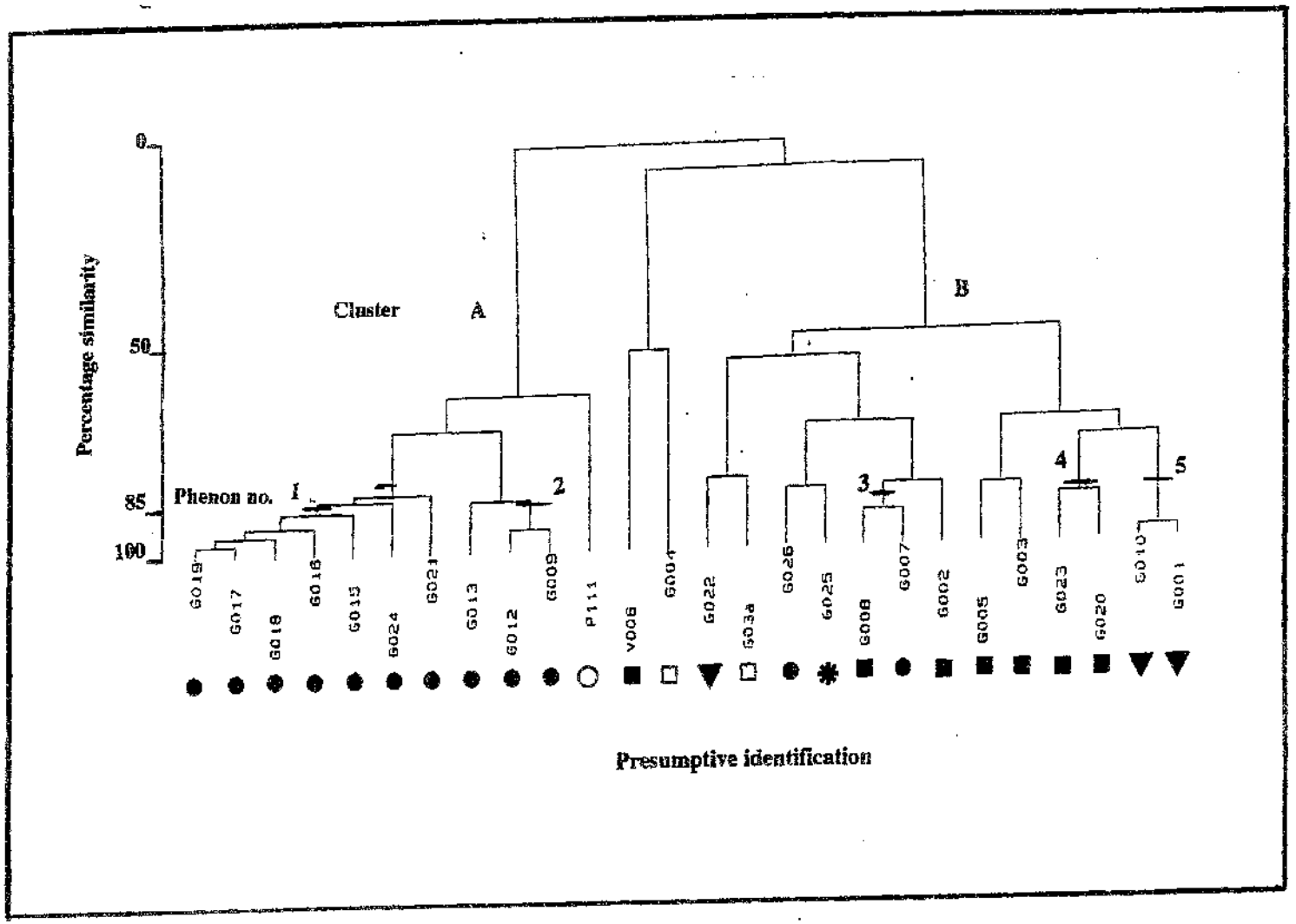

Percentage similarity
0
50
85
100
Cluster
A
B
Phenon no.
1
2
3
4
5
G019
G017
G018
G016
G015
G024
G021
G013
G012
G009
P111
V006
G004
G022
G034
G026
G025
G008
G007
G002
G005
G003
G023
G020
G010
G001
Presumptive identification

TABLE 10 : Summary of the percentage of isolates showing positive tests for all of the phenons associated with the cluster analysis of the group G bacterial isolates.

Tests	Phenons 1 (5)*	2 (2)	3 (2)	4 (2)	5 (2)
Growth on TCBS medium	100	100	100	100	100
Gram Stain	0	0	0	0	0
Fermentation of Glucose	100	100	100	50	100
Pigment	0	0	0	0	0
Motility	100	100	100	100	100
Swarming	100	100	0	0	0
Growth on CLED medium	20	0	0	0	0
Arginine Dihydrolase	0	0	100	100	0
Lysine Decarboxylase	100	100	100	50	0
Ornithine "	100	100	100	50	0
Nitrate Reduction	100	100	100	100	100
Oxidase Test	100	100	100	100	100
Indole Production	100	100	100	100	100
ONPG Hydrolysis	0	0	0	100	100
Voges Proskauer Test	100	100	0	0	0
Resistance to:					
O/129 (10 µg)	100	100	50	0	0
O/129 (150 µg)	0	50	50	0	0
Ampicillin	20	50	0	0	0
Hydrolysis of: Aesculin	0	0	0	0	0
Gelatin	100	100	100	100	100
Lecithin	100	100	100	100	0
Starch	100	100	100	100	100
Haemolysis (sheep rbcs)	100	100	0	0	0
Acid from: Arbutin	0	0	0	100	0
Salicin	0	0	0	0	0
Sucrose	100	100	100	100	100
Utilisation of:					
L-arabinose	100	0	0	0	0
D-cellobiose	80	100	100	100	100
D-galactose	100	100	100	100	100
D-melibiose	40	0	0	0	0
D-gluconate	100	100	100	50	100
D-glucuronate	0	100	0	100	0
L-citrulline	0	100	50	0	50
D-glucosamine	100	100	100	0	0
DL-3-hydroxybutyrate	80	100	0	0	0
Succinate	100	100	100	0	0
Resistance to:					
Chloramphenicol	0	0	0	0	0
Erythromycin	100	100	100	100	100
Fusidic acid	60	100	50	100	100
Methicillin	100	100	100	50	50
Novobiocin	0	0	0	0	0
Penicillin G	100	100	50	0	0
Streptomycin	80	100	100	100	100
Tetracycline	0	0	0	0	0
Ampicillin	100	100	0	0	0
Cephaloridine	100	100	100	100	100
Colistin Sulphate	0	0	0	0	0
Gentamicin	40	0	100	100	0

*Number of isolates per phenon

FIGURE 5 Cluster analysis of bacterial isolates from Conwy (labelled ✱) and Reculver (unlabelled), analysed by Jaccard Similarity Coefficient (S_J) linked with the UPGMA method and the program Bacterial Identifier (Bryant *et al.*, 1986). A reference strain V006 (*V. anguillarum* 6) was included in *Vibrionaceae.*

Legend:

Bacterial isolate presumptive family or genera

- ● *Cytophaga/Flavobacterium.*
- ○ Enterobacteriaceae.
- △ *Micrococcus.*
- ✱ Moraxellaceae.
- ■ *Pseudomonas.*
- □ Vibrionaceae.

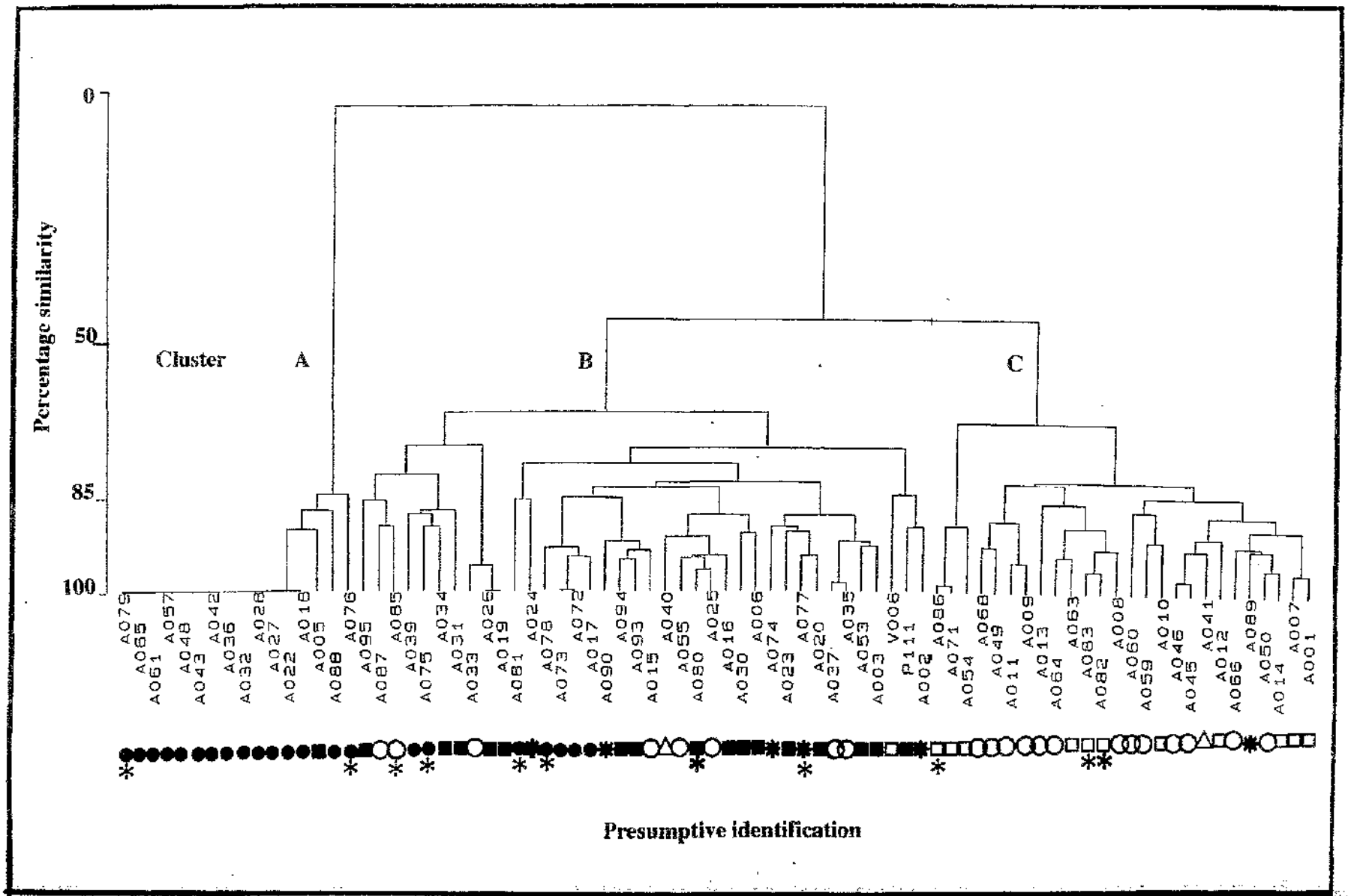

Percentage similarity
0
50
85
100
Cluster
A
B
C
A079
A065
A061
A057
A048
A043
A042
A036
A032
A028
A027
A022
A016
A005
A088
A076
A095
A087
A085
A039
A075
A034
A031
A033
A026
A019
A081
A024
A078
A073
A072
A017
A090
A094
A093
A015
A040
A055
A080
A025
A016
A030
A006
A074
A023
A077
A020
A037
A035
A053
A003
V006
P111
A002
A086
A071
A054
A068
A049
A011
A009
A013
A064
A063
A083
A082
A008
A060
A059
A010
A046
A045
A041
A012
A066
A089
A050
A014
A007
A001
Presumptive identification

FIGURE 6 Cluster analysis of bacterial isolates from Conwy (labelled ✱) and Reculver (unlabelled), analysed by Simple Matching, Similarity Coefficient (S_{SM}) linked with the UPGMA method and the program Bacterial Identifier (Bryant *et al.*, 1986). A reference strain V006 (*V. anguillarum* 6) was included in *Vibrionaceae*.

Legend:

Bacterial isolate presumptive family or genera

- ● *Cytophaga/Flavobacterium.*
- ○ Enterobacteriaceae.
- △ *Micrococcus.*
- ✱ Moraxellaceae.
- ■ *Pseudomonas.*
- □ Vibrionaceae.

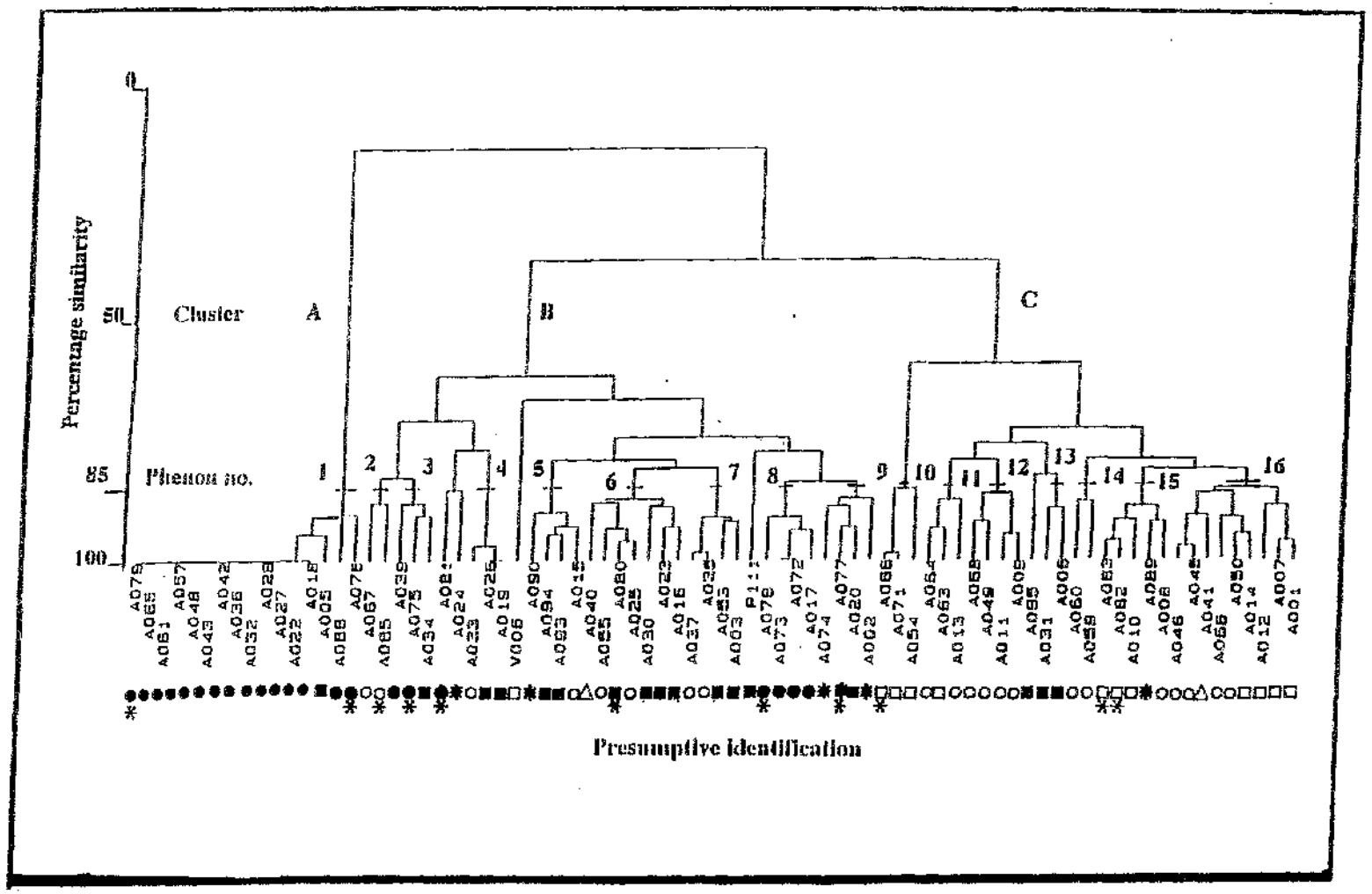

Percentage similarity
0
50
85
100
Cluster
A
B
C
Phenon no.
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Presumptive identification

TABLE 11 : Summary of the percentage of isolates showing positive tests for phenons numbered 1 to 8 associated with the cluster analysis of group A (Conwy and Reculver) isolates.

Tests	Phenons							
	1 (16)*	2 (2)	3 (3)	4 (4)	5 (4)	6 (7)	7 (4)	8 (4)
Growth on TCBS medium	0	100	33	0	0	14	0	0
Gram Stain	0	0	0	0	0	14	0	0
Fermentation of Glucose	0	50	0	0	0	43	50	0
Pigment	93	50	100	100	0	0	0	100
Motility	93	50	33	100	75	86	100	0
Swarming	0	100	0	0	0	0	0	0
Luminescence	76	0	0	0	0	0	0	0
Growth on CLED medium	0	0	0	0	25	0	0	0
Arginine Dihydrolase	0	50	0	0	50	86	0	50
Lysine Decarboxylase	0	0	33	0	100	100	0	100
Ornithine "	0	0	0	0	25	71	0	0
Nitrate Reduction	87	0	33	33	25	0	0	0
Oxidase Test	93	0	66	100	75	71	25	100
Indole Production	100	100	100	0	75	86	75	100
ONPG Hydrolysis	0	0	33	0	0	57	25	100
Voges Proskauer Test	0	0	0	0	0	0	0	0
Resistance to:								
O/129 (10 µg)	100	100	100	100	100	100	100	100
O/129 (150 µg)	13	100	100	100	100	86	100	75
Ampicillin	13	100	0	0	100	43	100	25
Polymyxin B	87	100	66	0	25	29	50	75
Hydrolysis of: Aesculin	0	100	0	0	0	0	0	0
Elastin	13	100	33	0	0	43	25	25
Gelatin	93	0	33	33	100	86	100	75
Lecithin	0	0	0	0	0	0	25	0
Starch	93	0	33	100	0	86	100	25
Xanthine Decomposition	0	0	0	0	25	14	0	0
Haemolysis (sheep rbcs)	0	50	0	0	25	0	0	25
Acid from: Arbutin	7	0	0	0	0	14	0	25
Salicin	0	0	0	0	0	0	0	0
Sucrose	7	50	33	0	75	0	25	100
Utilisation of:								
L-arabinose	7	100	33	0	100	100	50	100
D-cellobiose	0	100	100	100	100	100	100	100
D-galactose	0	100	100	100	75	100	100	100
D-melibiose	0	100	100	0	100	100	100	100
D-gluconate	0	100	66	0	100	86	100	100
D-glucuronate	0	100	100	100	100	86	75	100
L-citrulline	0	50	0	100	0	100	100	100
L-leucine	0	50	66	100	25	86	75	100
D-glucosamine	0	50	0	100	75	86	75	100
DL-3-hydroxybutyrate	0	50	33	0	100	86	100	100
Succinate	0	0	0	0	100	71	100	100
Resistance to:								
Chloramphenicol	0	50	0	0	0	14	0	0
Erythromycin	19	100	66	0	75	14	25	75
Fusidic acid	6	50	0	0	25	86	75	50
Methicillin	100	100	66	0	100	100	75	50
Novobiocin	94	100	33	100	75	86	50	0
Penicillin G	100	100	33	0	100	100	100	100
Streptomycin	100	100	66	100	100	100	50	100
Tetracycline	6	100	33	0	100	86	60	25
Ampicillin	0	50	66	0	50	29	75	75
Cephaloridine	94	100	33	0	25	100	75	75
Colistin Sulphate	94	50	33	100	0	14	25	0
Gentamicin	94	50	66	100	25	29	0	75

*Number of isolates per phenon.

TABLE 12 : Summary of the percentage of isolates showing positive tests for phenons 9 to 16 associated with the cluster analysis of group A (Conwy and Reculver) isolates.

Tests	Phenons							
	9 (4)*	10 (3)	11 (3)	12 (4)	13 (2)	14 (2)	15 (5)	16 (9)
Growth on TCBS medium	0	100	66	100	66	100	60	100
Gram Stain	0	0	0	0	0	50	0	20
Fermentation of Glucose	0	100	100	100	0	100	100	90
Pigment	0	0	0	50	33	0	100	0
Motility	25	100	100	100	100	100	100	100
Swarming	0	100	0	0	0	0	0	50
Luminescence	0	0	0	0	0	0	0	0
Growth on CLED medium	0	0	0	0	0	0	0	0
Arginine Dihydrolase	0	66	100	50	66	50	100	50
Lysine Decarboxylase	0	100	0	25	33	0	0	10
Ornithine "	0	33	0	25	0	0	0	0
Nitrate Reduction	50	66	100	0	66	50	100	90
Oxidase Test	100	100	33	100	100	0	0	50
Indole Production	75	100	100	0	100	100	100	40
ONPG Hydrolysis	50	33	100	100	0	100	60	100
Voges Proskauer Test	0	66	0	100	0	0	0	0
Resistance to:								
O/129 (10 µg)	100	100	100	0	100	0	80	80
O/129 (150 µg)	100	100	66	100	33	0	80	60
Ampicillin	25	100	0	50	100	50	40	90
Polymyxin B	50	0	0	50	66	50	0	10
Hydrolysis: Aesculin	0	0	0	50	33	0	0	0
Elastase	25	0	66	0	33	50	20	60
Gelatinase	75	66	33	25	100	0	80	80
Lecithinase	0	33	0	100	0	0	0	0
Starch	50	0	66	0	66	50	100	88
Xanthine Decomposition	0	33	33	50	33	100	40	80
Haemolysis (sheep rbcs)	25	100	100	50	33	100	100	90
Acid from: Arbutin	25	33	0	0	0	0	0	0
Salicin	0	66	0	0	0	0	0	0
Sucrose	75	33	100	0	0	0	40	0
Utilisation of:								
L-arabinose	100	0	0	25	100	50	100	90
D-cellobiose	100	0	66	25	33	100	100	100
D-galactose	100	100	66	100	66	100	80	90
D-melibiose	100	0	66	25	66	50	100	100
D-gluconate	75	100	100	100	100	100	20	100
D-glucuronate	75	100	0	100	33	100	100	60
L-citrulline	100	0	33	100	0	50	20	40
L-leucine	75	66	33	50	66	50	100	50
D-glucosamine	75	100	100	75	33	100	80	80
DL-3-hydroxybutyrate	50	0	66	75	33	100	100	90
Succinate	100	100	0	100	33	100	40	55
Resistance to:								
Chloramphenicol	0	0	0	0	0	0	0	0
Erythromycin	25	100	100	50	50	100	60	67
Fusidic acid	50	100	66	100	50	0	100	89
Methicillin	75	100	100	100	50	50	60	67
Novobiocin	75	0	0	25	0	50	20	0
Penicillin G	50	100	100	100	50	0	40	67
Streptomycin	75	100	100	75	100	50	80	100
Tetracycline	75	100	33	25	0	100	20	22
Ampicillin	75	0	0	0	50	0	20	44
Cephaloridine	50	100	100	100	50	50	80	67
Colistin Sulphate	25	0	0	0	0	0	20	0
Gentamicin	25	100	33	0	100	0	20	22

*Number of isolates per phenon.

(Fig. 6). Detailed analysis of these results is presented in section 4.

3.2.3 Taxonomic Analysis of the Conwy, Guernsey and Reculver Isolates.

Cluster analysis of all the bacterial isolates from Conwy, Guernsey and Reculver was carried out using both S_J and S_{SM}, similarity coefficients and the resulting dendrograms are presented in Figures 7 and 8 respectively.

Guernsey isolates clustered together in distinct regions of the dendrograms in relation to the Conwy and Reculver strains (Figures 7 and 8). The S_{SM} dendrogram was divided into 7 major clusters at the 85 % similarity. Phenon 1 was composed of presumptive *Cytophaga/Flavobacterium* from Conwy and Reculver. The next phenons, 2 and 3, contained all of the Guernsey bacterial isolates, along with presumptive vibrios from Reculver and Conwy (phenon 3). The remaining bacterial isolates from Conwy and Reculver were clustered as in Fig. 5 and 6 and are discussed later in section 4.

Analysis of the clusters at greater than 85 % similarity, using the S_J Coefficient, showed all of the Guernsey bacterial isolates placed in the final two phenons 6 and 7. One of the Reculver isolates, A31, shared similarity with G1 and G10 (phenon 7). The presumptive *V. alginolyticus* strains from all of the three locations were clustered together in phenon 6. Although the major clusters were different in location in both of the S_J and S_{SM} dendrograms (Fig. 7 and 8), the isolates present in the phenons showed a cognative pattern in similarity which was also related to that obtained by the individual analysis of the Guernsey (Fig. 3 & 4) and the Reculver-Conwy bacterial isolates (Fig. 5 & 6).

3.2.4 Analysis of bacteria from different origins.

Thirty one ecological, biochemical and nutritional tests were completed on a combination of 113 bacterial isolates from France (16), Spain (54) and United Kingdom (34 U.K., 10 Guernsey, 21 Reculver & 3 Conwy isolates) by a French group of researchers participating in the EC programme. Figure 9 shows the

FIGURE 7 Cluster analysis of bacterial isolates from Conwy (labelled ✱) Guernsey (labelled ★) and Reculver (unlabelled), analysed by Jaccard, Similarity Coefficient (S_J) linked with the UPGMA method and the programme Bacterial Identifier (Bryant *et al.*, 1986). A reference strain V006 (*V. anguillarum* 6) was included in *Vibrionaceae*.

Legend:

Bacterial isolate presumptive family or genera

● *Cytophaga/Flavobacterium.*

○ Enterobacteriaceae.

△ *Micrococcus.*

✱ Moraxellaceae.

■ *Pseudomonas.*

□ Vibrionaceae.

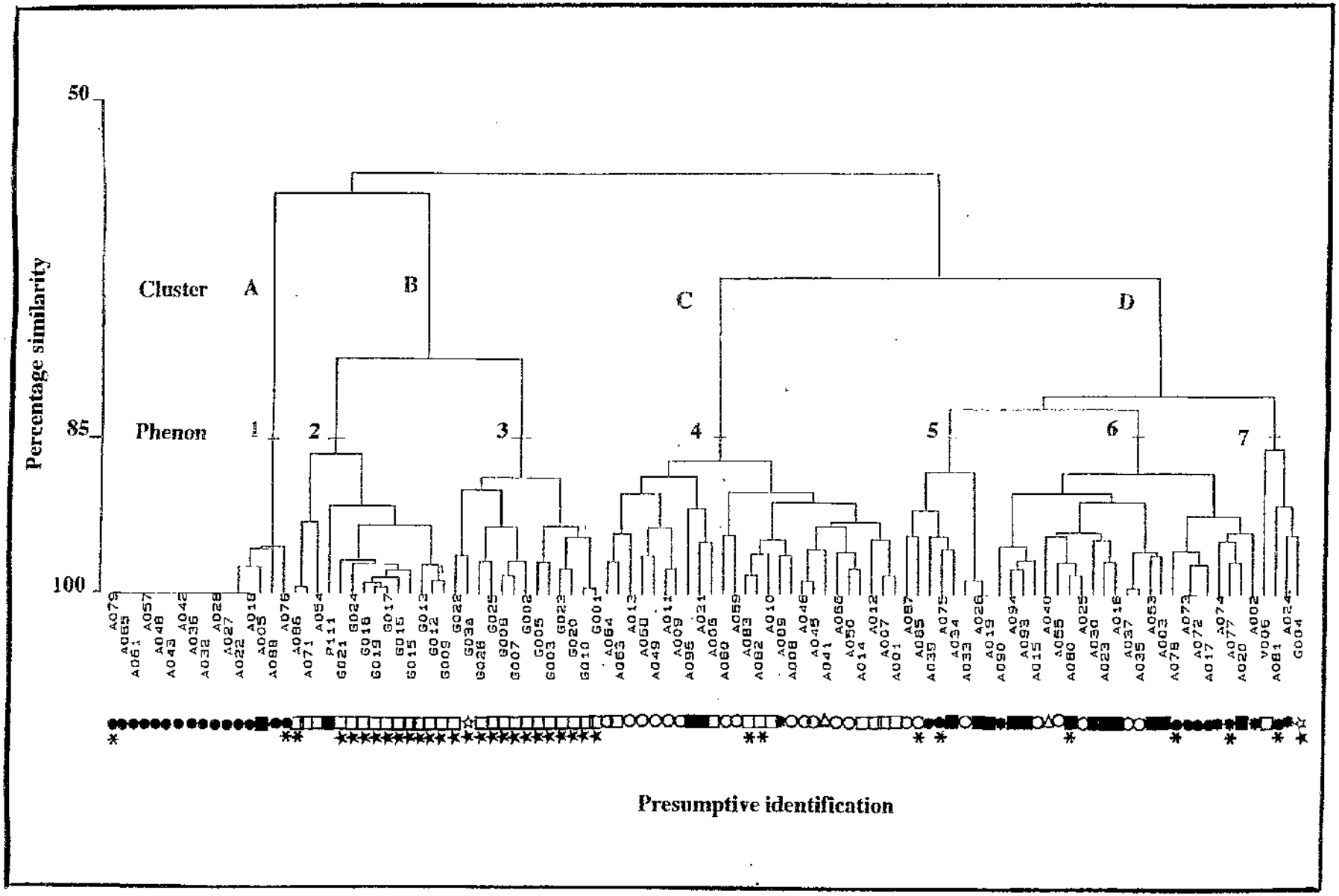

50
85
100
Percentage similarity
Cluster
A
B
C
D
Phenon
1
2
3
4
5
6
7
Presumptive identification

FIGURE 8 Cluster analysis of bacterial isolates from Conwy (labelled ✱) Guernsey (labelled ★) and Reculver (unlabelled), analysed by Simple Matching, Similarity Coefficient (S_{SM}) linked with the UPGMA method and the programme, Bacterial Identifier (Bryant *et al.*, 1986). A reference strain V006 (*V. anguillarum* 6) was included in *Vibrionaceae*.

Legend:

Bacterial isolate presumptive family or genera

- ● *Cytophaga/Flavobacterium.*
- ○ Enterobacteriaceae.
- △ *Micrococcus.*
- ✱ Moraxellaceae.
- ■ *Pseudomonas.*
- □ Vibrionaceae.

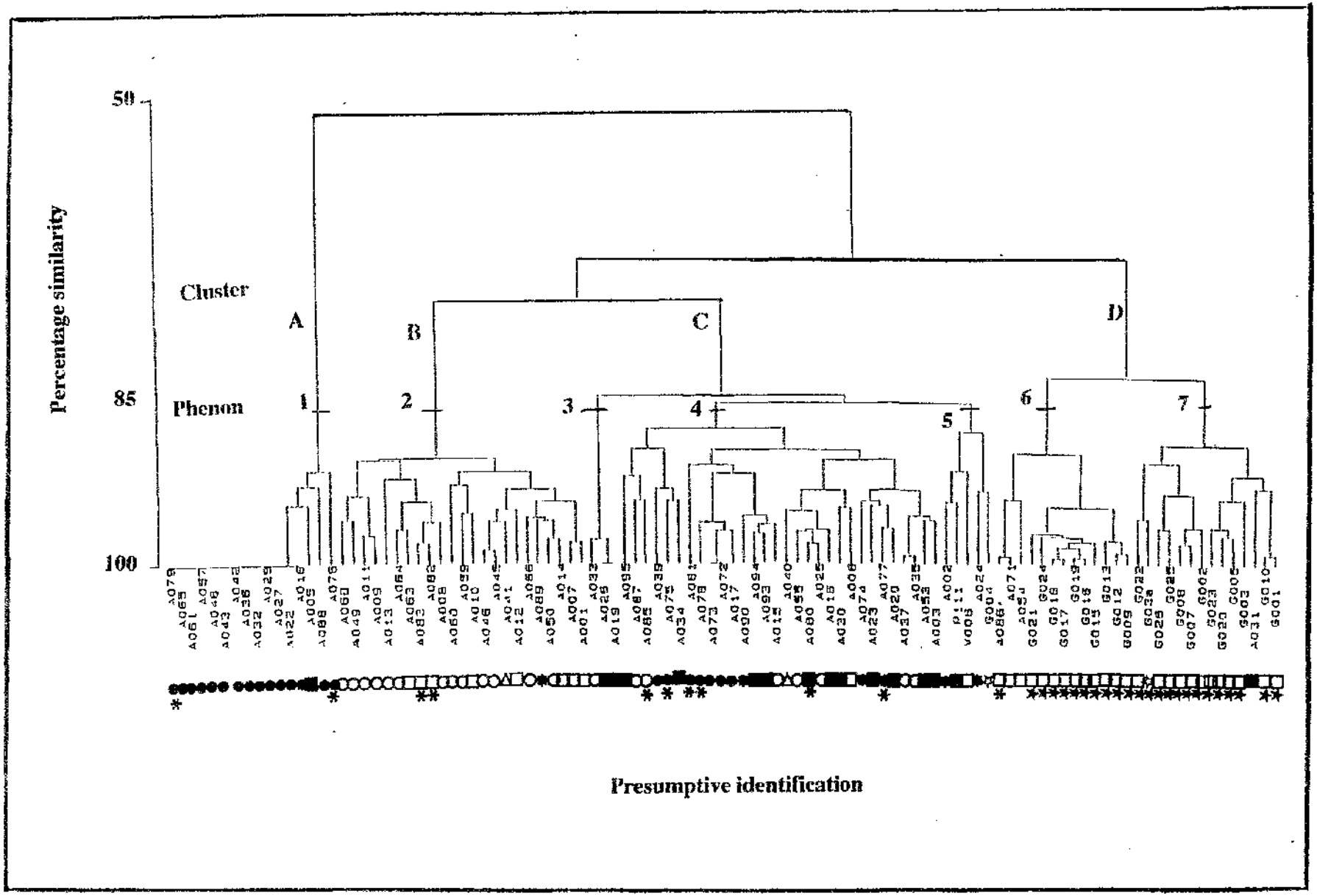

Percentage similarity
50
85
100
Cluster
A
B
C
D
Phenon
1
2
3
4
5
6
7
Presumptive identification

FIGURE 9 A simplified dendrogram of 113 isolates originating from France (16), Spain (54), United Kingdom (34) and reference bacterial strains (9) as produced by a group of French researchers.

Legend:

Colony pigmentation


Red pigmented


Yellow spreader

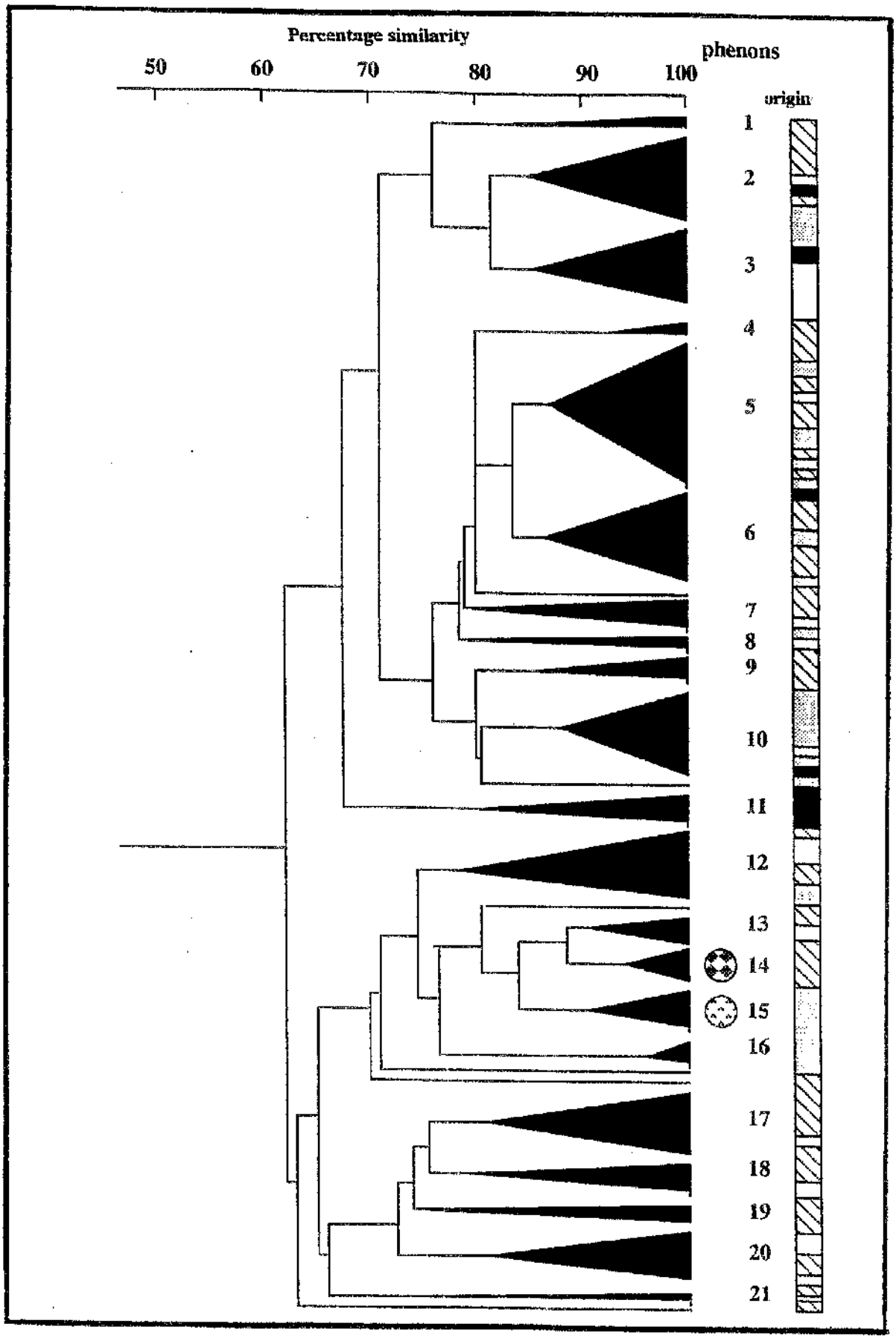
Origin of the bacterial isolates


France


Spain


United Kingdom


Reference bacteria


Percentage similarity
50
60
70
80
90
100
phenons
origin
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21

dendrogram produced after cluster analysis was performed on the tests results and 21 phenons were obtained.

Isolates from the U.K. were found in 3 large clusters associated with phenons 2, 3, 10, 15 and 16. The remainder of the isolates were distributed between 13 much smaller clusters. All of the isolates shared similarity with the Spanish strains and with some of the reference bacteria. French isolates were more frequently clustered with the Spanish strains, as compared with those from U.K. Approximately 95 % similarity was observed with isolates belonging to the same species; these similarities were within the 6 Spanish and 4 U.K. isolates and finally between 2 Spanish and 2 U.K. On the basis of analysed characteristics, the isolates examined represent a relatively important diversity.

Low similarity was observed between the isolates and the reference strains. Seven French and U.K. strains, and 8 Spanish and 4 U.K. isolates were the only strains that showed a significant relationship ($\geq$ 90 % similarity) with *V. alginolyticus* and *V. splendidus* respectively. The remainder of the isolates belonging to *Vibrionaceae* showed low similarities ($\geq$ 70 %) with reference strains of the species *V. alginolyticus, V. damsela, V. mediterranei, V. parahaemolyticus, V. splendidus* and *V. tubiashii*. These results are in agreement with previous phylogenetic studies (Nicolas, 1992b) which showed that a large number of bacterial strains from hatcheries correspond to new bacterial species.

3.3 DEVELOPMENT OF THE HAEMOCYTE CYTOTOXICITY ASSAY.

The principal aim of the experimental work carried out in this section was to develop a bioassay to be used in the screening of bacterial isolates from areas of disease outbreaks, to determine their toxicity and thus identify potential pathogenic strains.

3.3.1 Larval Viability Assay.

Initially, *C. gigas* larvae viability assays were used to determine the effects of larval exposure (2 day old larvae) to overnight cultures (20 ^{o}C) in marine broth of potential

pathogenic bacteria. Experimental results of larval growth and survival after 72 h incubation at 20 ^{o}C, with the potential pathogen, *V. anguillarum* NCIMB 6 (V6) or the possibly non-pathogenic bacterium, *Pseudomonas* 1-1-1 (P1-1-1), at bacterial concentrations of 1 or 10 c.f.u. per larva were recorded. The larvae were monitored over 72 h, and Figures 10 a, b and c show the effect of bacteria (24 h in marine broth culture) on the oyster larval survival. Approximately 90 - 100 % of the control larvae (that were not exposed to added bacteria) and larvae exposed to P1-1-1 (1 or 10 c.f.u. per larva), survived the 72 h incubation (Fig. 10 a).

Larvae exposed to 1 or 10 c.f.u. of V6 per larva showed a different pattern of survival compared to the control and the probably non-pathogenic P1-1-1. Almost 90 % of these larvae survived for up to 48 h, but there was a significant decrease in viability thereafter; only 10 % (10 c.f.u. of V6 per larva) and 15 % (1 c.f.u. of V6 per larva) survived up to 72 h at 20 ^{o}C.

Bacterial growth was monitored during the 72 h incubation at 20 ^{o}C. There was an increase in bacterial numbers for both bacterial types, from 10^2 to 10^4 per ml, for 1 c.f.u. of P1-1-1 per larva and 10^2 to 10^5 per ml, for 10 c.f.u. of P1-1-1 and for 1 and 10 c.f.u. of V6 per larva (Fig. 10 b).

Shell sizes were recorded throughout the exposure of the larvae and it was noted that although there was little or no significant difference between the shell sizes of test and control larvae (Fig. 10 c), the larvae incubated with 10 c.f.u. of V6 per larva exhibited reduced shell growth over 72 h at 20 ^{o}C and their shell sizes measured less than the control.

3.3.2 Experimental Infections with Bacterial Strains.

Experimental infections were performed with *Vibrio* isolates from France, Spain and the United Kingdom, under similar conditions. Oyster (*C. gigas*) or scallop (*Pecten maximus*) larval cultures were inoculated with 2 day old isolate cultures in marine broth, at 10^4 - 10^5 c.f.u. per ml^{-1}, final concentration, in triplicate or sometimes in

FIGURE 10 The effects of bacteria on oyster larvae.

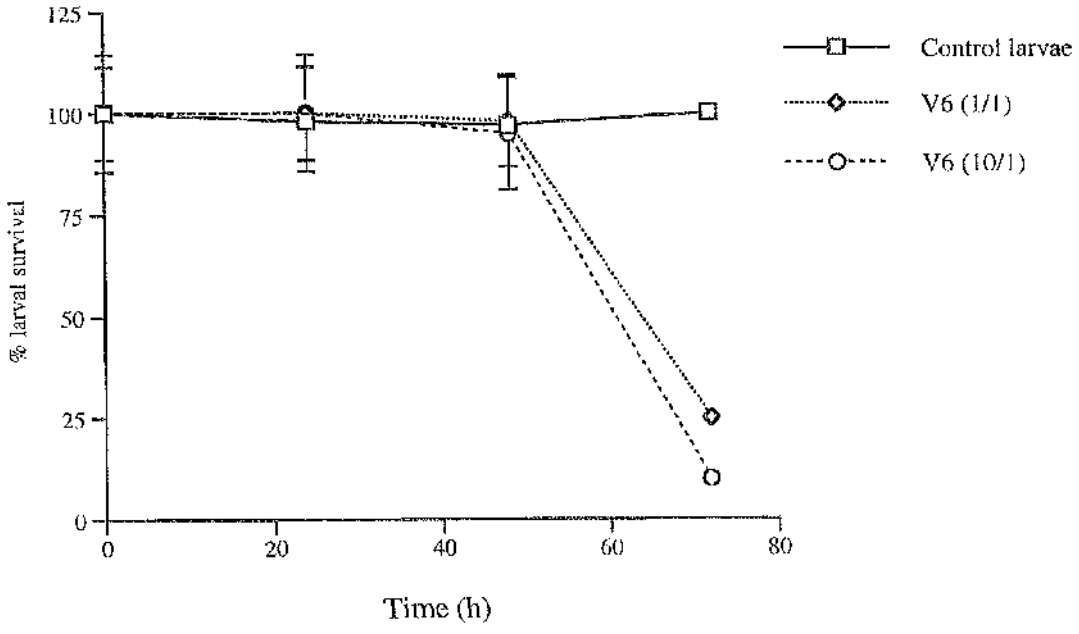
Oyster larvae (10 larvae/ml) were suspended in sterile sea water and incubated with different concentrations of bacteria at 20 °C for 72 h. Tests were carried out in duplicate.

a Oyster larval survival after exposure to 1 or 10 c.f.u. of *V. anguillarum* NCIMB 6 (a i) or of *Pseudomonas* 1-1-1 (a ii) per larva.

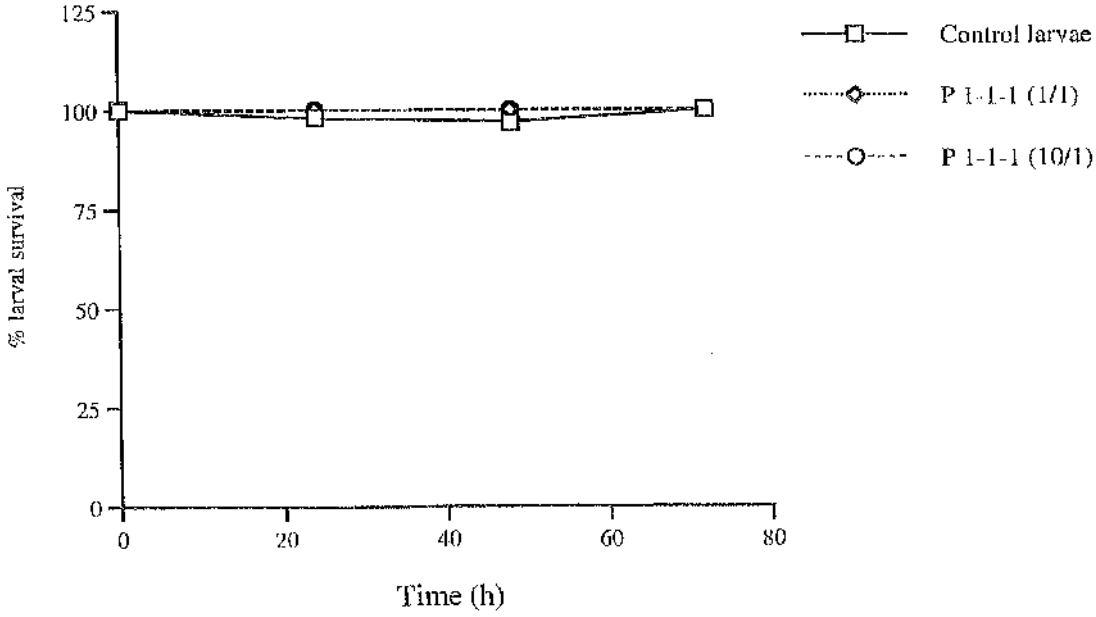
b Bacterial growth throughout the incubation of the oyster larvae with the *V. anguillarum* NCIMB 6 (b i) or *Pseudomonas* 1-1-1 (b ii) (1 or 10 c.f.u. per larva).

c Effects of *V. anguillarum* NCIMB 6 (c i) or *Pseudomonas* 1-1-1 (c ii) on the shell sizes of the oyster larvae (1 or 10 c.f.u. per larva).

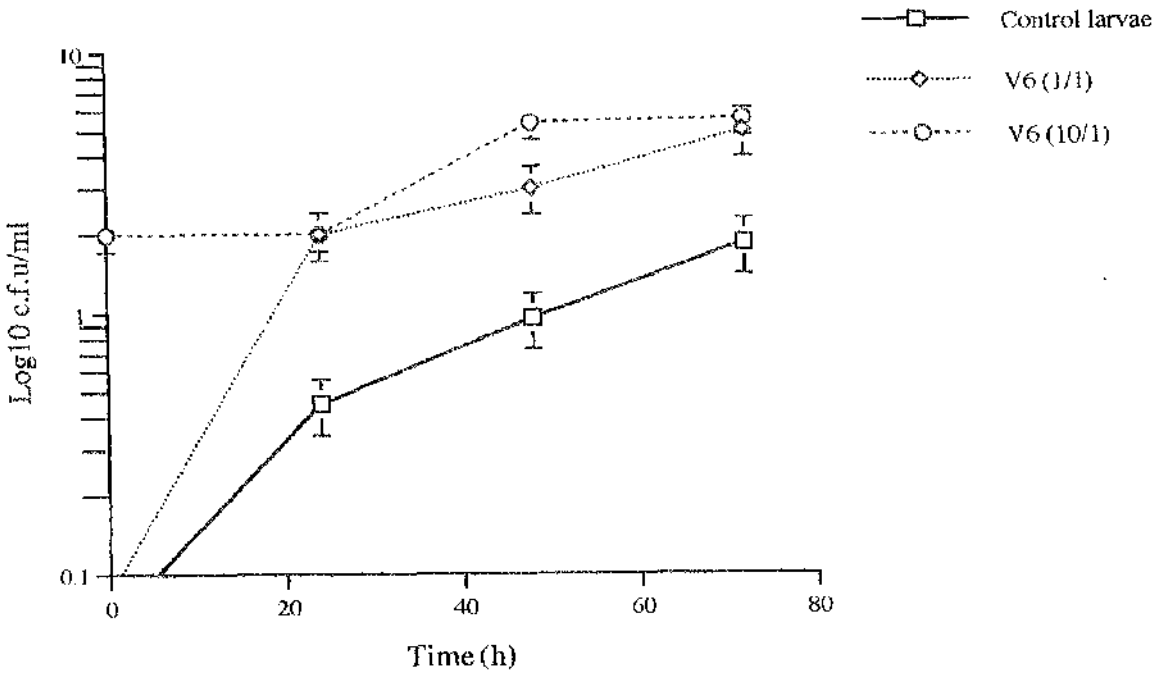
10 a i


125
100
75
50
25
0
% larval survival
0
20
40
60
80
Time (h)
Control larvae
V6 (1/1)
V6 (10/1)

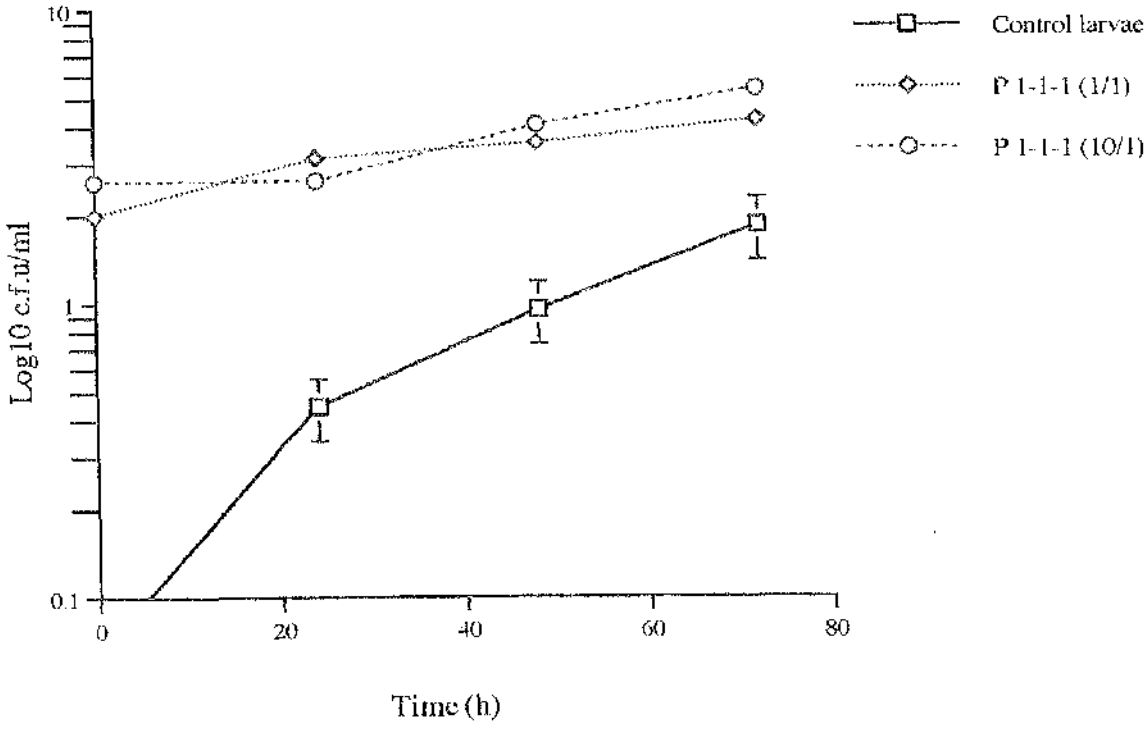
10 a ii


125
100
75
50
25
0
% larval survival
0
20
40
60
80
Time (h)
Control larvae
P 1-1-1 (1/1)
P 1-1-1 (10/1)

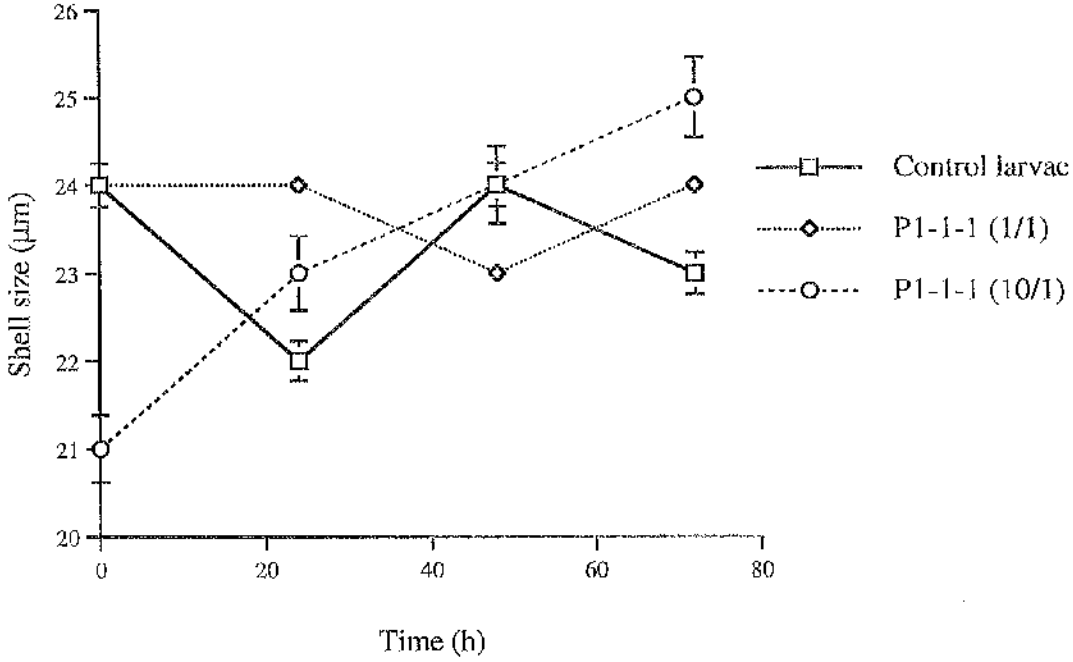
10 b i


Control larvae
V6 (1/1)
V6 (10/1)
Log10 c.f.u/ml
10
0.1
0
20
40
60
80
Time (h)

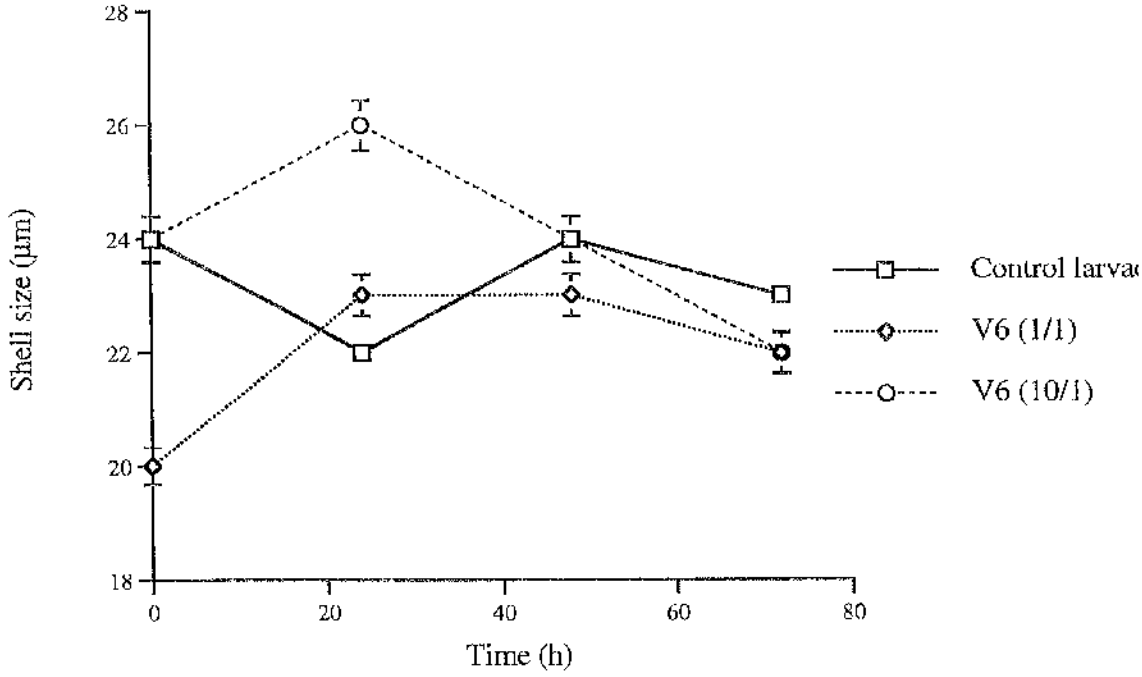
10 b ii


Control larvae
P 1-1-1 (1/1)
P 1-1-1 (10/1)
Log10 c.f.u/ml
10
0.1
0
20
40
60
80
Time (h)

10 c i


26
25
24
23
22
21
20
Shell size (µm)
0
20
40
60
80
Time (h)
Control larvae
P1-1-1 (1/1)
P1-1-1 (10/1)

10 c ii


28
26
24
22
20
18
Shell size (µm)
0
20
40
60
80
Time (h)
Control larvae
V6 (1/1)
V6 (10/1)

duplicate. Sea water was renewed every day.

Oyster larvae (12 day old, *C. gigas*) were inoculated with 3 U.K. isolates (G1, G13 from Guernsey and A1 from Reculver). Only G13 exhibited virulence in 2 replicates from day 5 (Experimental infection 1). In the second experimental infection, French (V322) and Spanish isolates (GR86, GR82, BZ162 and BZ168) were inoculated into the 12 day old *C. gigas* larvae (13 day old). No mortality occurred after 6 days.

The third experiment with 18 day old *C. gigas* larvae was again inconclusive. With the French inoculum of 10^5 bacterial cells ml^{-1} of V322, all the larvae died in 48 h. At 10^4 bacterial cell ml^{-1}, the Reculver isolates A45 and A10 provoked 30 % mortality in 3 days while the French isolates (V110, V322) and the Spanish isolate GR82, did not produce a lethal effect in 3 days. This assay was stopped early because the larvae reached metamorphosis.

In two other assays using scallop larvae, isolate A1 (Reculver) exhibited moderate virulence from day 5 and G1 (Guernsey isolate) from day 6 but the larvae in the controls without chloramphenicol also began to die. In the last experimental infection only the isolate V365 provoked mass mortality from day 3, the other inoculated isolates killed 30 and 48 % of the larvae by day 6.

3.4 DEVELOPMENT OF THE HAEMOCYTE ASSAY.

The larval viability assay was time consuming and did not facilitate the rapid screening of large numbers of potentially toxic bacteria, therefore a bivalve haemocyte cytotoxicity assay was considered.

The interaction of vibrios with *Mytilus edulis* at cellular level was investigated previously by Nottage and Birkbeck (1990), who showed that the differences in virulence of two strains of *V. alginolyticus* for the oyster larvae reflected differences in the toxicity of washed bacterial cells to haemocytes of *M. edulis*. The ratio of

bacteria to haemocyte used by Nottage *et al.* (1989) was high (50 to 500:1) and the assay depended on the uptake of the vital stain neutral red. A number of alternatives to the neutral red assay of Nottage *et al.* (1989) were considered.

3.4.1 Haemocyte Viability Assays.

Haemocyte viability was detected after exposure to different bacteria by the use of molecular probes that varied in their principle of action. The following results were obtained.

3.4.1.1 Neutral red dye.

The neutral red method is based on the principle that viable cells will take up neutral red dye by active transport and incorporate the dye into the lysosomes, whereas non-viable cells will not take up the dye. This vital dye was used to assess the percentage of haemocytes surviving exposure to various bacterial ratios (10, 100, 1000 c.f.u. per haemocyte); the method was essentially as described by Nottage and Birkbeck (1990). Initially, visual observation of *Mytilus* haemocytes interacting with bacteria, when spread on microscope slides, was performed. Experiments using different *Vibrio* spp. (V1338, V1339, V1340, V2164, V2165 or V2166) or a control strain P1-1-1, were performed with bacteria suspended in FSW (Fig. 11). More than 90 % of the control haemocytes survived the 3 h incubation at 20 ^{o}C. When the haemocytes were incubated with *Vibrio* spp. V1339, V2164 or V2165 at 10 c.f.u. per haemocyte, approximately 80 to 100 % survival was observed (Fig. 11 a). At 100 c.f.u. per haemocyte, there was approximately 50 % reduction in haemocytes incubated with V1339, whereas an average of 60 % of the haemocytes survived after exposure to V2164 or V2165 (Fig. 11 a).

No visual toxic effect was observed when the control strain P1-1-1, was incubated at 10, 100 or 1000 c.f.u. per haemocyte, with greater than 90 % of the haemocytes surviving exposure to this bacterium (Fig. 11 a & b). *Vibrio* species 1338 or 1340 had little effect on the haemocyte survival (Fig. 11 b). The following

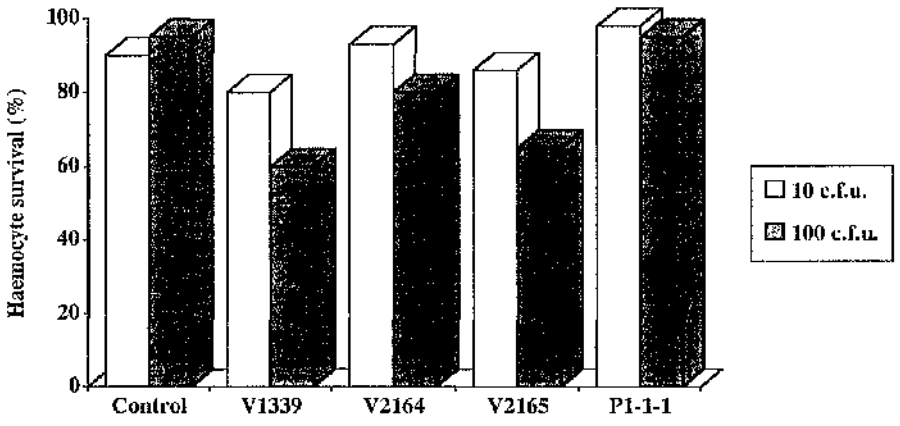
FIGURE 11 Visual observation of haemocyte survival indicated by the ability of the haemocyte to take up neutral red dye, after incubation with various bacteria suspended in filtered sea water and at different c.f.u. per haemocyte.

a Vibrios V1339, V2164, V2165 or P1-1-1 were tested at bacterial ratios of 10 or 100 c.f.u. per haemocyte.

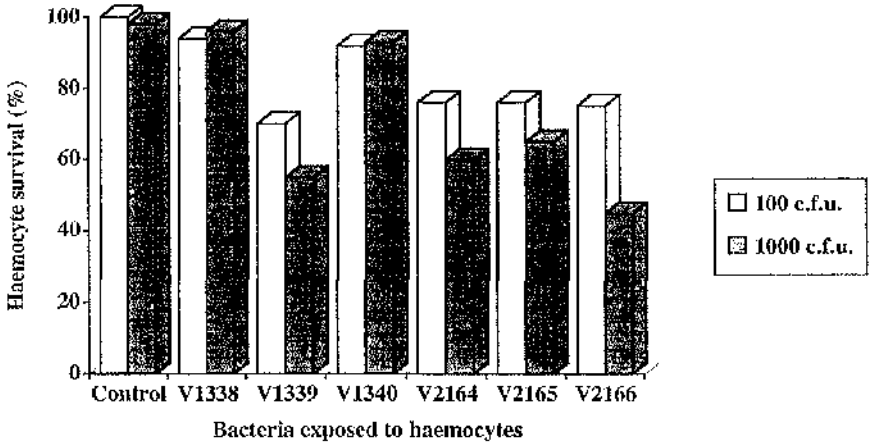
b Vibrios V1338, V1339, V1340, V2164, V2165 or V2166, were tested at 100 or 1000 c.f.u. per haemocyte.

Tests were carried out in duplicate and control tests was included for every mussel used in the assay (individual mussel haemocytes were not pooled). Cocultures of bacteria suspended in FSW and haemocytes (10^6/ml) were incubated for 3 hours. Afterwards 0.05% (w/v) neutral red was added to the mixture and further incubated for 30 minutes at 20 °C. The number of live haemocytes per 100 was recorded.

11a


Haemocyte survival (%)
100
80
60
40
20
0
Control
V1339
V2164
V2165
P1-1-1
10 c.f.u.
100 c.f.u.

11b


Haemocyte survival (%)
100
80
60
40
20
0
Control
V1338
V1339
V1340
V2164
V2165
V2166
Bacteria exposed to haemocytes
100 c.f.u.
1000 c.f.u.

Vibrio spp., V2164, V2165, V1339 or V2166, were more toxic to the haemocytes with increasing c.f.u. per haemocyte (Fig. 11 a, b).

An objective measurement of the effects of various *Vibrio* or P1-1-1 on *Mytilus* haemocytes was obtained by recording absorbance (540 nm) and fluorescence (400 nm) readings to determine the level of dye uptake by live haemocytes. High or low absorbance or fluorescence indicated haemocyte cell survival or death respectively.

When *Vibrio* spp. (V1338, V1339, V1340, V2164, V2165 or V2166) or P1-1-1 were screened for their toxicity to *Mytilus* haemocytes (Fig. 12 a); they did not exhibit significant toxicity to the haemocytes. The background reading was found to be higher than the readings for both the control or tests with bacteria. The toxicity of V1339 was investigated further at 10, 100 or 1000 c.f.u. per haemocyte but no significant decrease in haemocyte survival was observed (Fig. 12 b).

3.4.1.2 XTT

A further dye to be tested was XTT (2, 3 -bis [2-methoxy-4-nitro-5-sulfophenyl]-2-H-tetrazolium-5 carboxanilide inner salt). The XTT system measures the level of activity of mitochondrial dehydrogenases in living cells (Roehm *et al.*, 1991). Mitochondrial hydrogenases of viable cells cleave the tetrazolium ring in XTT, yielding orange formazan crystals which are soluble in aqueous solutions. The bioreduction of XTT is inefficient but can be potentiated by the addition of an electron coupling agent such as phenazine methosulphate (PMS) to the reaction (Roehm *et al.*, 1991). The haemocytes were incubated for 3 h with XTT at 20 ^{o}C.

From a suspension containing 10^6 haemocytes ml^{-1}, volumes of 40, 80 and 100 µl were incubated with different concentrations of XTT (20, 40 or 60 % of stock solution of 5 mg ml^{-1}). Figure 13 a shows that there was an increase in absorbance readings with increasing haemocyte and XTT concentrations. Test bacteria suspended in filtered haemolymph (FH) were introduced into the assay in the presence of

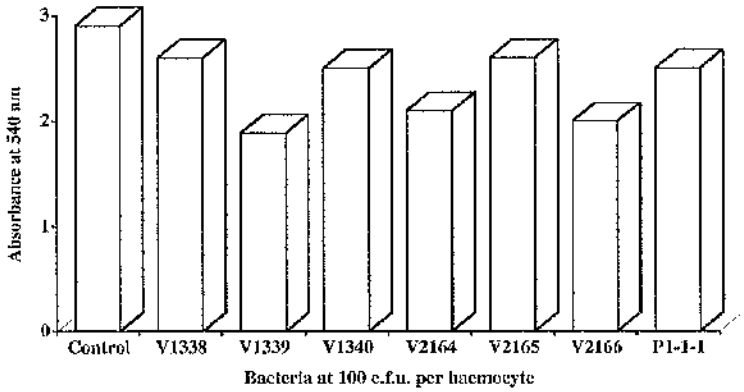
FIGURE 12 The effect of incubation of *Mytilus* haemocytes with various bacteria for 3 h at 20 °C, determined by neutral red absorbance at 540 nm or fluorecence at 400 nm.

a Effect of *Vibrio* spp. (V1338, V1339, V1340, V2164, V2165 and V2166) or P1-1-1, at 100 c.f.u. per haemocyte.

b The effect of three different ratios of V1339 (10, 100 or 1000 c.f.u. per haemocyte) suspended in filtered sea water, on *Mytilus* haemocytes.

Tests were carried out in duplicate and control tests was included for every mussel used in the assay (individual mussel haemocytes were not pooled). Cocultures of bacteria suspended in FSW and haemocytes (10^6/ml) were incubated for 3 hours. Afterwards 0.05% (w/v) neutral red was added to the mixture and further incubated for 30 minutes at 20 °C. The number of live haemocytes was determined by neutral red absorbance 540 nm or fluorescence 400 nm.

12a


Absorbance at 540 nm
3
2
1
0
Control
V1338
V1339
V1340
V2164
V2165
V2166
P1-1-1
Bacteria at 100 c.f.u. per haemocyte

12b

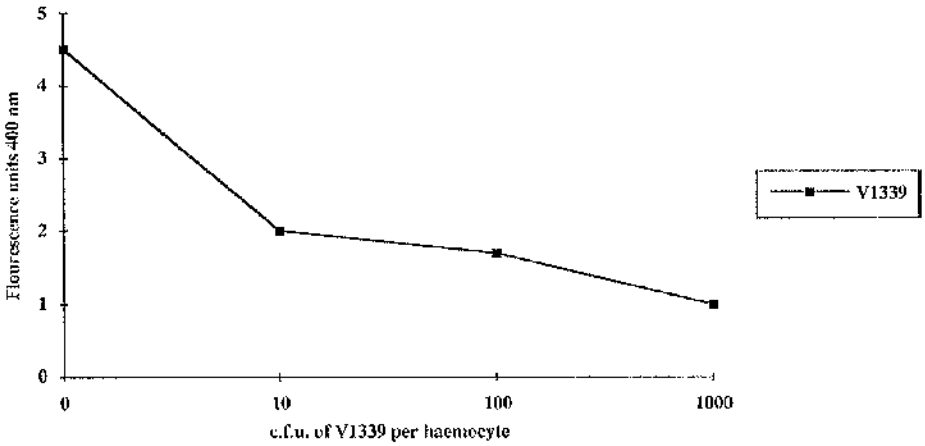

Flourescence units 400 nm
5
4
3
2
1
0
0
10
100
1000
c.f.u. of V1339 per haemocyte
V1339

FIGURE 13 The effect of Vibrios on *Mytilus* haemocytes. XTT was used to determine the number of live haemocytes after incubation with the different bacteria for 45 min at 20 °C.

a The effect of different concentrations of haemocytes and XTT solution on the production of formazan (A450 nm); 40, 80, or 100 µl of a suspension of haemocytes (10^6 ml^{-1}) were incorporated in the test with 20, 40 or 60 % concentrations of the stock solution of XTT (5 mg ml^{-1}).

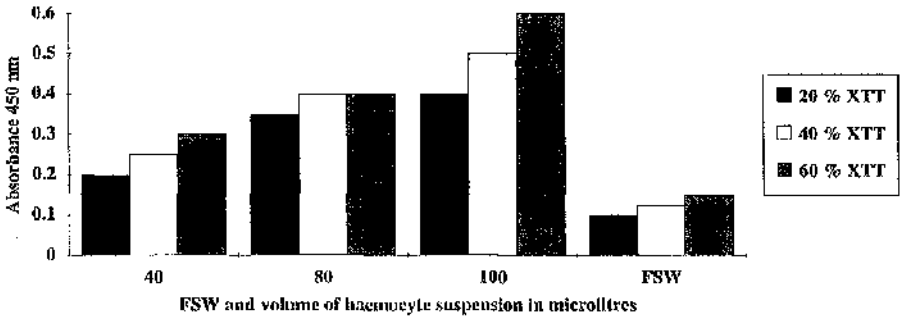
b, c, d Effect of *Vibrio* V1339, V2981 or V2165, suspended in FH or in FSW, on *Mytilus* haemocyte survival as determined by the production of formazan (A450 nm) in the presence of XTT solution.

c The effect of different concentrations of phenazine methosulphate (PMS) and XTT solution on the production of formazan (A450 nm) in the presence or absence (*) of penicillin-streptomycin (200 µg ml^{-1} and 0.0005 % respectively) solution in FSW.

f The effect of different concentrations of phenazine methosulphate (PMS) and XTT solution on the production of formazan (A450 nm) in the presence or absence (*) of penicillin-streptomycin (200 µg ml^{-1} and 0.0005 % respectively) solution in FH.

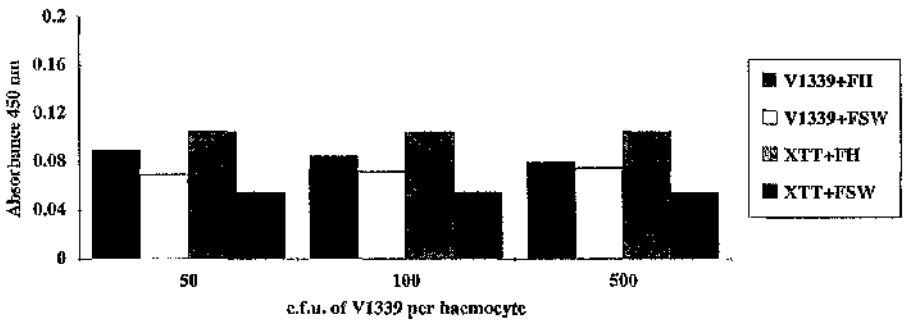
Initially, test bacteria and haemocytes were incubated for 3 hours, then 200 µg ml^{-1} XTT was added and the mixture was further incubated for 45 minutes. Tests were carried out in duplicate and appropriate controls were incorporated for all the mussels assayed.

13a



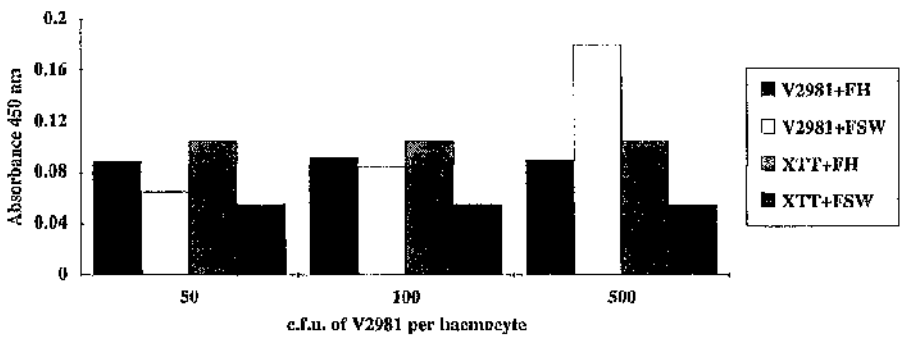
FSW and volume of haemocyte suspension in microlitres

13b



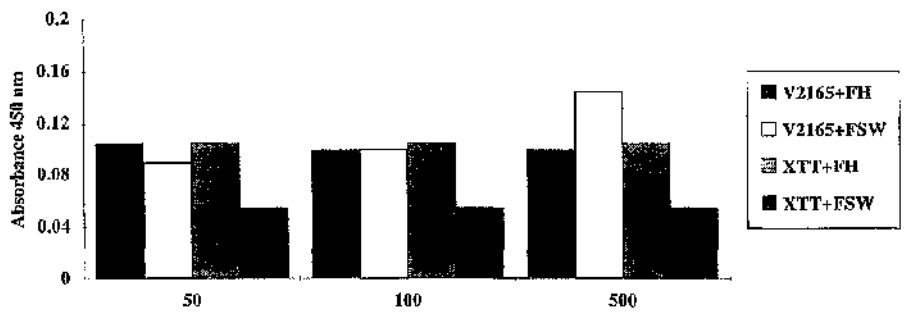
c.f.u. of V1339 per haemocyte

13c



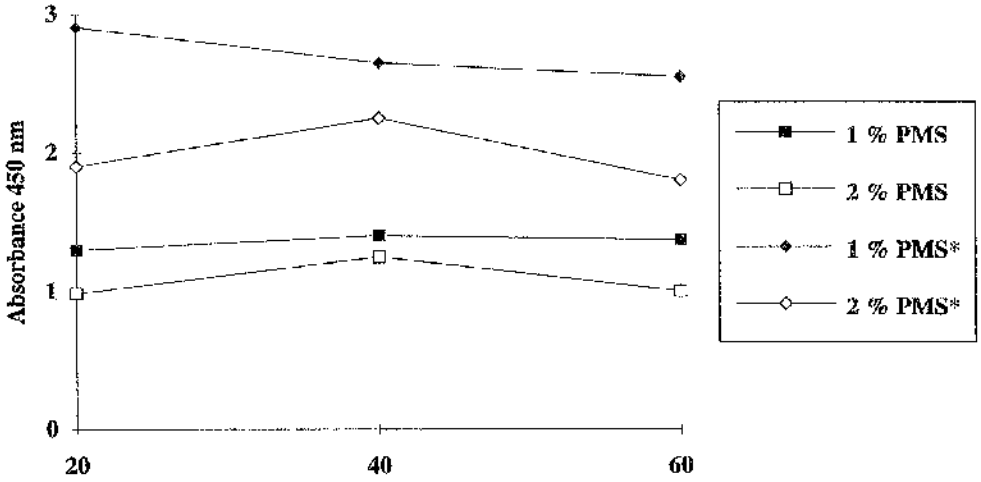
c.f.u. of V2981 per haemocyte

13d

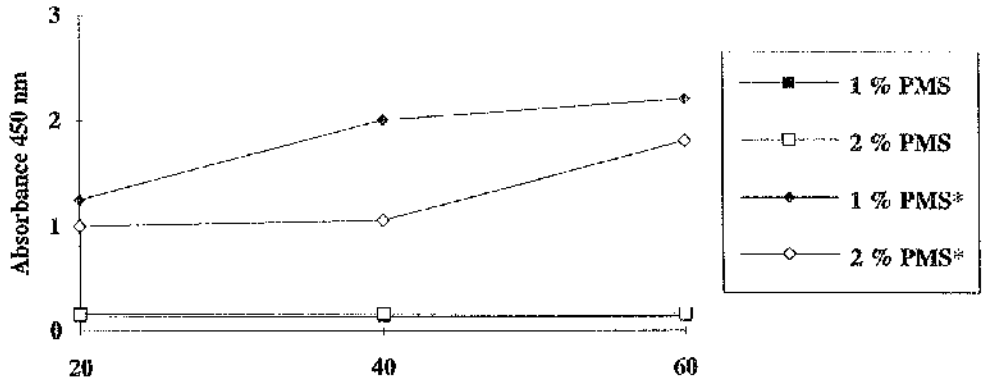


c.f.u. of V2165 per haemocyte

13e FSW



13f FH



XTT % of incubation medium (working solution 1 mg/ml)

haemocytes, no significant change in absorbance readings was observed when compared to the live haemocyte controls (Fig. 13 b, c, d). When the bacteria were suspended in FSW, V2981 and V2165 showed increased absorbance with increasing c.f.u. per haemocyte from 50 to 1000 (Fig. 13 b, c & d).

Due to the effect of the live bacteria on the absorbance readings, penicillin-streptomycin (final concentrations of 200 μg ml^{-1} and 0.0005 % respectively) was used after the incubation of haemocytes without bacteria. The potentiating effect of PMS on XTT was also examined (Roehm *et al.*, 1991) after 45 min incubation at 20 °C. Using FSW as a diluent, the absorbance readings were reduced with increasing concentrations of PMS from 1 to 2 % (Fig. 13 e). A reduction in the absorbance readings was obtained in the presence of penicillin-streptomycin (Fig. 13 e). When FH was used as a diluent (Fig. 13 f), the absorbance readings increased with increasing concentrations of XTT but only in the absence of penicillin-streptomycin and with 1 % PMS rather than penicillin-streptomycin, when present, reduced the haemocyte absorbance level to a minimum (Fig. 13 f). Therefore, penicillin-streptomycin was not used further because of its toxicity to the haemocytes.

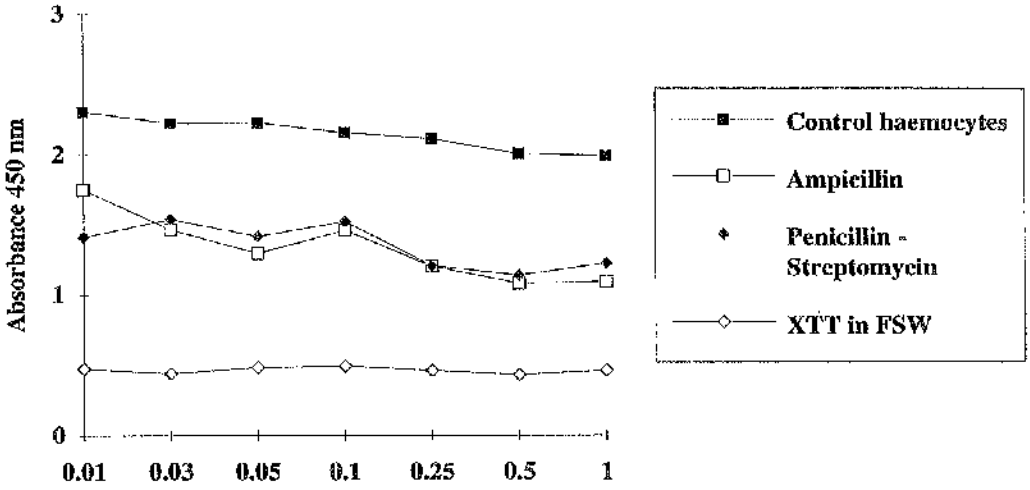
3.4.1.3 Toxicity screening of antibacterial agents.

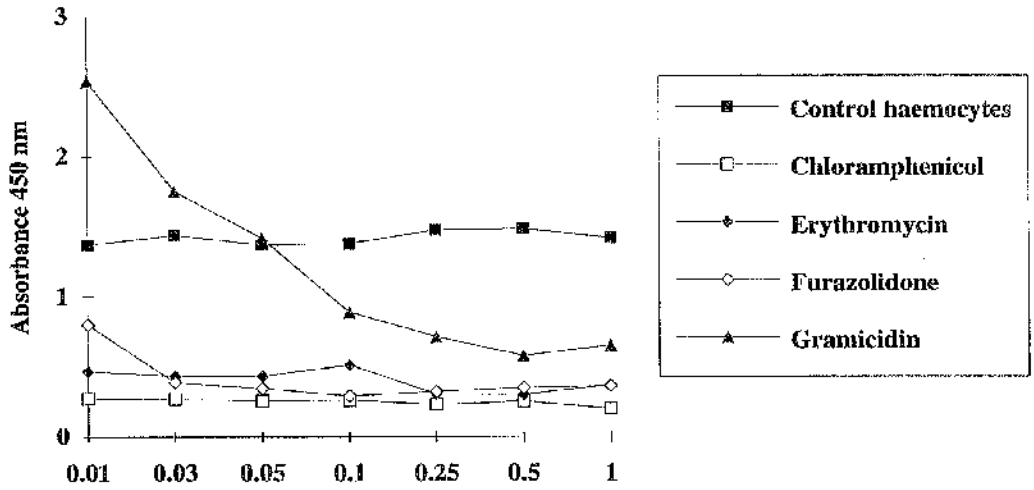
Other antibacterial agents were considered to inhibit bacterial growth before the addition of XTT. The test antibacterial agents were suspended in FSW and their toxicity to *Mytilus* haemocytes was investigated. Toxicity of the antibacterial agents was monitored over the concentration range 0.01 to 1 mg ml^{-1} for 45 min incubation at 20 °C when XTT was added.

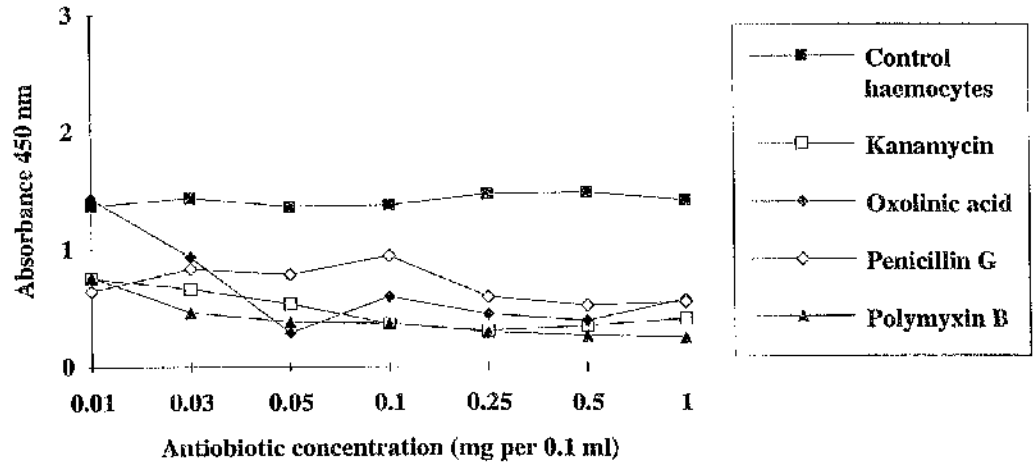
Test antibiotics which were toxic to the haemocytes were ampicillin, chloramphenicol, erythromycin, furazolidone, kanamycin, penicillin G, penicillin-streptomycin, polymyxin B, spectinomycin, streptomycin and sulphamethozone; vancomycin appeared toxic only at concentrations above 0.25 mg ml^{-1} (Fig. 14). Few antibiotics were low in toxicity over the range 0.01 to 0.05 mg ml^{-1};

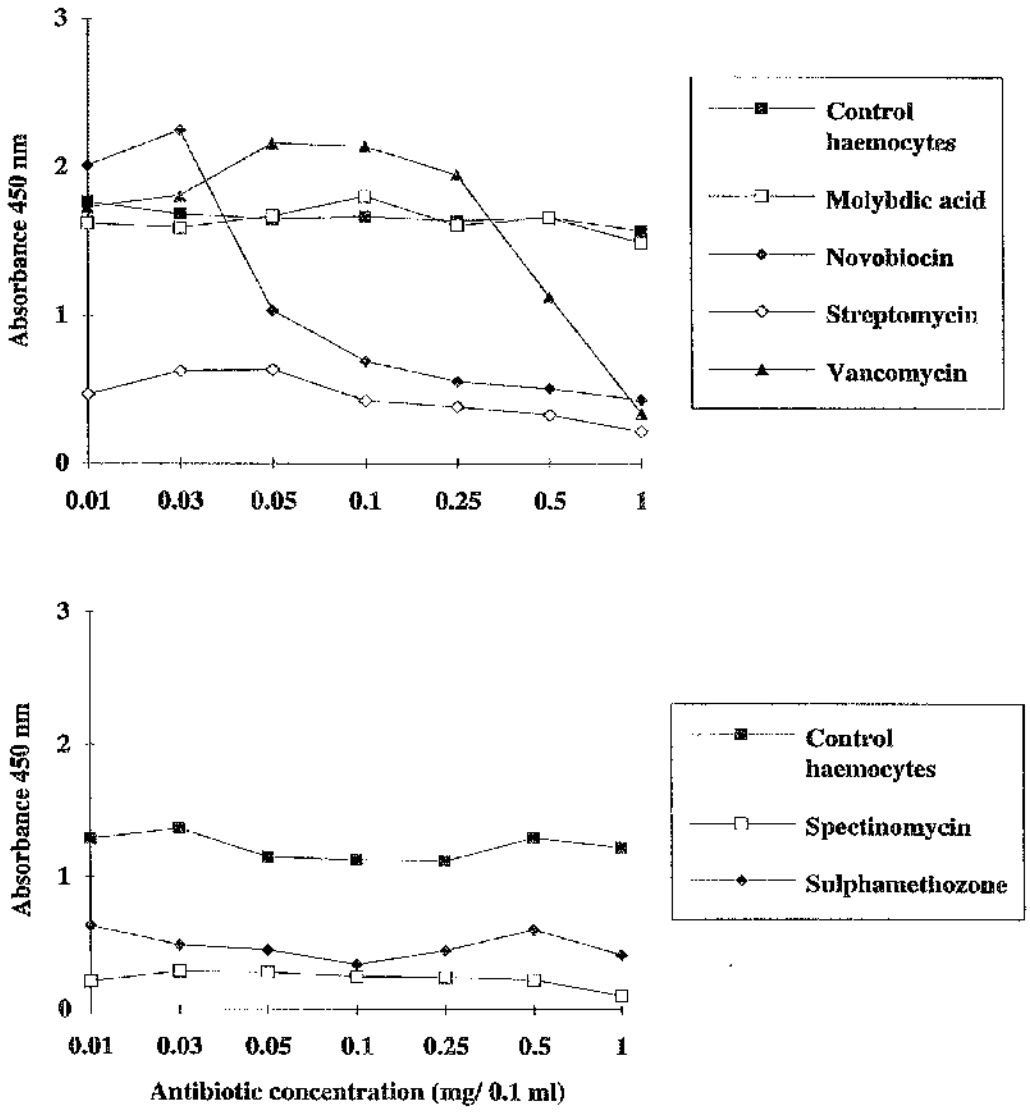
FIGURES 14 Antibiotic toxicity screening using *Mytilus* haemocytes, XTT and antibiotic concentrations ranging between 0.01 and 1 mg/0.1 ml.

Haemocytes from *Mytilus edulis* (10^6 ml^{-1}) were incubated in FSW containing different concentrations of antibiotic for 3 hours at 20°C. XTT (200 µg ml^{-1}) was then added and the mix was further incubated for 45 minutes at 20 °C. Tests were carried out in duplicate and controls were included for each mussel assayed.


Absorbance 450 nm
3
2
1
0
0.01
0.03
0.05
0.1
0.25
0.5
1
Control haemocytes
Ampicillin
Penicillin - Streptomycin
XTT in FSW


Absorbance 450 nm
3
2
1
0
0.01
0.03
0.05
0.1
0.25
0.5
1
Control haemocytes
Chloramphenicol
Erythromycin
Furazolidone
Gramicidin


Absorbance 450 nm
3
2
1
0
0.01
0.03
0.05
0.1
0.25
0.5
1
Antiobiotic concentration (mg per 0.1 ml)
Control haemocytes
Kanamycin
Oxolinic acid
Penicillin G
Polymyxin B


Absorbance 450 nm
3
2
1
0
0.01
0.03
0.05
0.1
0.25
0.5
1
Control haemocytes
Molybdic acid
Novobiocin
Streptomycin
Vancomycin
Absorbance 450 nm
3
2
1
0
0.01
0.03
0.05
0.1
0.25
0.5
1
Antibiotic concentration (mg/ 0.1 ml)
Control haemocytes
Spectinomycin
Sulphamethozone

these included kanamycin, gramicidin, oxolinic acid and novobiocin. Molybdic acid was the only non-toxic test substance.

3.4.1.4 Calcein-AM and ethidium homodimer.

Since bacterial toxicity to haemocytes could not be demonstrated easily with neutral red dye or XTT, calcein-AM (CAM) or ethidium homodimer (ETHD) were investigated to quantify the toxic effect of the bacteria on *Mytilus* haemocytes.

Live haemocytes are distinguished by the presence of intracellular esterase activity. Esterase activity is measured by the intense green fluorescence generated by enzymatic hydrolysis of calcein-AM. The substrate (CAM) is membrane permeable and virtually non-fluorescent; it is also a poly-anionic molecule that is well retained within live cells.

Mytilus haemocytes were incubated with bacteria for 3 h at 20 ^{o}C, the CAM was added, incubation was continued for up to 45 min at 20 ^{o}C and afterwards the number of live haemocytes after exposure to the various bacterial concentrations was determined. Instead of obtaining a decrease in fluorescence, indicating bacterial toxicity to the haemocytes, there was a substantial increase in fluorescence with increasing concentrations of *Vibrio* spp. (V1338, V1339, V2165, V2981 or V6), compared with the control haemocytes (Fig. 15). This was considered to be due to interaction of CAM with bacteria in FSW as well as with the haemocytes.

ETHD was then considered because its mode of action was based on a different principle that determined cell death. Live cells exclude the nucleic acid stain, ETHD. The nuclei of cells with damaged membranes label very rapidly and fluoresce red. Haemocytes were exposed to different volumes of ethanol, to obtain the concentration at which death of all the haemocytes was achieved. After incubation of the haemocytes with ethanol, ETHD was added and the mixture was incubated for 45 min at 20 ^{o}C. Results showed a linear response in fluorescent signal with increasing concentrations of ethanol (Fig. 16 a), a plateau being reached at 20 % v/v ethanol at

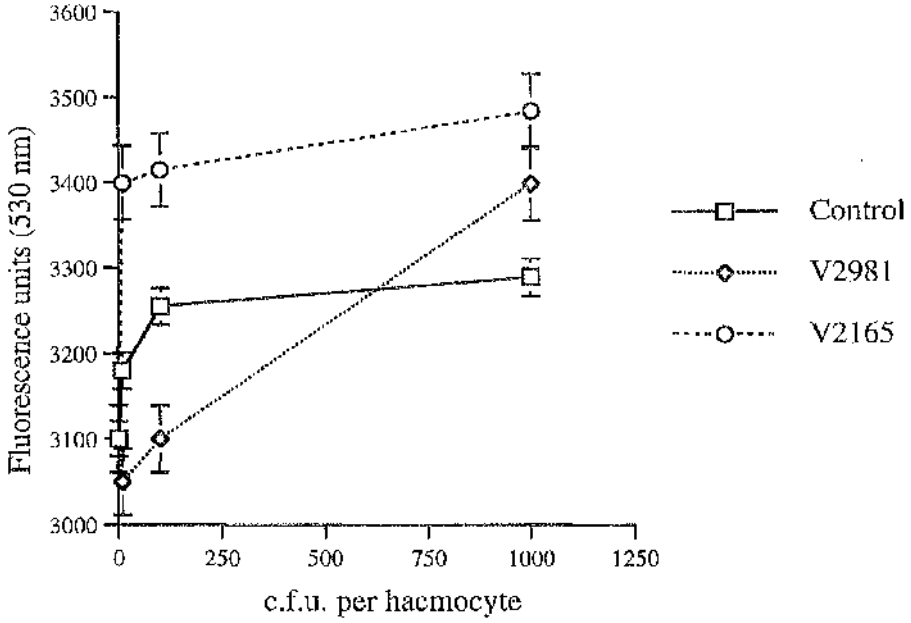
FIGURE 15 The effect of different vibrios on *Mytilus* haemocytes as determined using calcein-AM.

Bacteria were added at ratios of 0 to 1000 c.f.u. per haemocyte (10^6 ml^{-1}) and after 3 h incubation at 20 °C the fluorescence produced following further incubation for 45 min at 20 °C with CAM was measured (Excitation at 485 ± 20 nm and Emission ± 30 nm). Tests were carried out in duplicate.

a V2981, V2165

b V6, V1338 or V1339

15a


3600
3500
3400
3300
3200
3100
3000
Fluorescence units (530 nm)
0
250
500
750
1000
1250
c.f.u. per haemocyte
Control
V2981
V2165

15b

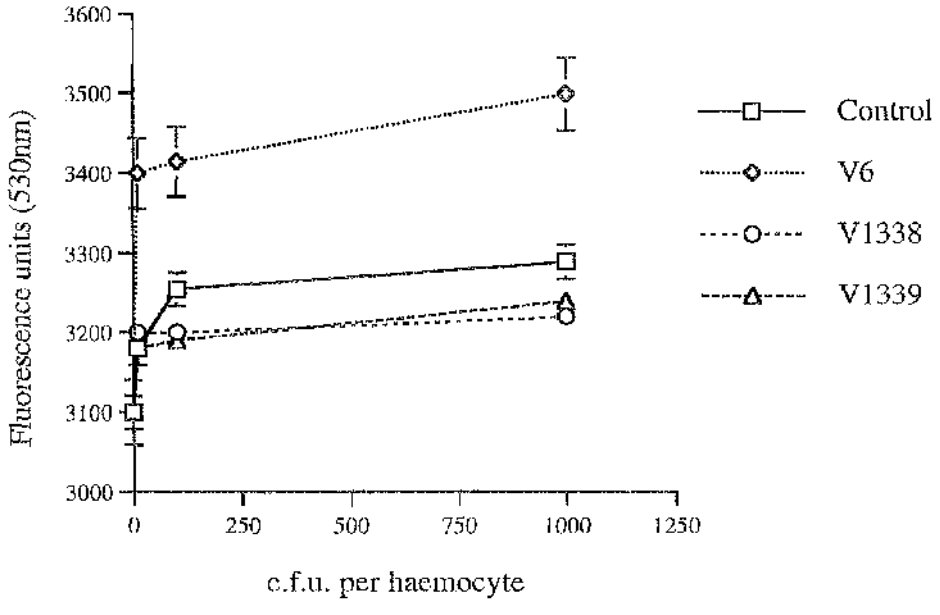
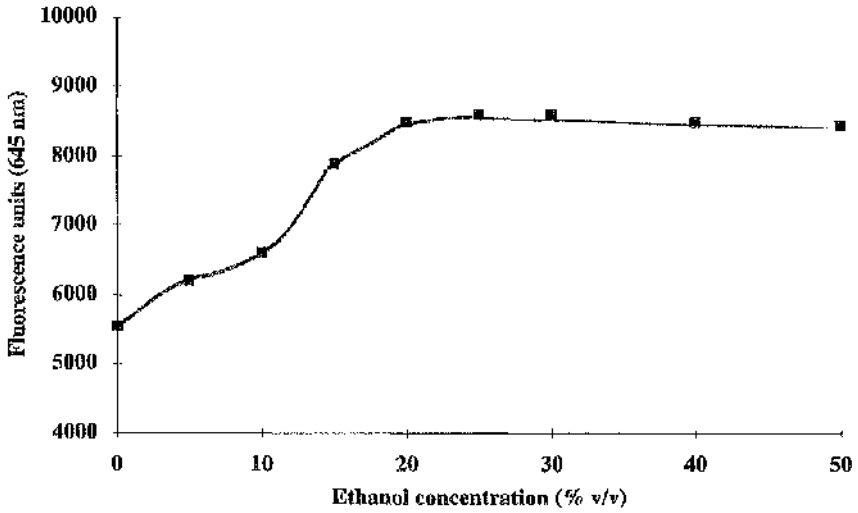

3600
3500
3400
3300
3200
3100
3000
Fluorescence units (530nm)
0
250
500
750
1000
1250
c.f.u. per haemocyte
Control
V6
V1338
V1339

FIGURE 16 Effects of increasing ethanol concentration on the viability of *Mytilus* haemocytes assessed by uptake of ethidium homodimer.

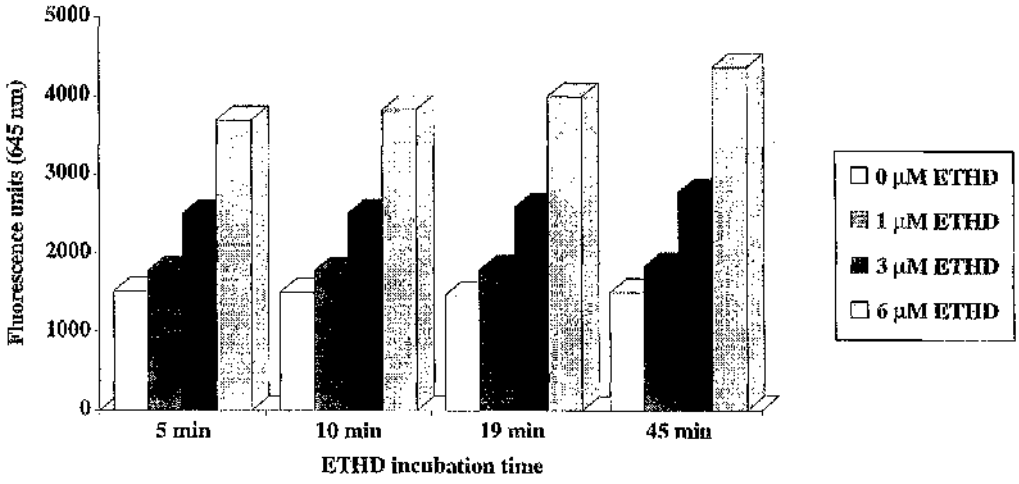
a Haemocytes (10^6 ml^{-1}) were exposed to various concentrations of ethanol, ethidium homodimer solution (final concentration 150 μM) was added and after 45 min at 20 °C the fluorescence due to DNA bound ethidium homodimer was measured.

b Effects of various ethidium homodimer concentrations and incubation times on the fluorescence signal produced by dead haemocytes (Excitation 485 ± 20 nm and Emission 645 ± 45 nm).

16a


10000
9000
8000
7000
6000
5000
4000
Fluorescence units (645 nm)
0
10
20
30
40
50
Ethanol concentration (% v/v)

16b


5000
4000
3000
2000
1000
0
Fluorescence units (645 nm)
5 min
10 min
19 min
45 min
ETHD incubation time
0 μM ETHD
1 μM ETHD
3 μM ETHD
6 μM ETHD

which concentration all cells were considered to be dead. Optimisation of the concentration of ETHD was carried out by incubating haemocytes with ethanol and afterwards exposing the haemocytes to three different concentrations of ETHD. The linear response shown in Fig. 16 b was obtained with increasing ETHD concentrations in FSW and by extending the length of incubation with ETHD.

Incubation time with ETHD and the effect of haemocyte concentration was taken into account in order to maximise the fluorescence signal. Experimental results, when plotted as volume of haemocyte suspension (10^6 haemocytes ml^{-1}) against incubation time with ETHD, showed a linear response with time up to 64 min, with increasing haemocyte concentration. From 64 to 180 min there was a decrease in the fluorescence signal (Fig. 17 a & b).

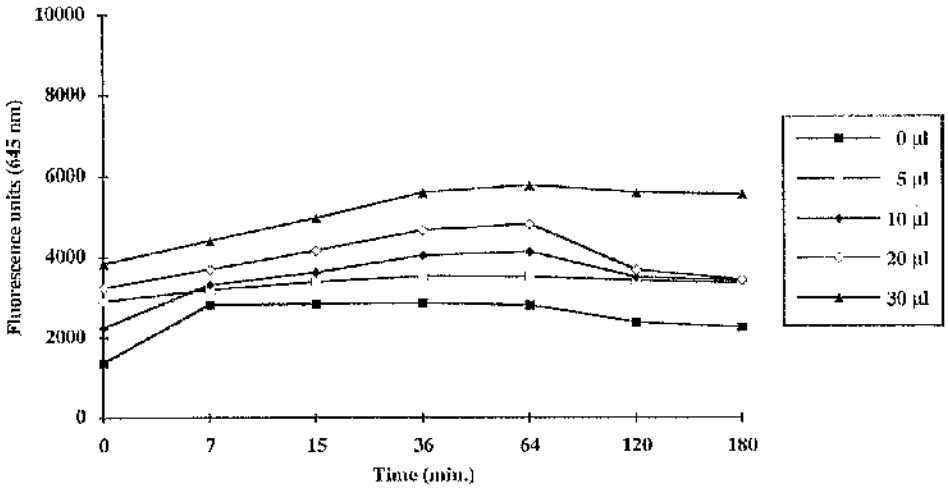
Using the above optimised conditions the ETHD assay was used in attempts to identify bacteria that were potentially toxic to bivalves. With *Mytilus* haemocytes the bacteria produced no effect when suspended in FSW and incubated with haemocytes for 3 h at 20 ^{o}C, even at 100 c.f.u. per haemocyte (Fig. 18 a). Substituting marine broth for nutrient broth containing 3 % NaCl, which was originally used by Nottage *et al.* (1989), did not enhance the bacterial toxicity, even with 50 - 500 c.f.u. bacteria per haemocyte (Fig. 18 b).

Various *Vibrio* spp. and P1-1-1 were screened for their toxicity to *Mytilus* haemocytes at different bacterial ratios (100 or 1000 c.f.u. per haemocyte). Figures 19 a and b, show the results for the bacteria (P1-1-1, V1197, V1338, V1339, V1340, V2166, V2981, V6, V5679, V91079, VB1 or VB51) screened. All were non-toxic with the exception of V1339 and VB51, which produced a toxic effect at 1000 c.f.u. per haemocyte, suspended in FSW.

FIGURE 17 Effect of haemocyte suspension volume and length of incubation with ETHD on the fluorescence signal.

(a) 0 - 30 µl of haemocyte suspension (10^6 haemocytes ml^{-1}) or **(b)** 40 - 80 µl haemocyte suspension were incubated with ETHD (final concentration 150 µM) for up to 180 min, the fluorescence signal (Excitation 485 ± 20 nm and Emission 645 ± 45 nm) being recorded at various intervals with the Cytofluor spectrofluorimeter.

17a


10000
8000
6000
4000
2000
0
Fluorescence units (645 nm)
0
7
15
36
64
120
180
Time (min.)
0 µl
5 µl
10 µl
20 µl
30 µl

17b

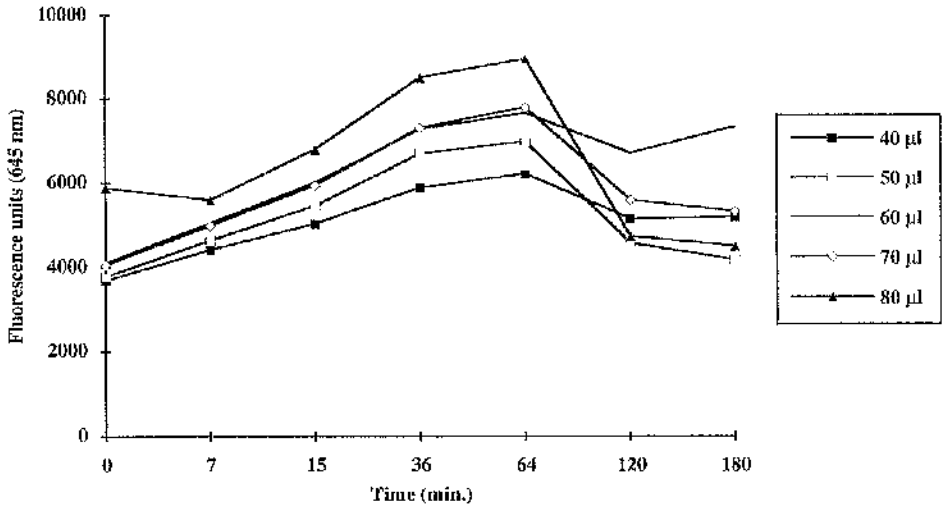
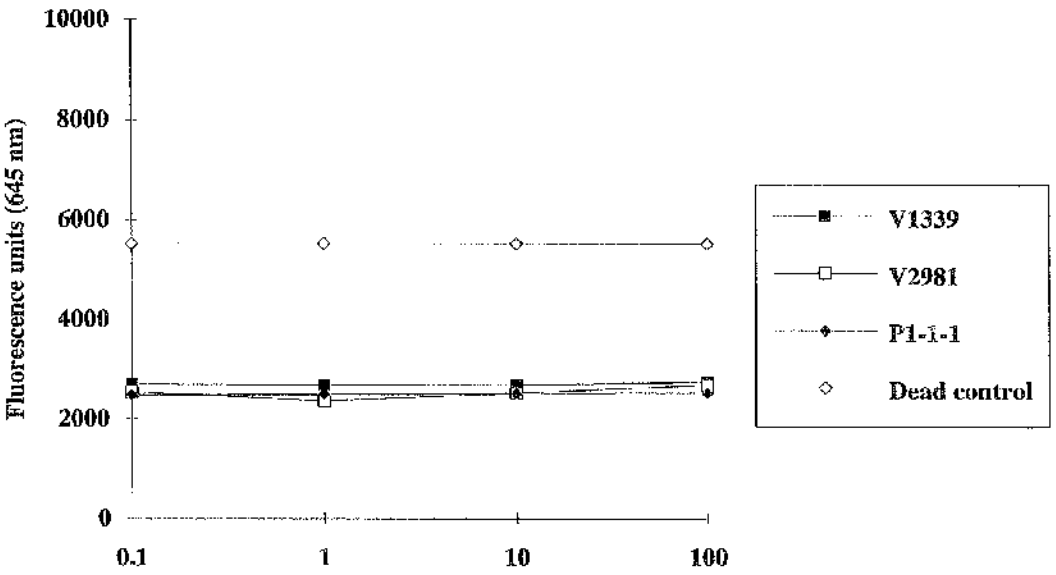

10000
8000
6000
4000
2000
0
Fluorescence units (645 nm)
0
7
15
36
64
120
180
Time (min.)
40 µl
50 µl
60 µl
70 µl
80 µl

FIGURE 18 The effect of increasing bacteria/haemocyte ratios on the toxicity of bacteria to haemocytes from *Mytilus edulis*.

a The effect of 24 hour marine broth cultures of bacteria (V1339, V2981 or P1-1-1) which were resuspended in FSW at 0.1 to 100 c.f.u. bacteria per haemocyte (10^6 ml^{-1}) and incubated for 3 hours. Ethidium homodimer solution (final concentration 150 µM) was then added and after 45 min at 20 ^oC the fluorescence due to DNA bound ethidium homodimer was measured. Duplicate tests were performed.

b The effect of 24 hour nutrient broth (with 3 % sodium chloride) cultures of V1339, V2981 or V2165 which were resuspended in FSW, to *Mytilus* haemocytes, at 50 to 500 c.f.u. per haemocyte and incubated for 3 hours. Ethidium homodimer solution (final concentration 150 µM) was added and after 45 min at 20 ^oC the fluorescence due to DNA bound ethidium homodimer was measured. Tests were carried out in duplicate.

18a Marine broth +FSW



18b Nutrient broth +FSW

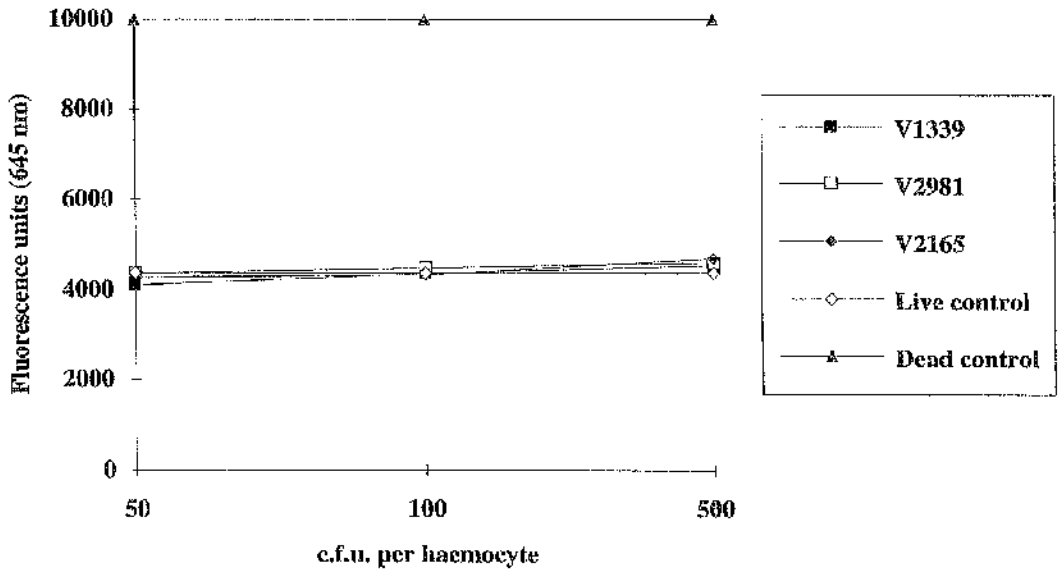
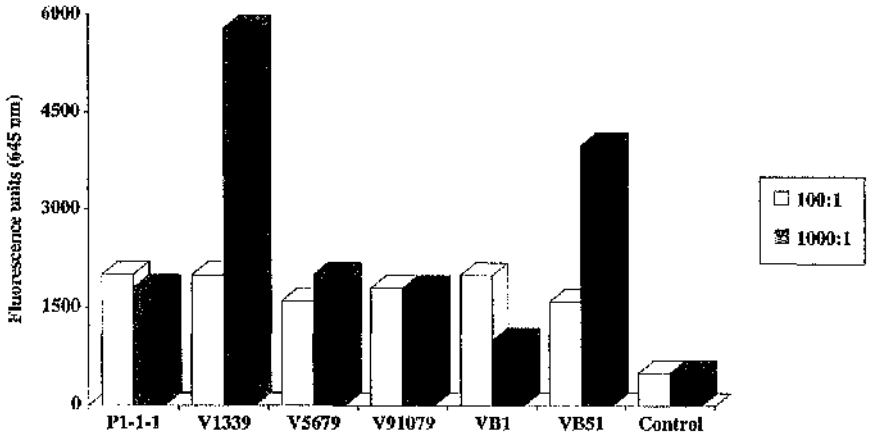


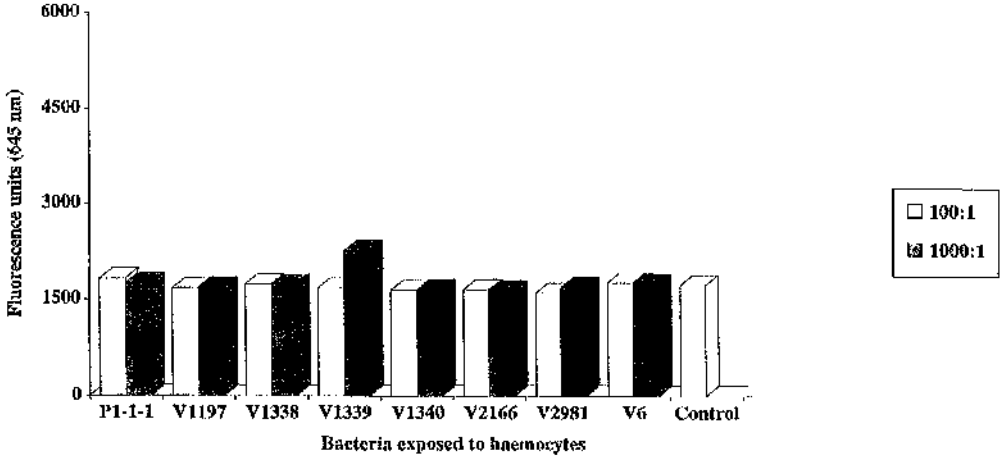
FIGURE 19 The effect of 100 and 1000 c.f.u. per haemocyte, on the toxicity of *Vibrio* spp. (V6, V1197, V1338, V1339, V1340, V2166, V2981, V5679, V91079, VB1 or VB51) or P1-1-1, suspended in FSW and when screened for their toxicity to *Mytilus* haemocytes using ETHD.

Bacteria and *Mytilus* haemocyte (10^6 ml^{-1}) suspensions were incubated for 3 hours at 20 °C. Haemocyte survival was estimated by measuring the fluorescence at 645 nm in the presence of ETHD (final concentration 150 μM).

19a FSW



19b FSW



This assay was repeated with V1339 or V2981, at ratios of 10, 50 or 500 c.f.u. per haemocyte respectively in FSW. There was no evidence of increased toxicity with increasing bacterial numbers, and fluorescence did not rise with extended incubation time (Fig. 20).

To overcome possible problems arising from haemocytes clumping, caffeine was incorporated into the assay; however, it did not have any effect on the fluorescence signal at concentrations of 0.001 to 10 mM (Fig. 21 a)

Background fluorescence of the diluents was recorded in the presence of live and control (dead) haemocytes; there was no effect on the haemocytes or the level of fluorescence (Fig. 21 b).

The effects of FH and FSW on the toxicity of *V. alginolyticus* 1339 to *Mytilus* haemocytes at 10 to 1000 c.f.u. per haemocyte were investigated (Fig. 22 a). V1339 was non-toxic when suspended in FSW at 10 c.f.u. per haemocyte. Fig. 22 a shows the gradual rise in toxicity with increasing c.f.u. of V1339 from 50 to 1000 per haemocyte and this was reflected in the increase in fluorescence by approximately 108 % at the maximum ratio of 1000 c.f.u of V1339 per haemocyte.

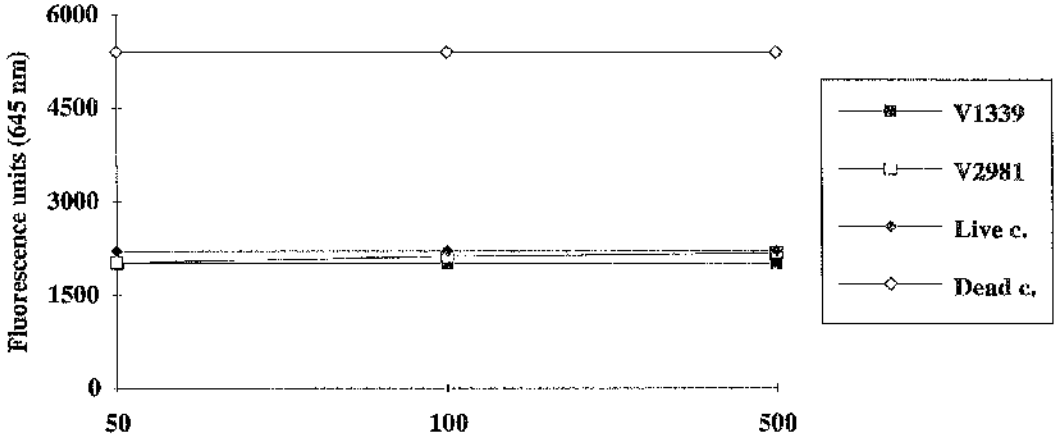
When V1339 was suspended in FH, the toxic effect of this bacteria on the haemocytes was more prominent. There was an increase in fluorescence by approximately 95 % at the maximum ratio of 1000 c.f.u. of V1339 per haemocyte respectively. This toxic effect produced by V1339 was evident with increasing bacterial numbers suspended in FH (Fig. 22 b).

Various *Vibrio* spp. (V1338, V1339, V2165, V2981 or V6) were screened for their toxicity to *Mytilus* haemocytes when incubated in FH at 0.1 to 100 c.f.u. per haemocyte. Most of the bacteria did not exhibit their toxicity at concentrations of 0.1, 1 or 10 c.f.u. per haemocyte. V2981 exhibited some degree of toxicity at 10 c.f.u. per haemocyte. The test bacteria V1338 or V2165, were less toxic at 100 c.f.u. per

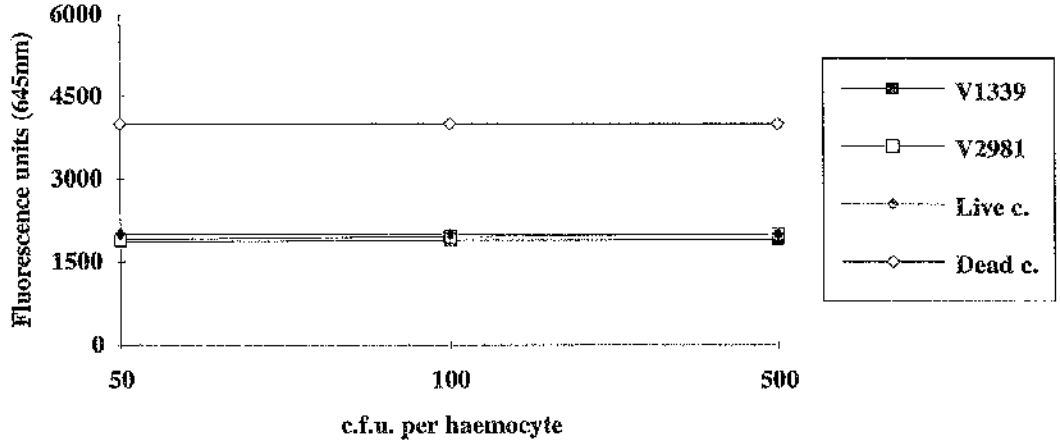
FIGURE 20 The effect of incubation time on the toxicity of V1339 or V2981 to *Mytilus* haemocytes.

Haemocytes (10^6 ml^{-1}) were incubated for (a) 3 h or (b) 6 h with V1339 or V2981 (50, 100 or 500 / haemocyte) and the subsequent viability of haemocytes assessed using ethidium homodimer. Live and dead controls were incorporated into the assay. Tests were performed in duplicate and appropriate controls were incorporated for each mussel used in the assay.

20a 3 h



20b 6 h



c.f.u. per haemocyte

FIGURE 21 The effect of caffeine and buffers on *Mytilus* haemocytes.

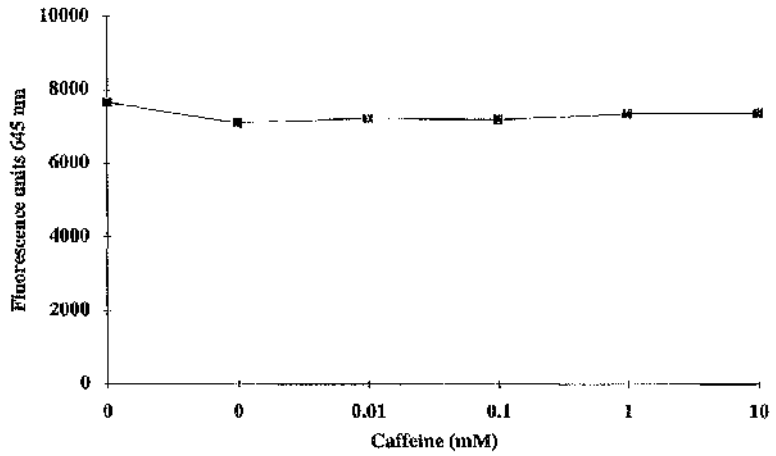
a The effect of incubation of *Mytilus* haemocytes (10^6 ml^{-1}) with different concentrations of caffeine (0.001 to 10 mM), on the fluorescence signal produced by ETHD.

b The effect of different buffers (FH, MPS or ETOH) on *Mytilus* haemocytes when incubated for up to 195 min at 20 °C.

Legend:

- MPS and *Mytilus* haemocytes.
- FH and *Mytilus* haemocytes.
- ETOH and *Mytilus* haemocytes.
- *Mytilus* haemocytes only.

21a


10000
8000
6000
4000
2000
0
Fluorescence units 645 nm
0
0
0.01
0.1
1
10
Caffeine (mM)

21b

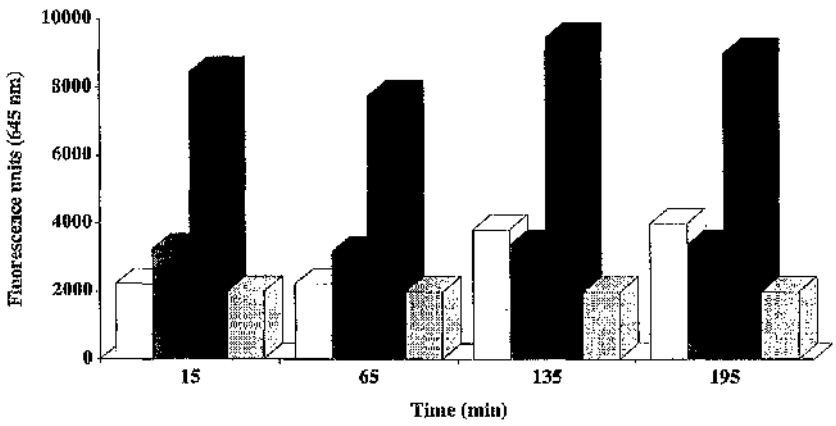
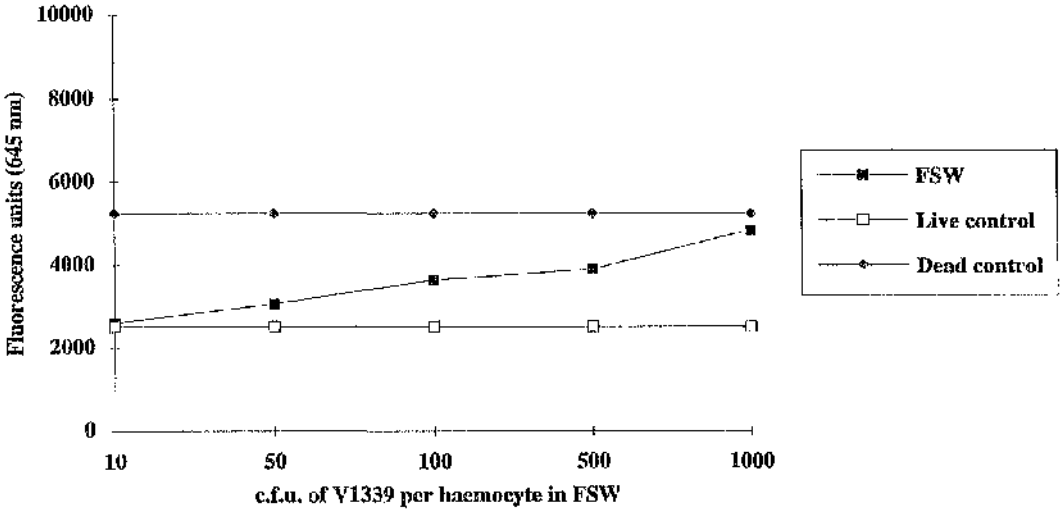

10000
8000
6000
4000
2000
0
Fluorescence units (645 nm)
15
65
135
195
Time (min)

FIGURE 22 The effect of incubation media, FSW (22 a) or FH (22 b), on the toxicity of V1339 to *Mytilus* haemocytes at 10 to 1000 c.f.u. per haemocyte and incubated for 3 h at 20 °C.

Cocultures of bacteria and haemocytes (10^6 ml^{-1}) were suspended in FSW or FH.. Haemocyte survival was estimated by measuring fluorescence at 645 nm in the presence of ETHD (final concentration 150 μM) when further incubated for 45 min.

22a


10000
8000
6000
4000
2000
0
Fluorescence units (645 nm)
10
50
100
500
1000
c.f.u. of V1339 per haemocyte in FSW
FSW
Live control
Dead control

22b

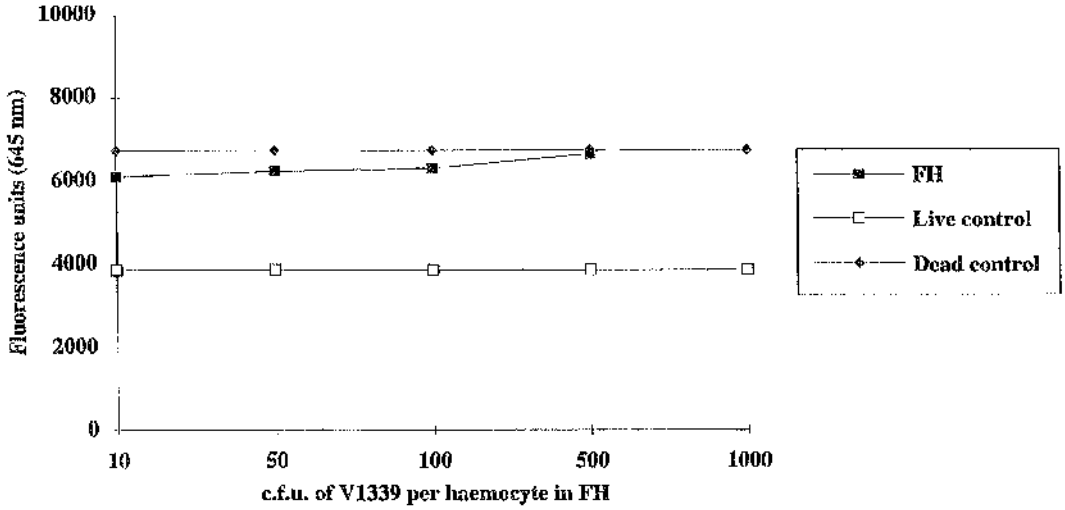
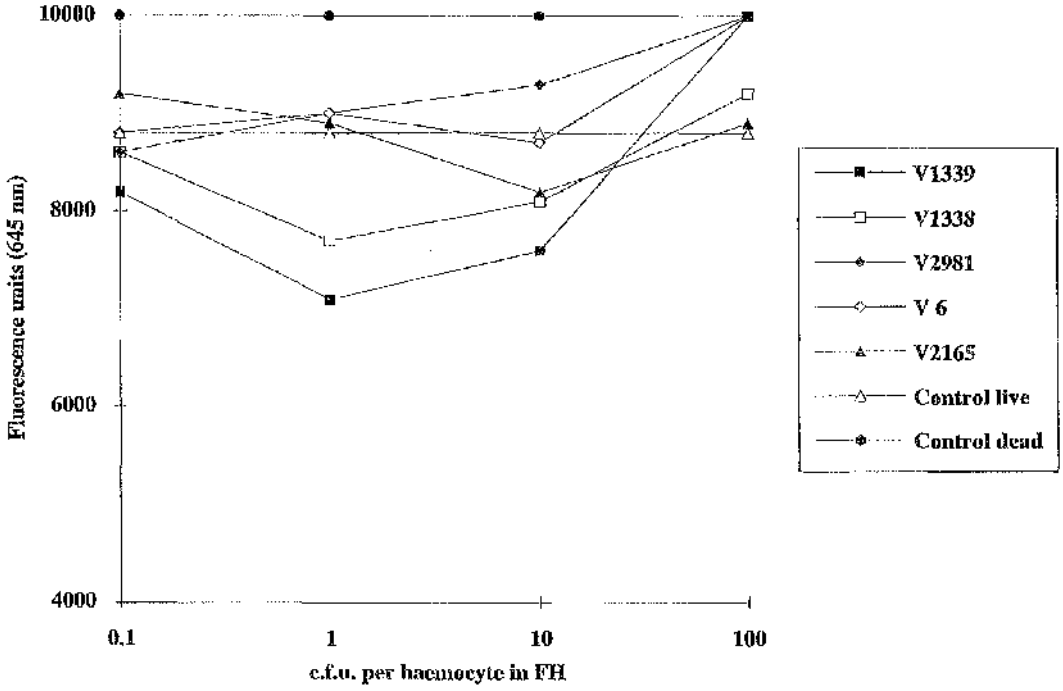

10000
8000
6000
4000
2000
0
Fluorescence units (645 nm)
10
50
100
500
1000
c.f.u. of V1339 per haemocyte in FH
FH
Live control
Dead control

FIGURE 23 The toxic effect of V1338, V1339, V2165, V2981 or V6 to *Mytilus* haemocytes when suspended in FH at bacterial ratios of 0.1 to 100 c.f.u. per haemocyte and incubated for 3 h at 20 °C.

Survival of *Mytilus* haemocytes (10^6 ml^{-1}) incubated with different vibrios in FH, was estimated by measuring the fluorescence at 645 nm in the presence of ETHD (final concentration 150 μg ml^{-1}).


10000
8000
6000
4000
Fluorescence units (645 nm)
0.1
1
10
100
c.f.u. per haemocyte in FH
V1339
V1338
V2981
V 6
V2165
Control live
Control dead

haemocyte, whereas the other bacteria (V6, V1339 and V2981) showed maximum toxicity at this level of exposure (Fig. 23).

3.5 PHOTOGRAPHIC RECORDS.

The use of indicator dyes did not show clearly the effect of bacteria on haemocyte function and it was decided to observe the bacteria/haemocyte interaction directly by microscopy; results were recorded using time-lapse and still photography.

3.5.1 Effect of Vibrios on *Mytilus* Haemocytes.

Mytilus haemocytes were allowed to adhere to plastic surfaces of 24-well Nunc plates and their movement was recorded for up to 4 h at 20 °C by time-lapse video recording. A high proportion of the haemocytes remained spread on the surface for several hours (Fig. 24 a) and displayed rapid sweeping movement across the plastic surface with occasional resting periods when cells remained rounded for several minutes before resuming active movement. When 10 or 50 c.f.u. per haemocyte of a possible non-pathogenic bacterium (P1-1-1) in FH was added, movement of the haemocytes was unaffected for 4 h at 20 °C with apparent continuous phagocytic activity (Fig. 24 b). On the other hand, when the pathogenic *V. anguillarum* 2981 was added in FH at ratios of 10 or 50 c.f.u. per haemocyte, normal movement patterns continued for up to 60 to 90 min, after which the haemocytes became rounded (Fig. 24 c) and did not resume normal movement within the next 90 min. Time-lapse recording was used to determine the kinetics of the rounding process after which still photography was used. No rounding was observed when V2981 was added to haemocytes suspended in FSW (Fig. 24 d).

3.5.2 Effect of Filtered Haemolymph.

The response of haemocytes to different concentrations of bacteria in FH with time was recorded. For untreated haemocytes the percentage of cells which remained spread on the plastic surface gradually declined from 60 % to 30 % over 160 min

FIGURE 24 The effect of *Pseudomonas* 1-1-1 or V2981, suspended in FH or FSW, on haemocytes of *Mytilus edulis*.

Photograph **(a)** *Mytilus* haemocytes after incubation without added bacteria for up to 3 h at 20 °C. (Normal haemocytes)

Photographs **(b)** The effect of different concentrations of *Pseudomonas* 1-1-1 in FH on *Mytilus* haemocytes after incubation for 3 h at 20 °C. (➤ Minor rounding of haemocytes)

(b i) 10 c.f.u. of P1-1-1 per haemocyte.

(b ii) 50 c.f.u. of P1-1-1 per haemocyte.

Photographs **(c)** The effect of different concentrations of *V. anguillarum* 2981 in FH on *Mytilus* haemocytes after incubation for 3 h at 20 °C. (➤Major rounding of haemocytes)

(c i) 10 c.f.u. of V2981 per haemocyte.

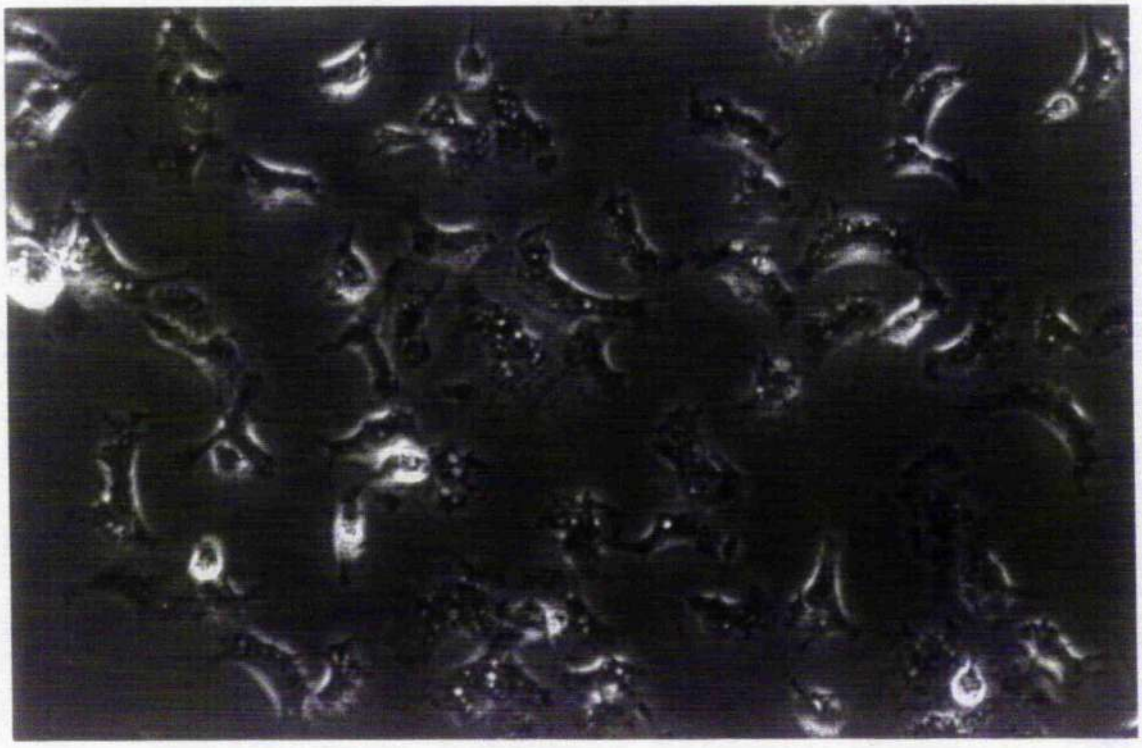
(c ii) 50 c.f.u. of V2981 per haemocyte.

Photographs **(d)** The effect of different concentrations of V2981 in FSW on *Mytilus* haemocytes after incubation for 3 h at 20 °C. (➤ Reduced rounding in the presence of FSW)

(d i) 10 c.f.u. of V2981 per haemocyte.

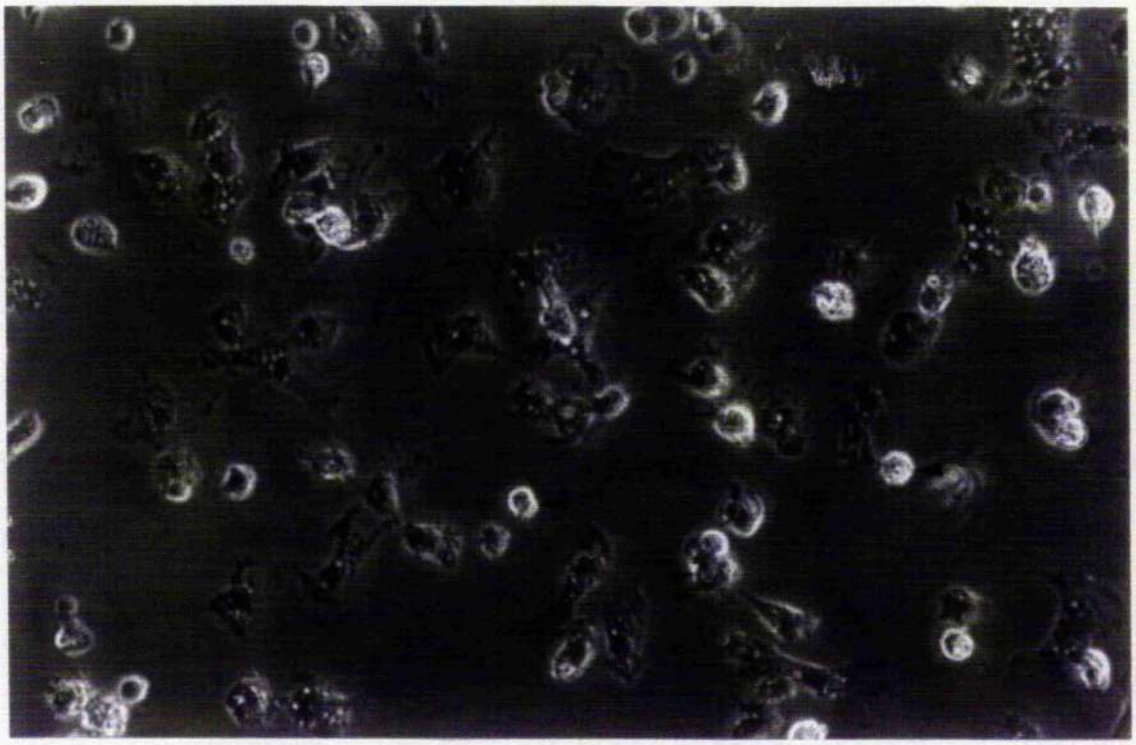
(d ii) 50 c.f.u. of V2981 per haemocyte.

A



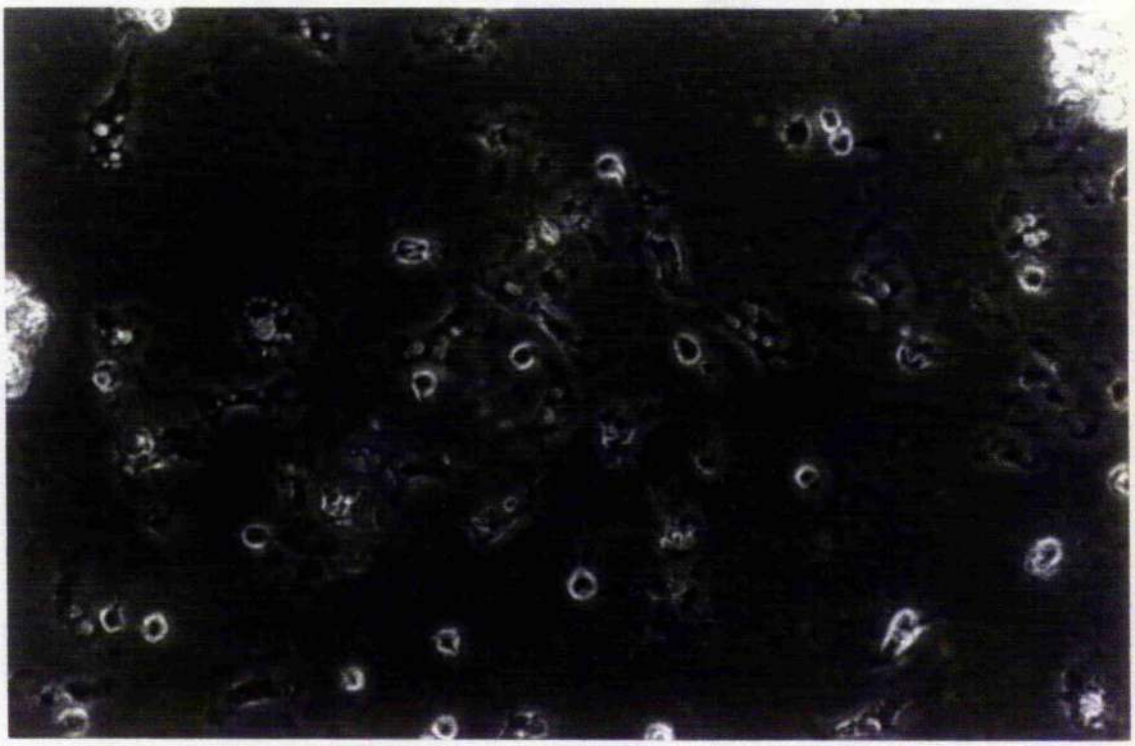
X40

B (i)

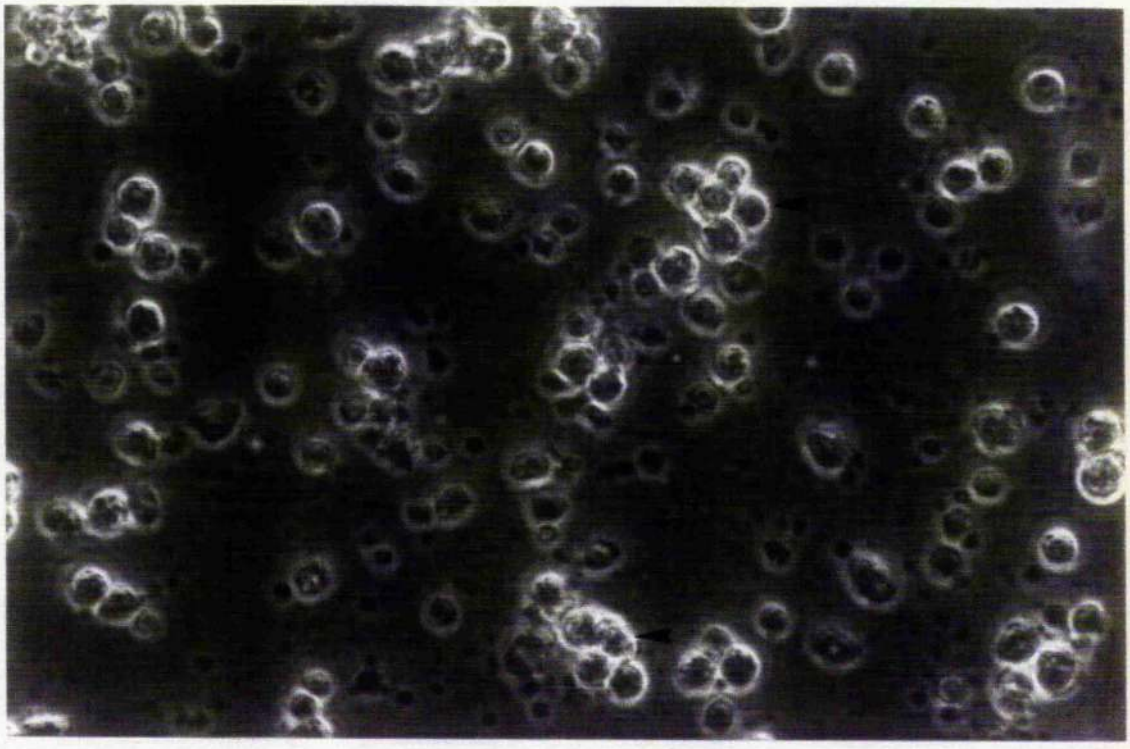


×40

B (ii)

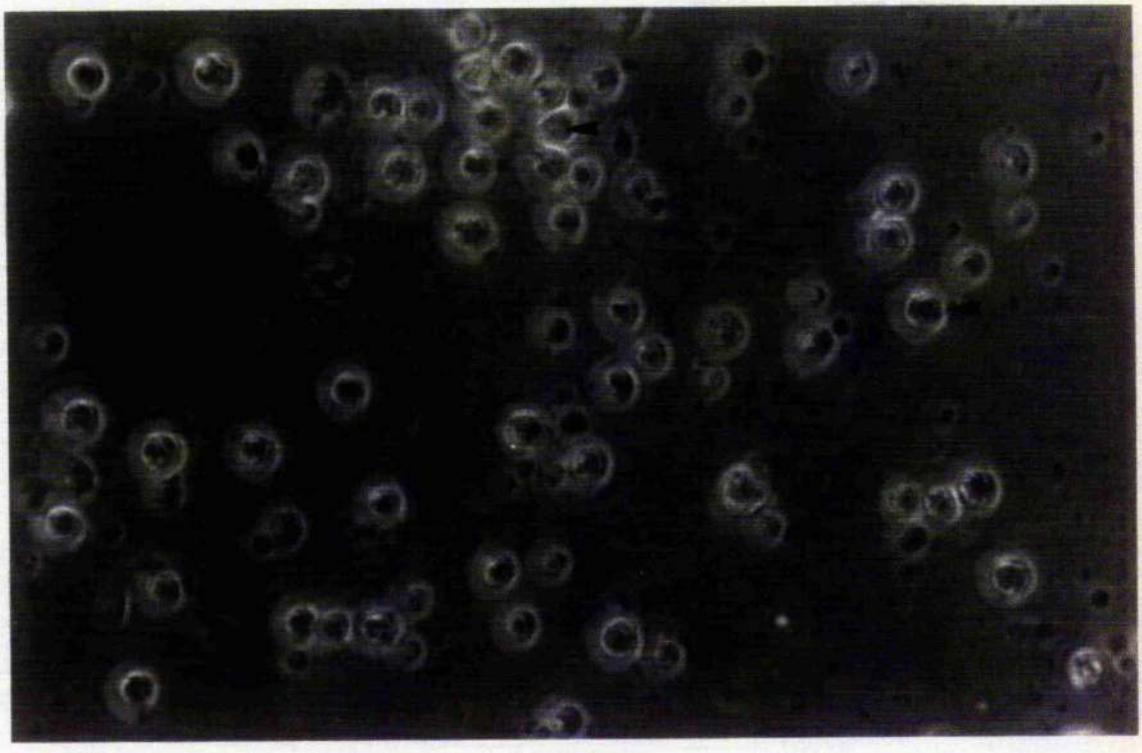


C (i)

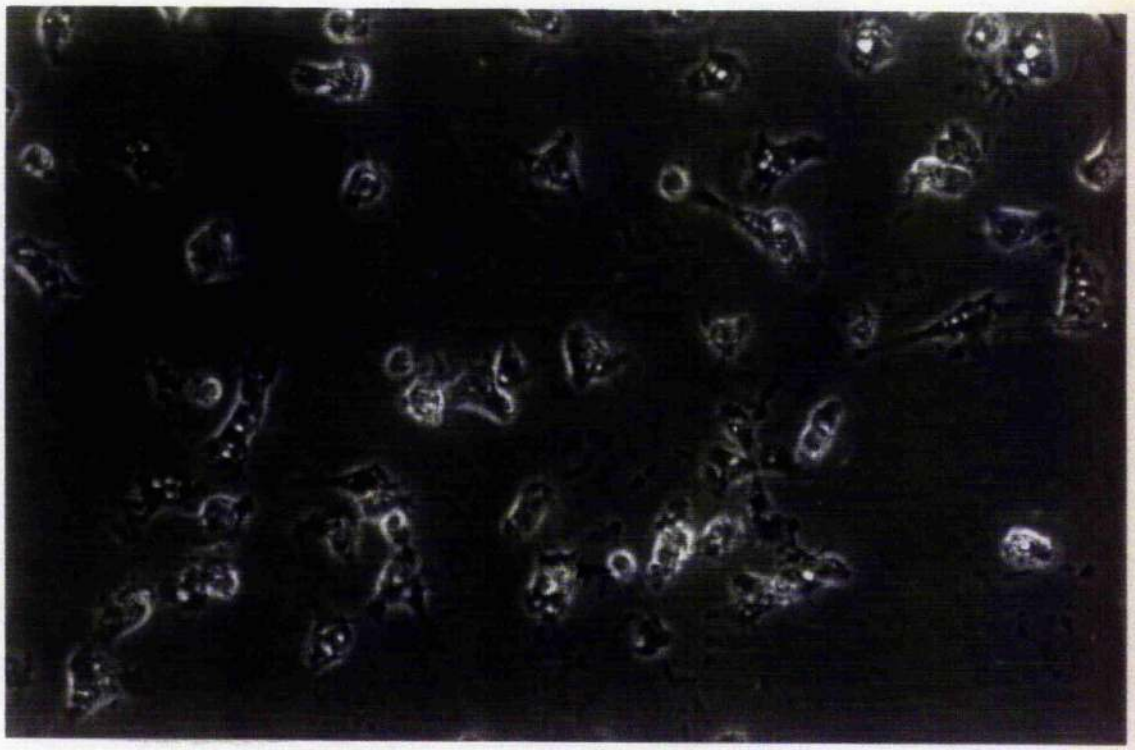


C (ii)

X40

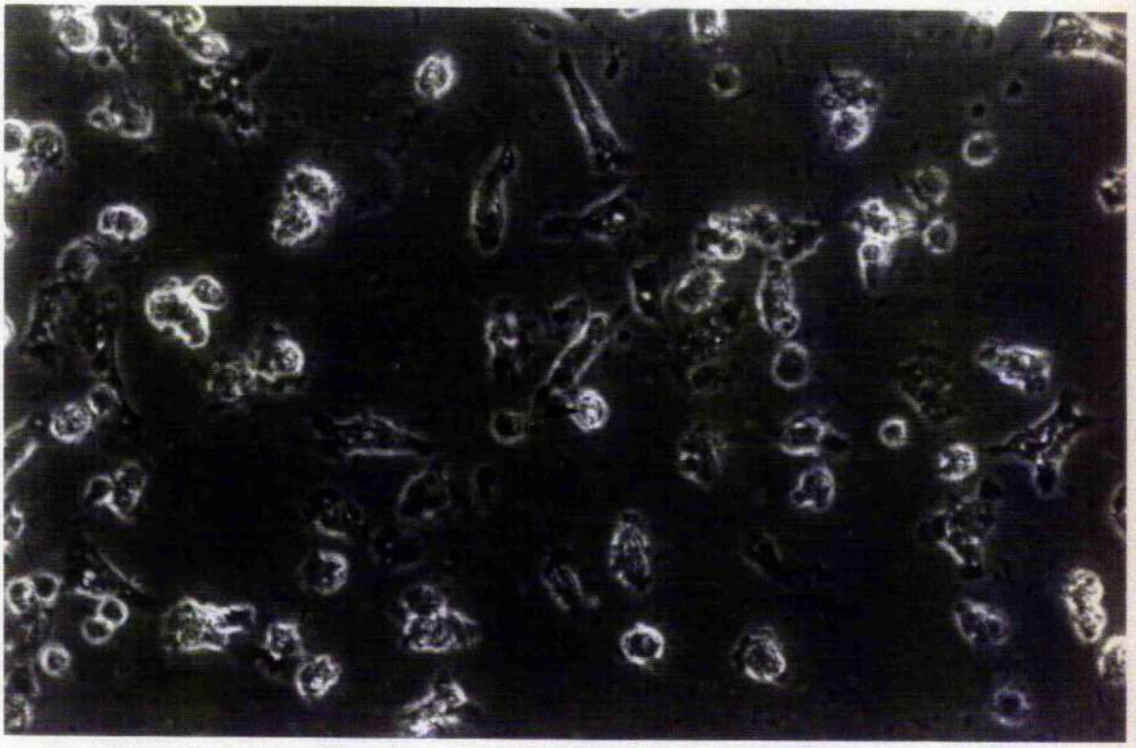


D (i)



D (ii)

X40



(Fig. 24 a). On exposure to V2981 at 10 to 500 c.f.u. per haemocyte, almost all the haemocytes were rounded by 150 min, and the rate of rounding was dose-dependent (Fig. 25 a). Reduction of spread cells to 50 % of the total control haemocyte number occurred in approximately 90, 120 and 150 min, at bacterial ratios of 500, 50 and 10 c.f.u. per haemocyte respectively (Fig. 25 a).

3.5.3 Effect of Bacterial and Filtered Haemolymph Dilution.

To determine whether bacteria alone were responsible for the toxic effect on *Mytilus* haemocytes, two different concentrations of V2981 bacteria were exposed to different dilutions of FH.

Initially, the haemocytes were mixed with FSW and FH separately. Results showed that the haemocytes were not affected by FSW but natural haemocyte shape change, i.e. rounding and spreading, occured in FH, therefore FH was used as a control for these tests. In the light of this last result, doubling dilutions of FH were mixed with V2981, to see whether the toxicity of the bacteria was enhanced with FH.

Diluting the FH 16-fold in FSW produced a similar effect to neat FH (Fig. 25 b). Dilutions greater than 1/16 showed reduced effect of V2981 towards the haemocytes. The use of 5 times the concentration of bacteria with doubling dilutions of FH produced an approximate 20 % increase in toxicity between the two bacterial concentrations (10 or 50 c.f.u. per haemocyte). This indicates that the toxicity was a function of both bacterial and FH concentration.

3.5.4 Kinetics of Haemocyte Rounding Induced by Bacteria.

The number of bacterium/haemocyte interactions required to cause irreversible haemocyte rounding was investigated using V2981 and haemocytes at ratios over the range of 0.1 to 10 c.f.u. per haemocyte, and determining the percentage haemocytes rounded after 150 min. When data was normalised to allow for the fraction of cells rounded in untreated control groups the response of haemocytes to bacteria closely

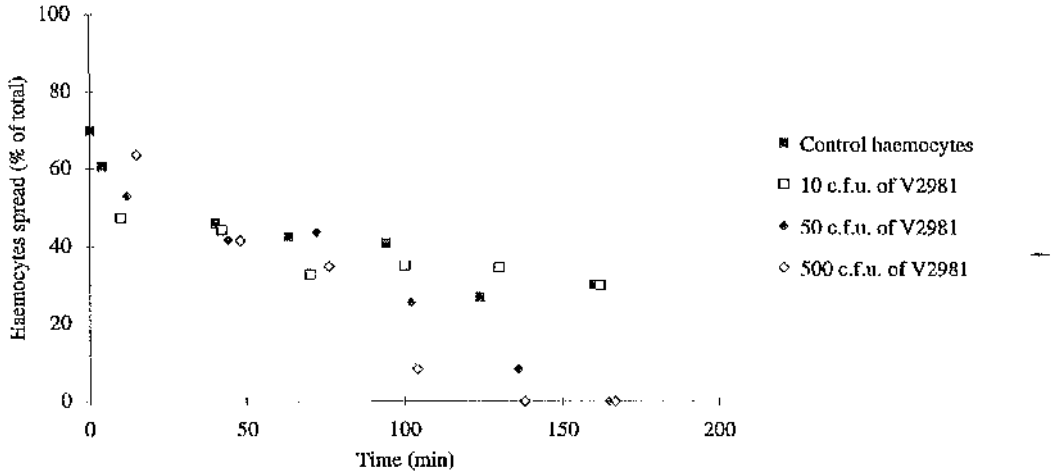
FIGURE 25 Kinetics of rounding of *Mytilus* haemocytes on interaction with *V. anguillarum* 2981 or *V. anguillarum* A7.

a Rounding of *Mytilus* haemocytes on interaction with 10, 50 or 500 c.f.u. of *V. anguillarum* 2981 per haemocyte.

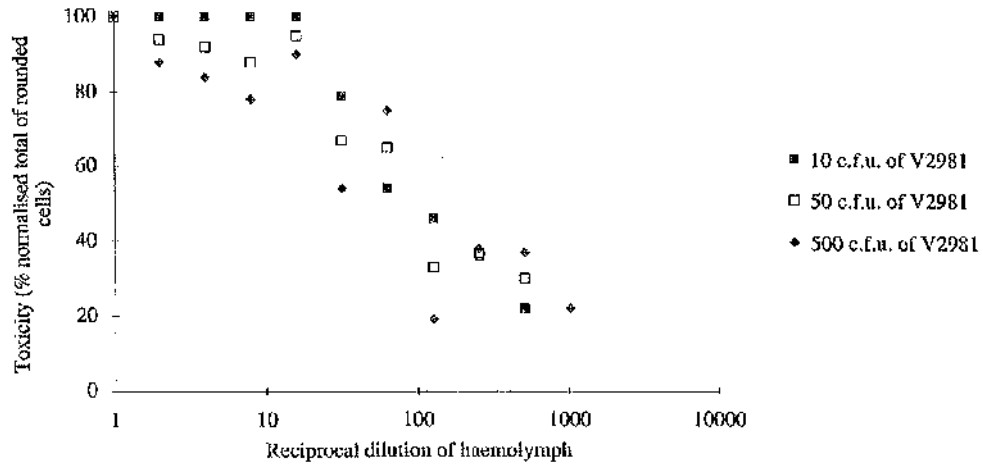
b The effect of dilution on the ability of FH to promote haemocyte rounding by V2981.

c Kinetics of rounding of *Mytilus* haemocytes induced by V2981 or *V. anguillarum* A7.

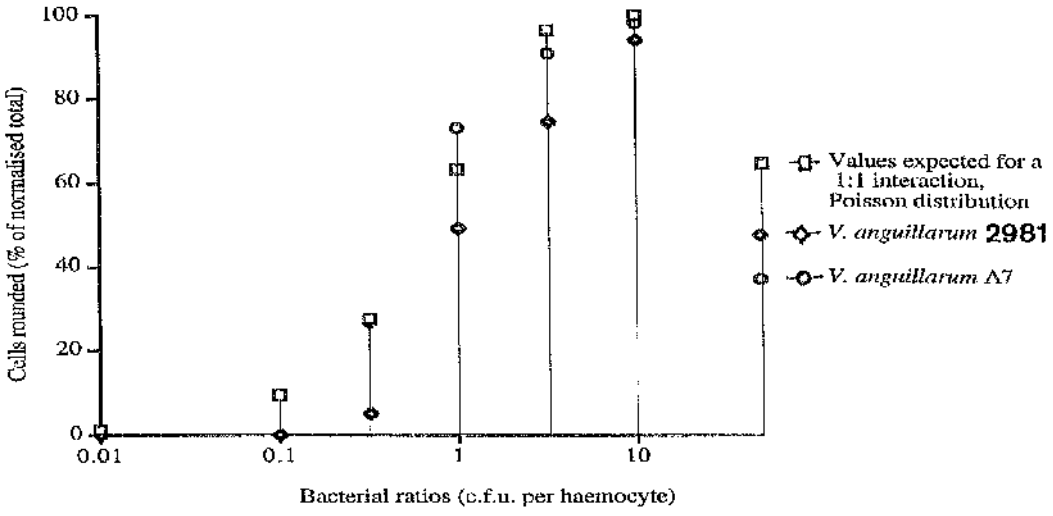
25a


Haemocytes spread (% of total)
100
80
60
40
20
0
0
50
100
150
200
Time (min)
Control haemocytes
10 c.f.u. of V2981
50 c.f.u. of V2981
500 c.f.u. of V2981

25b


Toxicity (% normalised total of rounded cells)
100
80
60
40
20
0
1
10
100
1000
10000
Reciprocal dilution of haemolymph
10 c.f.u. of V2981
50 c.f.u. of V2981
500 c.f.u. of V2981

25c


Cells rounded (% of normalised total)
100
80
60
40
20
0
0.01
0.1
1
10
Bacterial ratios (c.f.u. per haemocyte)
Values expected for a 1:1 interaction, Poisson distribution
V. anguillarum 2981
V. anguillarum Δ7

followed that expected for a one-hit interaction calculated from a Poisson distribution (Fig. 25 c). Similar results were obtained for *V. anguillarum* A7.

3.5.5 Toxicity of Vibrios to *Mytilus* Haemocytes.

Vibrio species were screened for their toxicity to *Mytilus* haemocytes by incubation with haemocytes for 150 - 180 min at 20 ^{o}C. Table 13, shows the toxicity of these 15 *Vibrio* spp. and *Pseudomonas* 1-1-1 at 50 c.f.u. per haemocyte. Most of the vibrios tested were highly toxic, in particular V2981 which induced maximum toxicity (100 % rounding of haemocytes) at 50 c.f.u. per haemocyte. Others, (V1337, V1338, V1339, V1340, V2164, V2165, V2166, V4979, V5679, V91079 or VB1) expressed high toxicity (> 70 % cell rounding). Partial toxicity (arbitrarily defined as < 55 % cell rounding) was expressed by V1336 and VB2. Two strains, V1179 and P1-1-1, were low (< 30 % cell rounding) in toxicity to *Mytilus* haemocytes.

3.6 SCREENING OF BACTERIAL ISOLATES FOR TOXICITY USING THE HAEMOCYTE BIOASSAY.

The main objective of this section was to screen the bacterial isolates from two shellfish hatcheries during disease outbreak (Guernsey and Reculver strains), and those from the experimental hatchery at Conwy that had no evidence of disease at the time of isolation of the bacterial strains. For that purpose, the above toxicity bioassay was used in the screening of the shellfish bacterial isolates. The toxic potential of these shellfish bacterial isolates was also compared with that presented in standard reference strains isolated from bivalves, so that the relationship among origin, pathogenicity and toxicity for haemocytes could be assessed in the strains studied. The toxicity (% normalised total of rounded haemocytes) of these strains was categorised as low (0 - 30 %), medium (34 - 66 %) and high (67 - 100 %).

TABLE 13 : Toxicity of standard bacteria to Mytilus haemocytes.

Strain ref. no.	Identification	Origin & reference	Toxicity % (50 c.f.u. per haemocyte)
1197	*V. anguillarum*	Horne et al. (1977)	28
1336	*V. tubiashii*	National Collection Of Marine Strains	53
1337	*V. tubiashii*	" " " " "	89
1338	vibrio sp.	" " " " "	93
1339	*V. alginolyticus*	" " " " "	100
1340	*V. tubiashii*	" " " " "	100
2164	*V. tubiashii*	" " " " "	91
2165	*V. tubiashii*	" " " " "	100
2166	*V. tubiashii*	" " " " "	96
2981	*V. anguillarum*	Austin et al. (1988)	100
4979	*V. anguillarum*	" " " "	87
5679	*V. anguillarum*	" " " "	79
91079	*V. anguillarum*	Horne et al. (1977)	96
B1	*V. anguillarum*	" " " "	98
B2	*V. anguillarum*	" " " "	54
P1-1-1	*Pseudomonas*	Wardlaw & Unkles (1978)	30

3.6.1 Toxicity of Bacterial Isolates From Guernsey.

Bacterial isolates from Guernsey were exposed to *Mytilus* haemocytes to determine their potential toxicity. None of the 23 isolates were highly toxic to the haemocytes, they exhibited low (65 %) or medium (35 %) toxicity (Table 14 and Appendix I). When the bacteria were characterised biochemically and identified, they fell into three major groups, tentatively identified as one of the following: *V. alginolyticus, V. anguillarum* or *V. tubiashii* and there were three odd strains partially identified as *Aeromonas sobria* (G4), *A. salmonicida* (G3a) and *V. furnissii* (G25).

The most toxic bacterial group were the presumptive *V. anguillarum*; all 5 strains (G3, G5, G8, G20 & G23) were of medium toxicity (Fig. 26 a) and all isolates showed > 90 % similarity to *V. anguillarum,* except for G3 which showed only 78 % similarity.

Twelve isolates tentatively identified as *V. alginolyticus* varied in toxicity. Two (G7 and G15) produced medium toxicity and 10 were low in toxicity, (G9, G12, G13, G16, G17, G18, G19, G21, G24 & G26). Seven of these 12 isolates (G9, G12, G13, G15, G17, G18 & G26) were closely identified as being *V. alginolyticus*, with identification scores greater than 90 % similarity. The remaining 5 isolates in this group were 60 to 86 % identified. The isolate G7 scored 70 % and therefore was not identified but when cluster analysis was carried out it was shown to fall into the *V. anguillarum* group and its toxicity reflects this group also, rather than the group of bacteria closely related to *V. alginolyticus* (Fig. 26 a).

There was a group of three isolates which were almost identified as *V. tubiashii* with identification scores above 90 % (Appendix I). One of the three isolates, G10 was medium in toxicity whereas the other two, G1 and G22 produced low toxicity. Three odd, partially-identified bacterial isolates which were moderately or non-toxic (*A. salmonicida* (G3a), *A. sobria* (G4) and *V. furnissii* (G25)) were also found in this group of isolates. These isolates showed 40 to 80 % similarity

TABLE 14 : Distribution of isolates in relation to their toxicity to Mytilus haemocytes, origin and identification.

Bacterial groups	Origin & group	Levels of toxicity		
		Low	Medium	High
Acinetobacter	R (AI)		51	
Cytophaga/Flavobacterium	R (AI)	17, 39, 72, 73		18, 22, 27, 28, 32 36, 42, 43, 48 57, 61, 65, 88
	C (AII)	75, 76, 78, 81		79
Enterobacteriaceae	R (AI)	8, 15, 25, 33 37, 35, 45 49, 60, 66	50, 55 56, 87	9, 11, 13 64, 46
	C (AII)		85	
Micrococcus	R (AI)		40	41
Moraxellaceae	R (AI)	2, 24, 74, 90	89	
	C (AII)	77		
Pseudomonas/Alcaligenes	R (AI)	3, 5, 16, 19, 20 23, 34, 44, 53	26, 30	6, 91, 93, 94
	C (AII)	80		
Vibrio	R (AI)	10	1, 96	7, 12, 14 54, 63, 71
	C (AII)	83, 86		82
	G (G)	1, 9, 12, 13, 16 17, 18, 19, 21 22, 24, 25, 26	3, 5, 7 8, 10, 15 20, 23	
Aeromonas	G (G)	4, 3a		

C: Conwy, G: Guernsey, R: Reculver.

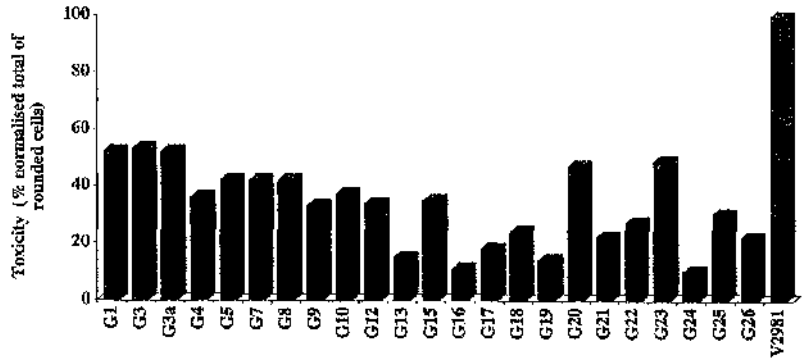
FIGURE 26 Toxicity of bacterial isolates from various shellfish hatcheries to haemocytes at 50 c.f.u. per haemocyte.

a Guernsey isolates.

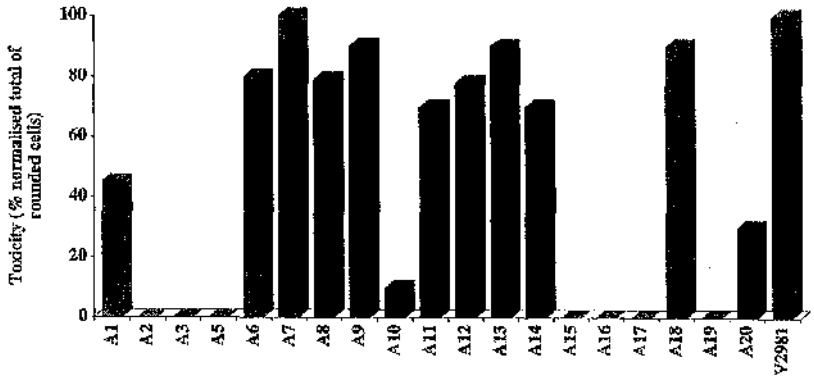
b, c, d & e Reculver isolates.

f Conwy isolates.

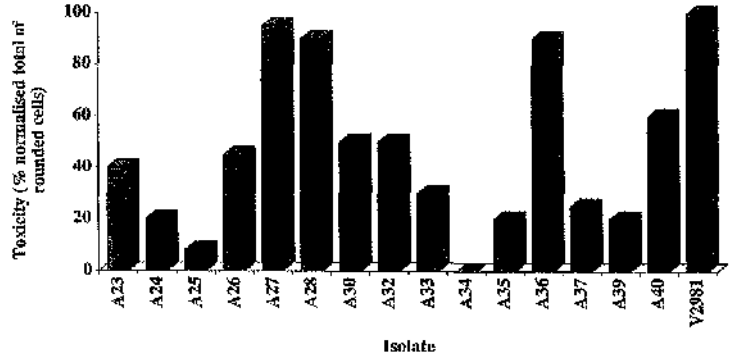
26a


Toxicity (% normalised total of rounded cells)
100
80
60
40
20
0
G1
G3
G3a
G4
G5
G7
G8
G9
G10
G12
G13
G15
G16
G17
G18
G19
G20
G21
G22
G23
G24
G25
G26
V2981

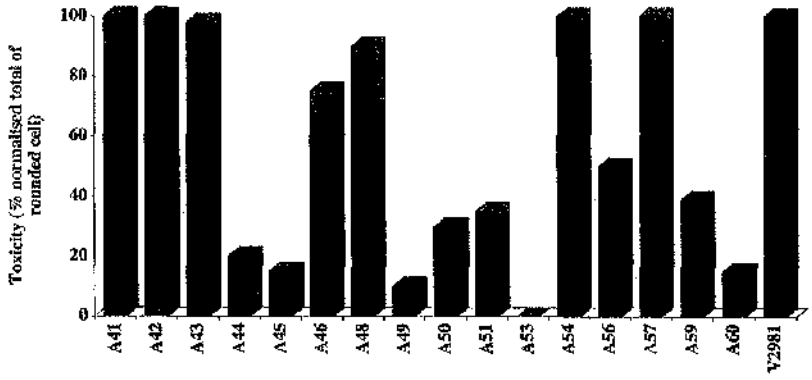
26b


Toxicity (% normalised total of rounded cells)
100
80
60
40
20
0
A1
A2
A3
A5
A6
A7
A8
A9
A10
A11
A12
A13
A14
A15
A16
A17
A18
A19
A20
V2981

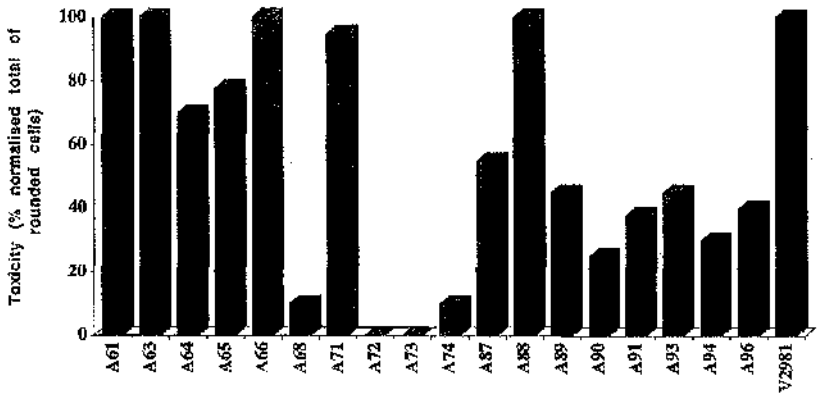
26c


Toxicity (% normalised total of rounded cells)
100
80
60
40
20
0
A23
A24
A25
A26
A27
A28
A30
A32
A33
A34
A35
A36
A37
A39
A40
V2981
Isolate

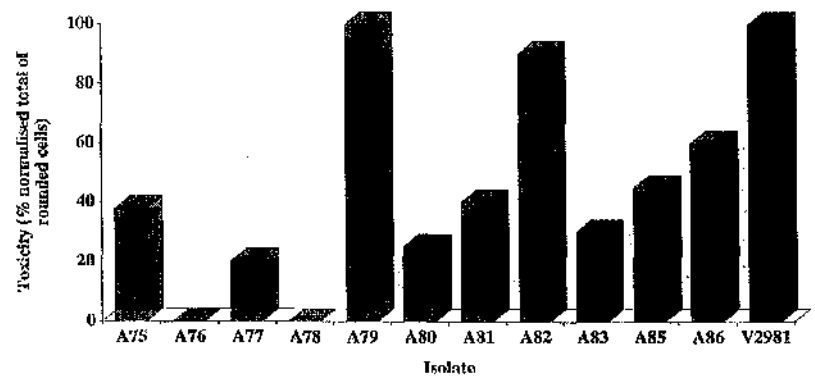
26d


Toxicity (% normalised total of rounded cell)
100
80
60
40
20
0
A41
A42
A43
A44
A45
A46
A48
A49
A50
A51
A53
A54
A56
A57
A59
A60
V2981

26e


Toxicity (% normalised total of rounded cells)
100
80
60
40
20
0
A61
A63
A64
A65
A66
A68
A71
A72
A73
A74
A87
A88
A89
A90
A91
A93
A94
A96
V2981

26f


Toxicity (% normalised total of rounded cells)
100
80
60
40
20
0
A75
A76
A77
A78
A79
A80
A81
A82
A83
A85
A86
V2981
Isolate

The bacteria isolated from Guernsey, which exhibited medium toxicity, were found in the sea water, quarry water and treated larval samples. The sea water contained presumptive *V. alginolyticus* (G7), *V. anguillarum* (G8) and *V. tubiashii* (G10). Four tentative *V. anguillarum* (G3, G5, G20 and G23), isolated from quarry water, displayed medium toxicity. A moderately toxic presumptive *V. alginolyticus* (G15), was isolated from treated larvae.

Overall the sea water contained tentative *V. alginolyticus* (G7, G9, G24, G26), *V. anguillarum* (G8), *V. tubiashii* (G10) and *V. furnissii* (G25). The quarry water had the following presumptive bacteria isolated from it, *V. anguillarum* (G3, G5, G20, G23), *V. tubiashii* (G1, G22) and an occasional bacteria, *A. salmonicida* and *A. sobria*. No *V. alginolyticus* was isolated from the quarry water. The bacteria isolated from un-treated larvae (G12, G13) and treated larvae (G15, G16, G17, G18, G19) were all related to *V. alginolyticus*.

3.6.2 Toxicity of Bacterial Isolates From Reculver.

A total of 68 Reculver bacterial isolates (Group AI) were tested for their toxicity to *Mytilus* haemocytes. The majority of the isolates were either high (43 %) or low (41 %) in toxicity. Approximately 16 % of the isolates exhibited moderate toxicity (Tables 14 & 15, Figures 26-b, c, d & e). The pattern of toxicity, in relation to the bacterial group or family to which the Reculver isolates were related to, was examined.

The bacterial strains that were low in toxicity were partially identified as Enterobacteriaceae (66 %), *Cytophaga/Flavobacterium* (40 %), Moraxellaceae (80 %), *Micrococcus* (33 %), *Pseudomonas* (56 %) and *Vibrio* (23 %). The percentage of moderately toxic strains were tentatively identified as follows, *Acinetobacter* (100 %), Enterobacteriaceae (34 %), *Micrococcus* (33 %), Moraxellaceae (20 %), *Pseudomonas* (19 %) and *Vibrio* (12 %). Highly toxic bacterial strains were tentatively identified as *Cytophaga/Flavobacterium* (60 %), *Micrococcus* (33 %),

TABLE 15 : Distribution of isolates from different origins in relation to their toxicity to Mytilus haemocytes.

Origin of isolates	Levels of toxicity		
	Low	Medium	High
Reculver (Group AI)	2, 3, 5, 8, 10 15, 16, 17, 19 20, 23, 24, 25 33, 34, 35, 37 39, 44, 45, 49 53, 60, 66, 72 73, 74, 90	1, 26, 30 40, 50, 51 55, 56, 87 89, 96	6, 7, 9, 11 12, 13, 14, 18 22, 27, 28, 32 36, 41, 42, 43 46, 48, 54, 57 61, 63, 64, 65 71, 88, 91, 93 94
% of toxicity	41%	16%	43%
Guernsey (Group G)	1, 3a, 4, 9, 12 13, 16, 17, 18 19, 21, 22 24, 25, 26	3, 5 7, 8 10, 15 20, 23	
% of toxicity	65%	35%	
Conwy (Group AII)	75, 76, 77, 78 80, 81, 83, 86	85	79, 82
% of toxicity	73%	9%	18%

Pseudomonas (25 %) and *Vibrio* (65 %) (Figure 26 b, c, d & e). Toxic strains such as *Cytophaga/Flavobacterium* and *V. neresis* were isolated from different hatchery water and larval samples. Other moderate to highly toxic bacteria were also found on different occasions in hatchery water (*Pseudomonas, V. anguillarum* & *V. alginolyticus*), treated larvae (*Pseudomonas,* Phenon 36 & *V. alginolyticus*) and untreated larvae (*Micrococcus, Acinetobacter, V. anguillarum*). *Cytophaga/ Flavobacterium* and *Pseudomonas* were obtained from algae.

3.6.3 Toxicity of Bacterial Isolates From Conwy.

Of the 11 bacterial isolates from Conwy (Group AII), 73 % were not toxic, and 9 and 18 % were moderately to highly toxic respectively (Table 14 & 15, Fig. 26 f). The toxic bacteria were presumptively identified as related to *Vibrio* (A82) and *Cytophaga/Flavobacterium* (A79); these strains were isolated from *O. edulis* and *C. gigas* respectively (Appendix I).. The tentative non-toxic bacteria were partially identified as *Cytophaga/Flavobacterium* and *Moraxellaceae*. The only presumptive *Enterobacteriaceae* isolate present (A85) was moderately toxic (Fig. 26 f, Tables 14 & 15).

3.7 TOXICITY OF SELECTED BACTERIA TO DIFFERENT BIVALVE HAEMOCYTES TYPES.

The haemocyte toxicity assay was used to determine the effects of 9 selected bacteria on haemocytes isolated from the following 7 bivalves, *Crassostrea gigas, Mytilus edulis, Mercenaria mercenaria, Ostrea edulis, Pecten maximus, Tapes decussatus* and *Tapes semidecussatus*. The chosen bacteria were, *V. anguillarum* 2981 (V2981),*V. splendidus* (V110), *V. alginolyticus* (V322), *V. marinarus* or *V. damsela* (V365), *V. ruditapes philippinarum* (Vrp), *V. palourde* (Vp1), *V. alginolyticus* 1339 (V1339), *V. anguillarum* NCIMB 6 (V6) and the Reculver isolate, *V. anguillarum* (A7). Bacteria were selected because of their toxicity to bivalve larvae and to *Mytilus* haemocytes. The toxicity (% normalised total of rounded haemocytes) of the strains was

categorised as low (0 - 33 %), medium (34 - 66 %) and high (67 - 100 %). Bacteria were tested at 50 c.f.u. per haemocyte and incubated with haemocytes for 150 min at 20 °C.

3.7.1 Clam Haemocytes.

Of the bacteria assayed at 50 c.f.u. per haemocyte, A7 and V2981 were highly toxic to all types of clam haemocytes (Fig. 27). Strain V365 was toxic to both *Mercenaria* and *T. semidecussatus* haemocytes (Table 16). Haemocytes of *T. decussatus* and *T. semidecussatus* were more susceptible to Vp1 than *Mercenaria* haemocytes (Table 17). Some of the test bacteria were particulary toxic to specific types of clam haemocytes such as *T. decussatus* (V1339 and V6) and *T. semidecussatus* (V322 and Vrp). *Vibrio* 1339 (isolated from moribund *Mercenaria* larvae) was the most toxic of the bacteria tested (Table 18).

3.7.2 Mussel, Oyster and Scallop Haemocytes.

Results showed that the majority of the test bacteria were toxic to haemocytes of *C. gigas* whereas only one isolate V110 was toxic to haemocytes of *Ostrea edulis* (Fig 28 a and b). Furthermore, haemocytes of *M. edulis* had the highest degree of sensitivity to the selected bacterial strains (A7, Vrp, V322, Vp1, V2981, V6, V1339 and V365) when compared to the other test haemocytes (Table 17 & Fig. 28 c). In contrast, haemocytes of *P. maximus* were the most resistant, being susceptible to one highly toxic strain, V365, an isolate from *P. maximus* larvae (Fig. 28 d & Table 16). Reculver isolate A7 from *Ostrea* larvae was one of the most toxic strains of those tested (Table 18).

3.8 ANALYSIS OF FACTORS INVOLVED IN THE TOXICITY.

3.8.1 The Effect of Temperature.

Haemocytes from the bivalve *M. edulis* were incubated with 10 or 50 c.f.u. of V2981 per haemocyte for 150 min, at four temperatures, 4, 10, 20, and 37 °C.

FIGURE 27 Toxicity of 9 test bacteria to haemocytes of *Mercenaria mercenaria, Tapes decussatus* and *Tapes semidecussatus.*

(a) *Mercenaria mercenaria.*

(b) *Tapes decussatus.*

(c) *Tapes semidecussatus.*

27a

Toxicity (% normalised total of rounded cells)
100
80
60
40
20
0
Control
V110
V322
V365
Vrp
Vp1
V1339
V2981
V6
A7

27b

Toxicity (% normalised total of rounded cells)
100
80
60
40
20
0
Control
V110
V322
V365
Vrp
Vp1
V1339
V2981
V6
A7

27c

Toxicity (% normalised total of rounded cells)
100
80
60
40
20
0
Control
V110
V322
V365
Vrp
Vp1
V1339
V2981
V6
A7
Bacteria

TABLE 16 : The toxic effect of bacteria on haemocytes from different bivalve species. The bacteria tested were 8 vibrios (V2981, V1339, V6, Vp1, V110, V322, V365, Vrp) and isolate A7 from Reculver.

Bacterial isolate	Bacterial host*	Toxicity levels of bacterial strains to haemocytes from the following bivalve species.						
		M. edulis	*M. mercenaria*	*P. maximus*	*T. semidecussatus*	*T. decussatus*	*O. edulis*	*C. gigas*
V2981	*(C. gigas/O. edulis)*	H	H	M	H	H	M	H
V1339	*(M. mercenaria)*	H	M	L	M	H	M	H
V6	(Cod)	H	L	M	M	H	L	H
Vp1	*(T. decussatus)*	H	M	L	H	H	L	L
V110	*(O. edulis)*	L	M	L	M	L	H	L
V322	*(C. gigas)*	H	L	L	H	L	L	H
V365	*(P. maximus)*	H	H	H	H	L	M	L
Vrp	*(T. philippinarum)*	H	L	L	H	M	M	L
A7	*(C. gigas)*	H	H	L	H	H	M	H

The standard bacteria were prepared in filtered haemolymph from each individual bivalve.

*Diseased hosts from which bacteria were isolated.

L : Low (0 - 33 % toxicity), M : Medium (34 - 66 % toxicity), H : High (67- 100 % toxicity).

TABLE 17 : Bivalve sensitivity to the test bacteria (V2981, V1339, V6, Vp1, V110, V322, V365, Vrp and isolate A7 from Reculver). The number of bacteria : haemocyte interactions classed as High (H), Medium (M) or low (L) in Table 16 is summarised.

	Bivalve sensitivity to standard bacterial strains.		
Bivalves	Low	Medium	High
C. gigas	4	0	5
M. edulis	1	0	8
M. mercenaria	3	3	3
O. edulis	3	5	1
P. maximus	6	2	1
T. decussatus	3	1	5
T. semidecussatus	0	3	6

The standard bacteria were prepared in filtered haemolymph from each individual bivalve.

TABLE 18 : Degree of bacterial toxicity to various bivalve haemocytes. The number of bacteria : haemocyte interactions classed as High (H), Medium (M) or low (L) in Table 16 is summarised.

Degree of bacterial toxicity to seven types of bivalve haemocytes.

Standard bacteria	Low	Medium	High
V2981	0	2	5
V1339	1	3	3
V6	2	2	3
Vp1	3	1	3
V110	4	2	1
V322	4	0	3
V365	2	1	4
Vrp	3	2	2
A7	1	1	5

FIGURE 28 Toxicity of 9 test bacteria to haemocytes of *Crassostrea gigas*, *Ostrea edulis*, *Mytilus edulis* and *Pecten maximus*.

(a) *Crassostrea gigas.*

(b) *Ostrea edulis.*

(c) *Mytilus edulis.*

(d) *Pecten maximus.*

28a

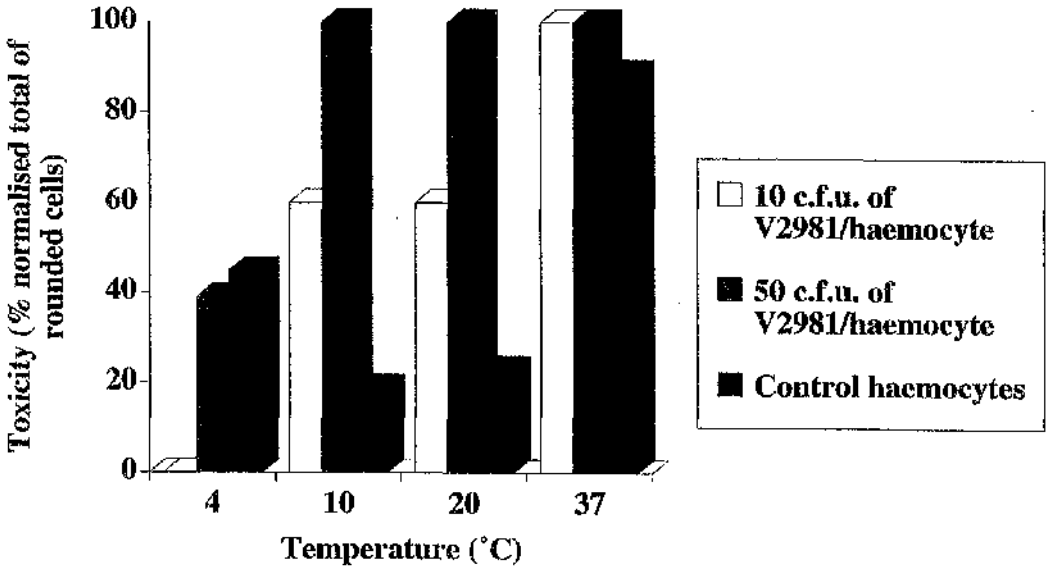
28b

28c

28d

FIGURE 29 Effect of incubation temperature on the toxicity of *V. anguillarum* 2981 to haemocytes of *Mytilus edulis*.

Haemocytes of *M. edulis* were mixed in filtered haemolymph with 10 or 50 c.f.u. *V. anguillarum* 2981 per haemocyte and after incubation for 150 min at various temperatures the percentage of cells rounded was determined. Results were normalised to take account of cell rounding which occurred in the control cells.


Toxicity (% normalised total of rounded cells)
100
80
60
40
20
0
4
10
20
37
Temperature (°C)
10 c.f.u. of V2981/haemocyte
50 c.f.u. of V2981/haemocyte
Control haemocytes

Control haemocytes were prepared without bacteria and these were also incubated at the same test temperatures.

Figure 29 shows the effect of temperature at 10 c.f.u. per haemocyte on the toxicity of the isolate V2981. At 37 °C haemocytes showed a general increase in cell rounding; however, at this temperature only 11 % of the total haemocytes used were viable, whereas, 55 % of the haemocytes were viable at 4 °C. Higher survivals of haemocytes were observed at 10 and 20 °C with 81 and 77 % haemocyte viability respectively.

The maximum toxic effect of the bacteria was observed at 10 to 20 °C. However, at 4 °C the toxicity of the bacteria increased with increasing bacterial numbers; the toxic effect (39 %) observed with 50 c.f.u. of V2981 per haemocyte, had disappeared completely with 10 c.f.u. per haemocyte. At 10 and 20 °C, the toxicity of V2981 was clearly evident with an average of 60 and 100 % of the haemocytes affected by the presence of 10 and 50 c.f.u. of V2981 per haemocyte respectively. Subsequently, all tests involving the study of bacterial toxicity to haemocytes were carried out at the optimum temperature of 20 °C.

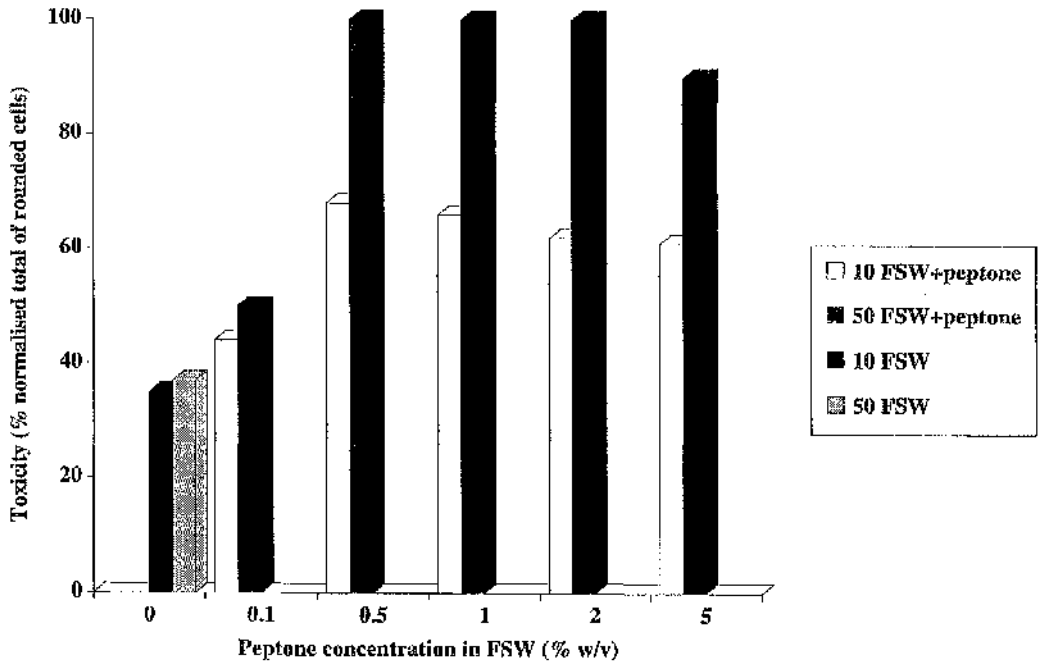
3.8.2 Effect of Peptone Concentration on the Toxicity of V2981 to *Mytilus* Haemocytes.

To determine whether bacteria cultured in low nutrient medium exhibited greater toxicity to *Mytilus* haemocytes than those cultured in normal laboratory media, isolate V2981, was cultured at 20 °C in sea water with low concentrations of peptone.

Preliminary experiments with isolate V2981 cultured in FSW without peptone indicated that the bacteria produced limited toxicity (37 %) in the presence of 10 and 50 c.f.u. bacteria per haemocyte (Fig. 30). Growth of bacteria in four concentrations of peptone (0.1 to 5 %) resulted in increased toxicity to haemocytes but higher concentrations resulted in slightly lower toxicity of V2981 than were found in 0.5 % peptone.

FIGURE 30 Effect of bacterial and peptone concentrations on the toxicity of the *V. anguillarum* 2981 to *Mytilus* haemocytes.

Effect of culture medium peptone concentration on the toxicity of *V. anguillarum* 2981 to *Mytilus* haemocytes. Bacteria were cultured in FSW with added peptone (0 - 5 %) and the resulting bacteria were tested for toxicity towards *M. edulis* haemocytes as ratios of 10 or 50 c.f.u. per haemocyte.


Toxicity (% normalised total of rounded cells)
100
80
60
40
20
0
0
0.1
0.5
1
2
5
Peptone concentration in FSW (% w/v)
10 FSW+peptone
50 FSW+peptone
10 FSW
50 FSW

3.8.3 The Toxic Effect of Lipopolysaccharide on *Mytilus* Haemocytes.

Because filtered haemolymph was necessary for the detection of a toxic effect of bacteria on *Mytilus* haemocytes, preliminary investigations were done to identify both the haemolymph molecule involved and the bacterial surface molecule recognised. Separation of haemolymph proteins by SDS-PAGE revealed two major polypeptides of 41 and 70 kDa respectively (Fig. 31). As lipopolysaccharide (LPS) was considered a possible receptor target on bacteria, FH was treated with LPS and analysed by SDS-PAGE.

Comparison of profiles of FH and FH treated with LPS did not reveal whether the 41 and 70 kDa bands were eliminated due to the complex pattern produced by LPS (Fig. 31). Partially purified LPS from V2981 (section 2.3.5) was mixed with FH (2 mg ml^{-1}) and tested for toxicity to *Mytilus* haemocytes. High levels of haemocyte rounding were observed suggesting that LPS or toxins contained in the LPS preparations were responsible for the effects.

3.8.4 Treatment of Filtered Haemolymph and Toxicity of V2981 to *Mytilus* Haemocytes.

To determine the approximate size of the factor promoting cytotoxicity, haemolymph fractions were prepared using microcentrifuge filters (Sigma) with nominal molecular weight limits of 10, 000 and 30, 000 da. Following centrifugation of the haemolymph through such filters, fractions containing molecules of M.W. less than 10, 000 and 30, 000 respectively were obtained (Table 19). These fractions diluted in FSW (1/16) and mixed with 10 or 50 c.f.u. of V2981 per haemocyte, produced more than 90 % toxicity indicating that the toxic factors involved probably have a molecular weight of < 10, 000 da.

Further analysis of the factor (s) involved heat treatment of the FH at 56°C and 100 °C for 15 min. The toxicity of 50 c.f.u. of V2981 per haemocyte in the presence of heat-treated haemolymph decreased by approximately 50 % indicating

FIGURE 31 SDS-PAGE analysis of filtered haemolymph before and after absorption with *V. anguillarum* 2981 lipopolysaccharide.

1 Standards (SDS-7, Sigma).

2 Neat filtered haemolymph with LPS.

3 Filtered sea water with LPS.

4 Filtered haemolymph (1:5).

5 Filtered haemolymph (1:2).

6 Filtered haemolymph (neat).

Filtered haemolymph was mixed with an equal volume of LPS solution and compared on SDS-PAGE. Analysis with filtered haemolymph at various concentrations with LPS solution and molecular weight standards.

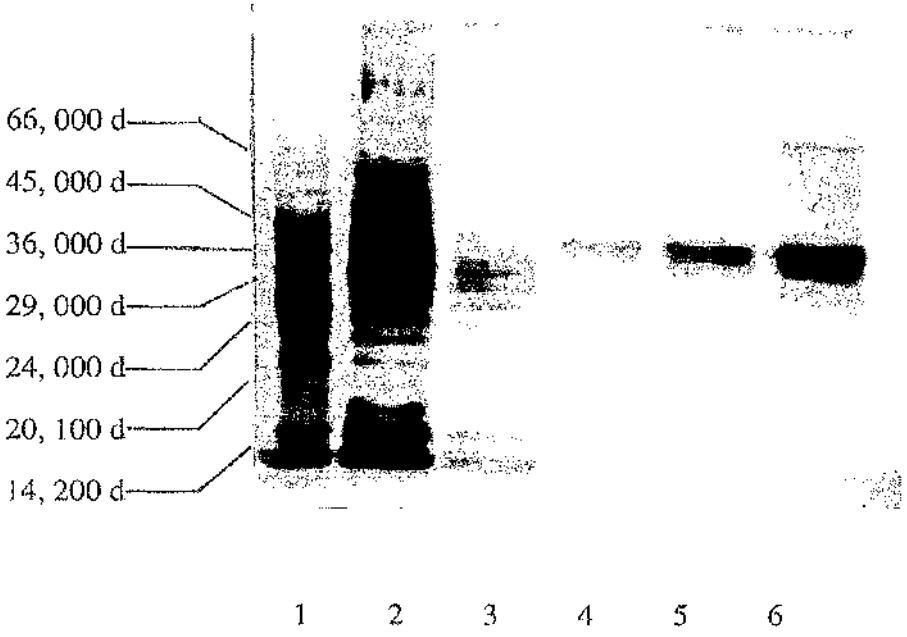

66, 000 d
45, 000 d
36, 000 d
29, 000 d
24, 000 d
20, 100 d
14, 200 d
1
2
3
4
5
6

TABLE 19 : Effect of heat treatment or ultra-filtration of FH, on the ability of FH to promote the toxicity of V2981 towards Mytilus haemocytes. Filtered haemolymph or treated fractions were mixed with Mytilus haemocytes and subsequent cell rounding was determined. Toxicity was expressed as the percentage of cells rounded, normalised cell rounding occurred in control cells.

Treatment of FH and subsequent toxicity of V2981 (% of normalised total of rounded cells) to Mytilus haemocytes.

Bacteria / haemocyte ratio	FH	Heat-treated FH		Ultra filtration	
		(56 ° C / 30 min.)	(100 ° C / 30 min.)	Fraction of FH* <10, 000	<30, 000
10	91%	45%	39%	93%	95%
50	100%	66%	60%	100%	99%

*:-Nominal molecular weight limit.

that the factor (s) involved were temperature sensitive and probably proteinaceous in nature (Table 19).

In addition to these experiments, FH was treated with trypsin (final concentration of 50 μg ml^{-1}) and incubated for 60 min at 25 °C before incubation with suspensions of V2981 (50 c.f.u. per *Mytilus* haemocytes). Cytotoxicity was greatly reduced when FH (neat) was treated with trypsin (neat), and the dose response was seen in the inhibition. Figure 32 gives further evidence that the factor was a protein. Haemocytes treated with trypsin produced no visible cytotoxic effect.

To determine whether FH played a positive role in the cytotoxicity induced by V2981, FH was pre-exposed to V2981 for 30 min, 1 or 24 h. at 20 °C. Following incubation, bacteria were removed by centrifugation, and the absorbed FH was filtered (0.2 μm) and used as a diluent for the cytotoxicity tests. Fresh V2981 bacteria were then suspended in FSW, treated FH or crude FH, and added to haemocytes at final ratios of approximately 10, 50 and 500 c.f.u. per haemocyte. Results using 30 min absorbed FH and FH were similar, with maximum cytotoxic activity with each concentration of bacteria tested (Fig. 33). As expected lower activity was observed with FSW as diluent (Fig. 33).

Similar experiments were carried out using V2981 suspended in FH or marine broth pre-exposed to V2981 for 1 or 24 h. Results (Table 20) showed that exposure of V2981 in bacteria-treated FH produced higher cytotoxic activity when compared to that of V2981 mixed with bacteria-treated marine broth after 1 h. These activities were similar when both FH and marine broth were exposed to bacteria for 24 h, although the levels of activity were not the same. Mixtures of crude FH and V2981 produced 100 % cytotoxicity after 1 and 24 h, whereas bacteria-treated FH and V2981 produced 40 % less activity when compared to the latter.

Similar experiments were carried out using the treated diluents (FH and marine broth) in the absence of V2981; the activity on the haemocytes of bacteria-

FIGURE 32 Effect of trypsin treatment of filtered haemolymph on the toxic effect of *V. anguillarum* 2981 to *Mytilus* haemocytes.

Filtered haemolymph was treated for 60 min at 25 °C with trypsin solution before mixing with bacteria which were then added to haemocyte suspensions and the cytotoxicity was subsequently determined.

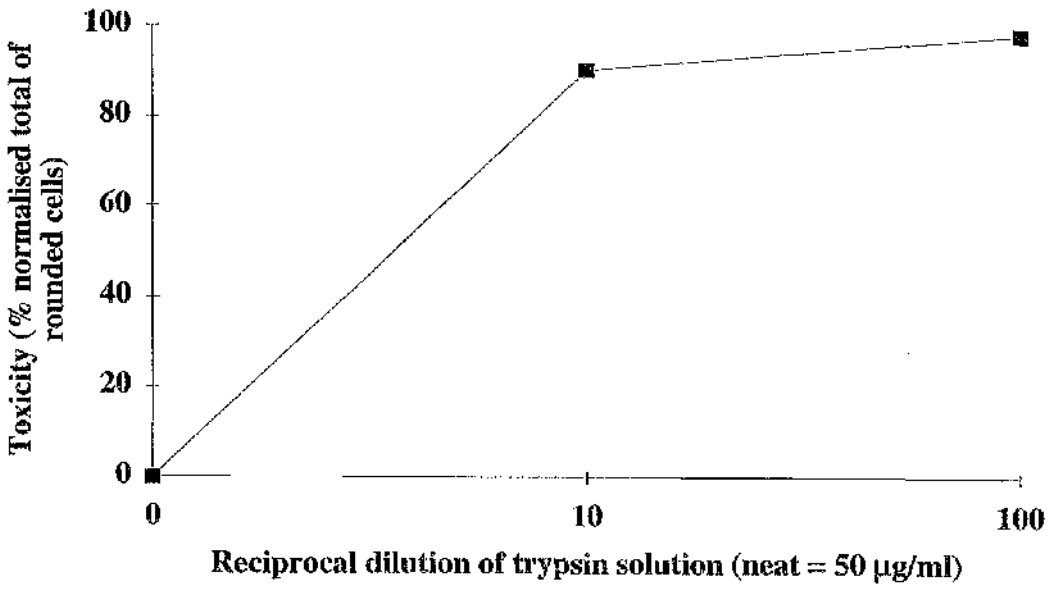

Toxicity (% normalised total of rounded cells)
100
80
60
40
20
0
0
10
100
Reciprocal dilution of trypsin solution (neat = 50 μg/ml)

FIGURE 33 The effect of FSW, untreated FH and bacteria-treated FH as diluents, on the toxicity of *V. anguillarum* 2981 to *Mytilus* haemocytes.

Bacteria were suspended in FSW, untreated filtered haemolymph (FH) or filtered haemolymph preabsorbed for 30 min at 20°C with bacteria (V2981) (BTFH) before testing for cytotoxicity towards *Mytilus* haemocytes at ratios of 10, 50 and 500 c.f.u. per haemocyte. Results were normalised to allow for rounding which occurred in control cells without added bacteria.

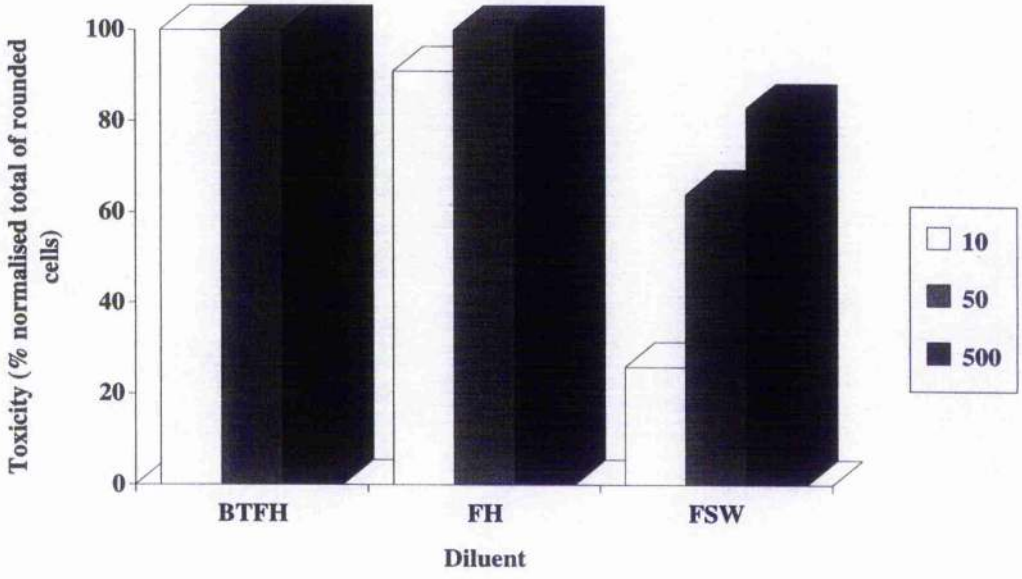

Toxicity (% normalised total of rounded cells)
100
80
60
40
20
0
BTFH
FH
FSW
Diluent
10
50
500

TABLE 20 : The effect of pre-treatment of FH, marine broth (MB) and V2981, on the toxicity of V2981 to Mytilus haemocytes. Pre-treatment of FH, MB and V2981 was carried out using mixtures of FH, MB and V2981, incubated at 20 °C for 1 and 24 h.

	Toxicity (% normalised total of rounded cells)	
Haemocyte treatment	Pre-exposure (1 h)	Pre-exposure (24 h)
Fresh V2981 + bacterial-treated FH	100	60
Fresh V2981 + bacterial-treated MB	24	59
Fresh V2981 + FH	100	100
Bacterial-treated FH	34	45
Bacterial-treated MB	15	29
FH	16	14

treated FH was more than twice that produced by crude FH, whereas 24 h bacteria-treated marine broth was only slightly toxic (Table 20).

3.9 HAEMOCYTE BIOASSAY SCREENING OF CULTURE SUPERNATES.

3.9.1 Culture Supernate of Control Bacterium, *Vibrio* 2981.

The culture supernate of *V. anguilarum* 2981 was investigated for its effects on mussel haemocytes. The toxic effect of bacterial culture supernate on haemocytes was observed by microscopy and death or physiological changes (rounding) occurred in haemocytes when compared to the control haemocytes without culture supernatants. Neat culture supernate produced rounding of 100 % of the haemocytes (100 % toxicity) and there was a linear response in haemocyte cell rounding when exposed to culture supernate (Fig. 34 a).

3.9.2 Other Culture Supernates.

The effect of culture supernates produced by 14 different isolates, from Conwy and Reculver (Fig. 34 b) was tested. Only one of the six Conwy bacterial culture supernates (A79), was toxic when tested neat and this effect was lost when the supernate was diluted 1/2. Half of culture supernates tested from the Reculver bacteria contained factor(s) which were toxic to *Mytilus* haemocytes. With the exception of A65 and the control V2981 supernates, which retained their toxicity to the haemocytes even after 1/2 dilution, supernates (A61, A63 and A64) when diluted 1/2, lost part of their toxic potential. The remainder of the Reculver bacterial culture supernates tested (A68, A72, A73 and A74) were not toxic.

3.9.3 Toxicity Studies of Culture Supernates.

3.9.3.1 Gill assay screening for bacterial toxicity.

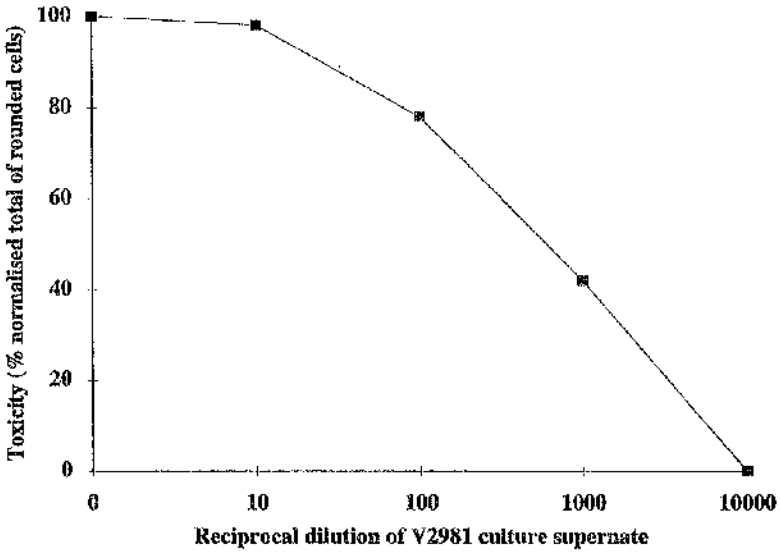
Previous work by Nottage and Birkbeck (1986) showed that marine bacteria release toxins into their culture supernates and which can be detected by their action on

FIGURE 34 Toxicity to *Mytilus* haemocytes of bacteria-free culture filtrates.

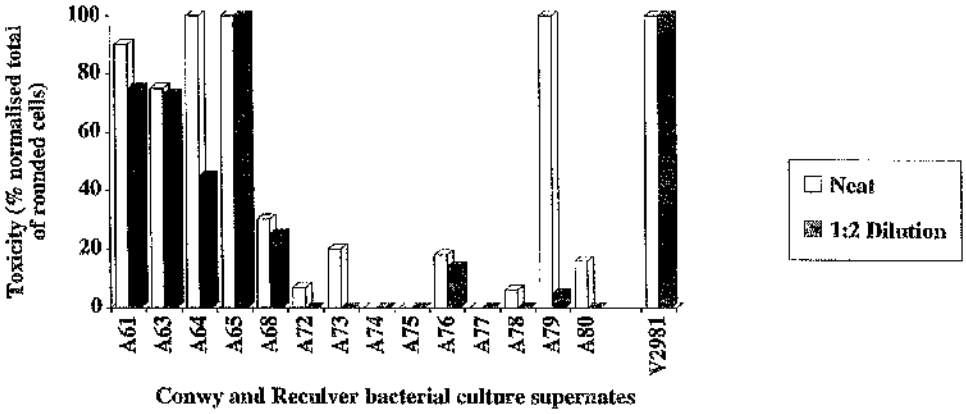
a Toxicity of serial dilutions of *V. anguilarum* 2981 culture supernate (in neat marine broth) incubated with *Mytilus* haemocytes for 150 min at 20 ^{o}C.

b The effects of different bacterial culture supernates on *Mytilus* haemocytes, neat and diluted 1/2 (prepared in neat marine broth). The culture supernates tested were from Conwy (A75 - A80) and Reculver (A61 - A74).

34a


Toxicity (% normalised total of rounded cells)
100
80
60
40
20
0
0
10
100
1000
10000
Reciprocal dilution of V2981 culture supernate

34b


Toxicity (% normalised total of rounded cells)
100
80
60
40
20
0
A61
A63
A64
A65
A68
A72
A73
A74
A75
A76
A77
A78
A79
A80
V2981
Conwy and Reculver bacterial culture supernates
Neat
1:2 Dilution

sections of *Mytilus* gill. The assay detects the inhibition of ciliary activity in *Mytilus edulis* gill segments due to ciliostatic toxin and the disintegration of the gill caused by proteases. Here the effect of native or heat-treated (100 °C for 10 min) culture supernate was tested.

3.9.3.1.1 Guernsey bacterial culture supernates.

These 23 bacterial culture supernates contained no heat-stable gill disintegration factor(s) (Table 21), but most untreated culture supernates did cause gill segment disintegration, including all bacteria tentatively identified as *V. alginolyticus*. The untreated culture supernates of the presumptive *Aeromonas*, *V. anguillarum*, *V. furnissii* and *V. tubiashii* also caused gill disintegration but to a lesser extent than that produced by the presumptive *V. alginolyticus* isolates (Table 21).

No ciliostatic toxin activity was found in culture supernates of *Aeromonas* spp. and *V. furnissii* but 67, 60 and 33 % of the heat-treated culture supernates of *V. alginolyticus, V. anguillarum* and *V. tubiashii* strains respectively were ciliostatic.

3.9.3.1.2 Reculver bacterial culture supernates.

None of the 68 culture supernates contained heat-stable factor(s) capable of disintegrating *Mytilus* gill segments (Table 22) but the majority of the isolates produced heat-labile factors with this activity, particularly the *Vibrionaceae*. Ciliostatic toxins were not present in culture supernates produced by *Moraxellaceae*. Various percentages of the other genera produced ciliostatic toxins particularly *Vibrionaceae* and *Micrococcus*.

3.9.3.1.3 Conwy bacterial culture supernates.

The *Vibrionaceae* culture supernates from Conwy isolates all contained factors, heat-stable ciliostatic toxin and heat labile factor(s) causing gill disaggregation (Table 23). Similarly 25 and 80 % of the *Cytophaga/Flavobacterium* culture supernates contained heat-stable ciliostatic toxin and heat-labile toxins respectively.

TABLE 21 Percentage of Guernsey bacterial isolates in each family or genus which produced toxins affecting *Mytilus* gill tissue.

Bacterial isolates	Percentage of positive tests			
	Heat-treated 100 °C/10 min.		Untreated	
	Ciliostatic toxin	Disintegration of gill	Ciliostatic toxin	Disintegration of gill
Aeromonas (2)*	0 %	0 %	0 %	50 %
V.alginolyticus (12)	67 %	0 %	75 %	100 %
V.anguillarum (5)	60 %	0 %	80 %	80 %
V.furnissii (1)	0 %	0 %	0 %	100 %
V.tubiashii (3)	33 %	0 %	100 %	66 %
Total numbers (%)	**12 (52 %)**	**0 (0 %)**	**16 (70 %)**	**20 (87 %)**

*Numbers represent the total number of isolates identified and tested for toxin production under the defined conditions.

TABLE 22 Percentage of Reculver bacterial isolates in each family or genus which produced toxins affecting *Mytilus* gill tissue.

Bacterial isolates	Percentage of positive tests			
	Heat-treated 100 °C/10 min.		Untreated	
	Ciliostatic toxin	Disintegration of gill	Ciliostatic toxin	Disintegration of gill
Cytophaga/Flavo. * (16)**	16 %	0 %	50 %	75 %
Enterobacteriaceae (7)	43 %	0 %	43 %	57 %
Micrococcus (4)	50 %	0 %	75 %	75 %
Moraxellaceae (4)	0 %	0 %	0 %	50 %
Pseudomonaceae (15)	13 %	0 %	40 %	73 %
Vibrionaceae (22)	82 %	0 %	82 %	91 %
Total numbers (%)	**26 (38 %)**	**0 (0 %)**	**38 (56 %)**	**52 (76 %)**

**Cytophaga/Flavobacterium*

***Numbers represent the total number of isolates identified and tested for toxin production under the defined conditions.

TABLE 23 Percentage of Conwy bacterial isolates in each family or genus which produced toxins affecting *Mytilus* gill tissue.

Bacterial isolates	Percentage of positive tests			
	Heat-treated 100 °C/10 min.		Untreated	
	Ciliostatic toxin	Disintegration of gill	Ciliostatic toxin	Disintegration of gill
*Cytophaga/Flavo.** (5)**	25 %	0 %	80 %	80 %
Enterobacteriaceae (1)	0 %	0 %	0 %	0 %
Moraxellaceae (1)	0 %	0 %	0 %	0 %
Pseudomonaceae (1)	0 %	0 %	0 %	100 %
Vibrionaceae (3)	100 %	0 %	100 %	100 %
Total numbers (%)	**4 (36 %)**	**0 (0 %)**	**7 (64 %)**	**8 (73 %)**

**Cytophaga/Flavobacterium*

***Numbers represent the total number of isolates identified and tested for toxin production under the defined conditions.

3.10 ANALYSIS OF BACTERIAL ISOLATES FROM LARVAL TURBOT.

The aim of this section of the work was to determine the toxicity of bacterial isolates from turbot using the haemocyte toxicity assay. Results were compared with those presented by reference strains, so that the relationship between origin, pathogenicity and toxicity for haemocytes could be assessed for the strains studied.

In previous studies by Dr. P. Munro, the influence of bacterial gut flora on larval survival had been studied. The composition of gut bacterial flora of turbot larvae had been shown to depend on rearing conditions (Munro & Birkbeck, 1993). Therefore, bacteria isolated from turbot larvae cultured under different rearing conditions were tested for toxicity to *Mytilus* haemocytes (Appendix I). Bacterial isolates (Table 24) from three rearing groups were compared, isolates from turbot larvae fed on rotifers reared at low density (extensively-reared) but from cultures previously grown at a low density source (R), those from turbot larvae fed on extensively-reared rotifers from a high density source (ER) and finally those from turbot larvae fed on intensively-reared rotifers from a high density source (IR). Analysis was carried out on 116 different isolates which had previously been characterised by Dr. P. Munro. *Vibrio* was the dominant genus and *V. alginolyticus* was the prinicipal strain isolated (Tables 24).

3.10.1 Cluster Analysis of the Turbot Bacterial Isolates.

Taxonomic analysis of the biochemical characteristics (Appendix III) of the 116 turbot bacterial isolates resulted in a dendrogram composed of 19 clusters (Fig. 35). *V. alginolyticus* was widely distributed within the dendrogram and predominately located within 4 of these clusters (2, 7, 17, 18). Also, *V. parahaemolyticus* and *V. campbellii* were prevalent within two (numbers 16, 19) and four (numbers 6, 10, 12, 14) individual clusters, respectively. The remaining clusters contained a variety of species from different origins (Tables 24 & 25).

TABLE 24 : Number of bacterial species isolated from turbot larvae reared under intensive or extensive rearing conditions.

Presumptive bacterial identification	Bacterial isolate groups		
	R	ER	IR
V. alginolyticus	-	8	14
V. parahaemolyticus	4	8	7
V. campbellii	4	2	5
V. pelagius	-	-	1
V. splendidus 1	2	-	1
V. splendidus 11	2	2	-
V. anguillarum	1	-	1
V. ordallii	1	-	-
V. phenon 6	1	-	1
V. phenon 36	-	-	1
Aeromonas	3	9	2
Enterobacteriaceae	3	-	-
Pseudomonas/Alcaligenes	-	-	1
Unidentified	13	11	8
Total (116)	34	40	42

R: Extensive from a low density source.
ER: Extensive from a high density source.
IR: Intensive from a high density source.

FIGURE 35 Cluster analysis of bacterial isolates from turbot hatcheries.

Legend:

- ▲ *V. alginolyticus.*
- ✱ *V. anguillarum.*
- ● *V. campbellii.*
- ◔ *V. costicola.*
- ●● *V. furnissii.*
- ✡ *V. ordallii.*
- △ *V. parahaemolyticus.*
- ■ *V. pelagius.*
- □ Phenon.
- ○ *V. splendidus* I & II.
- ✸ *V. tubiashii.*
- ☆ *Aeromonas.*
- ⟠ *Pseudomonas.*
- ♦ Enterobacteriaceae.

Amalgamation distance
0
1
2
3
Isolate code

Particular bacterial isolates within the three groups (R, ER, IR) shared some characteristics, these were located within clusters 1, 2, 4, and 7. Similarity solely between the ER and IR isolates was evident in clusters 5, 10, 14, 17, 18 and 19. Those isolates grouped within clusters 6, 11 and 12 were unique to the R group. Some R group strains shared partial similarity with ER (clusters 3, 9) and IR (clusters 13, 15, 16) isolates.

3.10.2 Toxicity of the Turbot Bacterial Isolates.

Table 25 summarizes the toxicity to *Mytilus* haemocytes of the bacteria isolated from turbot hatcheries. Out of 116 isolates approximately half exhibited low or medium toxicity (52 & 57 isolates respectively) and 7 strains were extremely toxic to *Mytilus* haemocytes.

There was a total of 34 isolates in the R group of which 28 were not toxic (Table 26 & 27). The remaining isolates (6) were moderately toxic and presumably belonging to *Aeromonas*, *V. campbellii* and *V. ordallii*. The ER group had 4 isolates which produced high levels of cytotoxicity to *Mytilus* haemocytes and were tentatively identified as *V. alginolyticus* (Table 26 & 27). Out of the 40 isolates tested on *Mytilus* haemocytes, 9 were not toxic and 27 were moderately toxic. The remaining bacteria, comprising of presumptive *V. campbellii*, *A. sobria* and *A. salmonicida*, were low in toxicity.

Bacteria in the IR group resembled those isolates from the ER group, both in toxicity to *Mytilus* haemocytes and in bacterial type (Table 26 & 27). Presumptive *V. alginolyticus* was the most toxic isolate and 15 strains were not toxic out of the group of 42 isolates. A further 6 isolates partially identified as *V. alginolyticus* produced low or medium toxicity (Table 24, 26 & 27).

3.10.3 Distribution of Bacterial Isolates in Relation to Their Toxicity.

Overall, the bacterial isolates produced 3 levels of toxicity groups, low (45 % of total species), medium (49 % of the total species) and high (6 % of the total species) (Table

TABLE 25 : Haemocyte toxicity of bacteria isolated from turbot hatcheries in relation to bacterial identity.

Species / family	Number and bacterial species with the following toxicity levels. Low	Medium	High
V. alginolyticus	5	11	6
V. campbellii	2	9	-
V. parahaemolyticus	12	7	-
V. pelagius	-	1	-
V. splendidus 1	2	1	-
V. splendidus 11	4	-	-
V. anguillarum	1	1	-
V. ordallii	-	1	-
V. phenon 6	1	1	-
V. phenon 36	-	1	-
Aeromonas	5	9	-
Enterobacteriaceae	3	-	-
Pseudomonas/Alcaligenes	-	1	-
Unidentified	17	14	1
Total	52	57	7

TABLE 26 : Number and toxicity levels of the bacterial strains from turbot hatcheries.

Turbot larvae isolate groups	Number of bacterial isolates in each toxicity group. Low	Medium	High
Group R	28	6	-
Group ER	9	27	4
Group IR	15	24	3
TOTAL	52	57	7

R: Extensive from a low density source.
ER: Extensive from a high density source.
IR: Intensive from a high density source.

TABLE 27 : Origin and level of toxicity of bacterial isolates from larval turbot rearing.

Isolate origin	Isolates with the following levels of toxicity		
	Low	Medium	High
Group R isolates from turbot larvae fed on extensively-reared rotifers from an extensive source (34 isolates)	R1, R2, R3, R11 R13, R14, R15, R16 R18, R19, R20, R21 R23, R24, R25, R27 R29, R32, R35, R36 R37, R38, R39, R42 R44, R45, 46, R47	R4, R5 R6, R8 R12, R40	
Group ER isolates from turbot larve fed on extensively-reared rotifers from an intensive source (40 isolates)	ER1, ER3, ER4 ER13, ER14 ER15, ER16 ER44, ER47	ER5(L), ER5(S), ER6, ER6(L) ER8(S), ER9, ER10, ER12 ER17(L), ER17(S), ER18, ER25 ER26, ER27, ER28, ER31 ER33, ER34, ER36, ER39 ER40, ER41, ER43, ER45 ER49, ER50, ER51	ER21, ER23 ER24, ER32
Group IR isolates from turbot larvae fed on intensively-reared rotifers from an intensive source (42 isolates)	IR8(R), IR10(L) IR10(S), IR12 IR14, IR18, IR19 IR20, IR21, IR26 IR28, IR31, IR32 IR38, IR50	IR2(L), IR2(S), IR3, IR4 IR6, IR7, IR8(SM), IR11 IR13, IR17, IR22, IR24 IR27, IR30, IR33, IR34 IR35, IR37, IR39(L), IR39(S) IR42, IR46, IR47, IR48	IR1, IR5 IR46(B)
Total number of isolates per toxicity grouping	52	57	7

Some isolates produced two colony types which were tested separately.
*Colony types : (L) Large, (S) Small, (R) Rough, (SM) Smooth, (B) Brown

26 & 27). Most of the non-toxic isolates were found in the R group (28 out of 34 isolates); although the R group had 6 of the moderately toxic bacterial isolates out of a total of 34 and no highly toxic bacteria, it had the highest larval survival rate. The ER group had medium (9 out of 40 isolates) and highly toxic (4 out of 40) bacteria; this group of turbot larvae had the lowest larval survival rate of the three groups. Finally, the IR group of isolates contained 15 low, 24 medium and 3 highly toxic bacteria out of a total of 42 isolates; this group of turbot larvae had a poorer rate of larval survival compared to that of R group which contained no highly toxic bacteria (Table 26 & 27).

3.10.4 Turbot Larval Survival.

Higher survival rates were found in larvae reared under extensive (low density) conditions compared to intensive (high density) rearing conditions during the investigation. The percentage of turbot larval survival rates for the groups R, IR and ER were 32.4, 11.2 and 4.6 % respectively.

3.10.5 Toxicity Screening of Turbot Bacterial Isolates from Hatcheries Located in Three Different Countries.

Bacterial isolates were obtained from larval turbot from 3 different locations: Norway, Scotland and Spain, on different occasions (Table 28). Among these isolates, NT16 and NT17 were of medium and low toxicity respectively; NT17 induced 90 % of haemocytes to increase their vesicle content dramatically (Fig. 36) when tested for toxicity to *Mytilus* haemocytes. Several physiological changes were observed when bacteria were exposed to haemocytes; only NT17 produced increased phagocytic-like activity of the haemocytes and without rounding of haemocytes. The isolate H3-13 was moderately toxic, whereas, H3-17, UN8 and BS9, were highly toxic to the haemocytes. The strain UN8 was isolated from unhealthy turbot larvae and BS9 was isolated from infected turbot juveniles (black spot infection).

Six Spanish isolates from turbot larvae were tested for cytotoxicity and these included two putative vibrios. The isolate STC11 (isolated from a control tank), was

very toxic whereas the remainder of the isolates including the two putative pathogenic vibrios were not toxic.

TABLE 28 : Toxicity to Mytilus haemocytes of bacterial strains isolated from turbot hatcheries in three countries.

	Isolates with the following levels of toxicity :		
Isolate origin	Low	Medium	High
Scotland	H3-1	H3-13	H3-17
	H3-8		
	H3-15		
	H3-20		
	UN7		UN8
	UN14		
	C4		
	C33		
	C39		
	C45		
	C46		
Norway	NT17	NT16	
Spain	STA11		STC11
	STB12		
	V. alginolyticus AR	*V. pelagius AR*	BS9
Total number of isolates	15	3	4

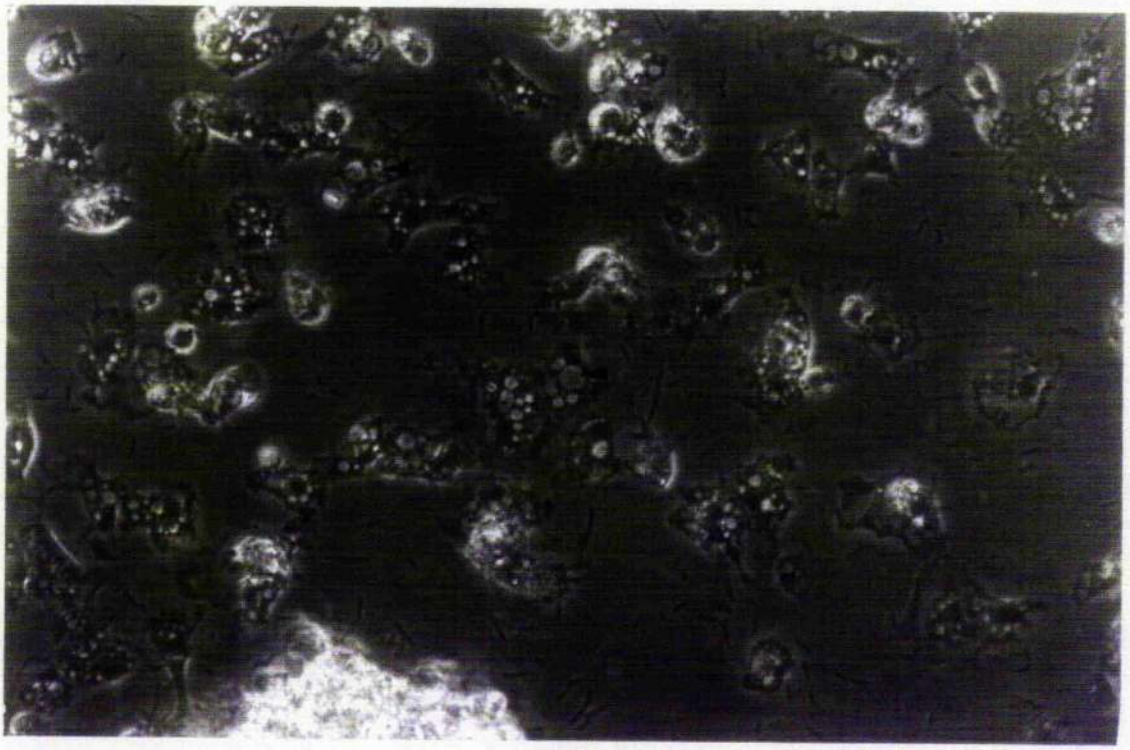
H, healthy larvae; Un, unhealthy larvae: C, copepod fed larvae; NT, Norwegian turbot; AR, STA, STB, STC, isolates obtained from Ms Anna Riaza, Prodenar, SA.

For details of isolation see Munro et al., 1993.

FIGURE 36 Vesiculation of haemocytes after incubation with bacterial isolate NT17.

(➡ indicates presence of bacteria possibly within the vesicles)

X40



Discussion

4.1 IDENTIFICATION AND NUMERICAL TAXONOMIC ANALYSIS OF BACTERIA ASSOCIATED WITH SHELLFISH.

Bacterial diseases are widespread in the marine environment, affecting all types of wild and reared fish and shellfish. Studies have indicated that a wide range of bacteria are involved and the pathological manifestations of these diseases are well documented (Sindermann, 1990; Austin & Austin, 1993; Couch & Fournie, 1993). Many authors have investigated outbreaks of disease in bivalve hatcheries and have identified causative organisms (Tubiash, *et al.*, 1965, 1970; Di Salvo *et al.*, 1978; Brown, 1981; Jeffries, 1982; Nicolas *et al.*, 1992b). A considerable amount of work has been accomplished in identifying the bacteria associated with shellfish diseases (Austin & Austin, 1993; Couch & Fournie, 1993). The work of Jeffries is of particular interest as this was carried out at Reculver, where pathogens isolated were subsequently identified tentatively as the species *V. tubiashii.* One would expect isolates from the hatcheries to contain bacteria from both normal hatchery flora as well as opportunistic and true pathogens.

Identification of these bacteria involves two broad approaches: those relying on serology and those based on phenotypic tests. In this study, identification of bacteria isolated from shellfish was done using the latter technique and it should be noted that conventional phenotypic tests were performed, rather than using the commercially available diagnostic kits (e.g. API20E) which are designed primarily for use with human pathogens and are not suitable for identification of vibrios (Dalsgaard *et al.*, 1996).

Bacterial strains were identified using methods described by Baumann *et al.* (1971); Furniss *et al.* (1979); Lee *et al.* (1979); Oliver (1982). These strains were isolated from bivalve larvae and water samples which were obtained from locations where shellfish are produced commercially (Guernsey and Reculver) and for research (Conwy). Samples from Reculver and Guernsey were collected during outbreaks of

suspected vibriosis. Thus, bacterial isolates were identified using numerical taxonomy where the phenotypic similarities among strains were organised by determination of Simple Matching and Jaccard Coefficients, combined with UPGMA clustering (Sneath & Sokal, 1973). Several clusters (phenons) were obtained from the resulting dendrograms. Type strains of recognised *Vibrio* spp. and some reference *Pseudomonas* strains were also used for comparative purposes. The phenons present in the dendrogams produced by simple matching analysis are described below in relation to their origin.

4.1.1 Analysis of clusters of bacterial isolates from Guernsey.

This study of 24 Guernsey isolates indicated that *Vibrio* was the principal genus and *Vibrio alginolyticus* was the dominant strain. Taxonomic analysis produced 3 major clusters A, B and C (Section 3.2.1, Fig. 4). Cluster A consisted of 2 phenons at the 85 % similarity level which were identified as *V. alginolyticus*. The characteristics of these isolates, such as their haemolytic pattern and ability to utilise single carbon sources, L-arabinose, D-melibiose and DL-3-hydroxybutyrate (Lee *et al.*, 1979; Baumann & Schubert, 1984; West & Colwell, 1984; Austin & Allen-Austin, 1985a, 1985b; Lee & Donovan, 1985; Bryant *et al.*, 1986; Holmes *et al.*, 1986; Collins *et al.*, 1989; Holt *et al.*, 1994), closely resembled those described for *V. alginolyticus*.

Phenon 1 contained isolates G15, G16, G17, G18 and G19, obtained from *C. gigas* treated with benzalkonium chloride. There were two strains in phenon 2, G9 and G12, isolated from sea water and *C. gigas* larvae respectively. The phenotypic profile of these strains showed close similarity to *V. alginolyticus* although they differed in their ability to lyse sheep erythrocytes.

Analysis of the bacteria in cluster B (Section 3.2.1, Fig. 4) revealed low similarities between isolates (approx. 50 % similarity) when compared to other clusters studied. This cluster included the type strain of *V. anguillarum* NCIMB 6^T.

Cluster C (Section 3.2.1, Fig. 4) contained 3 phenons that were quite heterogeneous, grouping different *Vibrio* and *Aeromonas* (Lee *et al.*, 1979; Popoff, 1984; Collins *et al.*, 1989) species. The strains G7 and G8, found in phenon 3, were isolated from sea water; being identified as *V. anguillarum* (Lee *et al.*, 1979; Baumann & Schubert, 1984; West & Colwell, 1984; Austin & Allen-Austin, 1985a, 1985b; Lee & Donovan, 1985; Bryant *et al.*, 1986; Holmes *et al.*, 1986; Collins *et al.*, 1989; Holt *et al.*, 1994) and *V. alginolyticus* respectively. Isolate G8 showed atypical responses when compared to *V. alginolyticus* in the swarming test and the utilisation of D-glucuronate and L-citrulline. G7 differed from *V. anguillarum* in its ability to produce decarboxylases (lysine & ornithine) and arginine dihydrolase, ONPG, acid from salicin and arbutin and in its ability to lyse sheep erythrocytes.

Phenon 4 was composed of two quarry water isolates, G20 and G23, which were both tentatively identified as *V. anguillarum*. Both isolates differed from the *V. anguillarum* type strain because they were non-haemolytic and unable to utilise L-arabinose, D-glucuronate, L-citrulline, D-glucosamine and succinate. They were also V-P negative and did not produce arginine dihydrolase or acid from arbutin.

Phenon 5, which was formed at 95 % similarity, contained isolates G1 and G10, from quarry and sea water respectively. These isolates were identified as *V. tubiashii*. Phenotypic results of G1 and G10 were in accord with the description of *V. tubiashii* except for their inability to express arginine dihydrolase and lecithinase (Lee *et al.*, 1979; Baumann & Schubert, 1984; West & Colwell, 1984; Austin & Allen-Austin, 1985a, 1985b; Lee & Donovan, 1985; Bryant *et al.*, 1986; Holmes *et al.*, 1986; Holt *et al.*, 1994).

4.1.2 Cluster analysis bacterial isolates from Reculver and Conwy.

Cytophaga/Flavobacterium group (Holmes *et al.*, 1984; Reichenback, 1989) was the principal bacterial group present in the 11 strains obtained from Conwy, whereas Enterobacteriaceae (Brenner, 1984) was the predominant genus present in the 67

isolates obtained from Reculver. It was evident from the taxonomic analysis (Section 3.2.2, Fig. 6) that the majority of the phenons showed the following characteristics: negative responses for Gram staining, swarming, luminescence and hydrolysis of sheep erythrocytes, aesculin and xanthine.

Cluster A (Section 3.2.2, Fig. 6) contained phenon 1; a group of 15 bacterial isolates from Reculver and 1 isolate (A79), from Conwy. A total of 13 isolates exhibited gliding motility, were pigmented, were identical in all tests performed (100 % similarity) and resembled the *Cytophaga/Flavobacterium* group. The remaining 3 showed approximately 90 % similarity and, according to (Holmes *et al.*, 1984; Reichenback, 1989), were presumptively identified as *Cytophaga/Flavobacterium* (gliding/non-motile, pigmented, oxidase positive and O-F negative). The tentative *Cytophaga* strains were long slender Gram-negative rods which grew on marine agar with a bright yellow pigment and had a thin spreading margin. These isolates were luminescent, non-haemolytic and incapable of producing decarboxylase (lysine, ornithine), arginine dihydrolase, elastase, lecithinase or utilising the 10 test single carbon sources. All the bacterial isolates in this phenon produced amylase, gelatinase and reduced nitrate.

Cluster B (Section 3.2.2, Fig. 6) was composed of 8 phenons (numbered 2 to 10) which contain partially identified bacterial isolates. Phenon 2 contained two strains, one from Conwy (A85) and the other from Reculver (A87). Their characteristics resembled those of the family Enterobacteriaceae. One distinct characteristic, their being oxidase negative, separated these isolates from other bacteria with similar morphological and biochemical characteristics. The isolates in this phenon produced amylase, elastase, gelatinase and hydrolysed aesculin. A characteristic which distinguished them from obligate aerobic bacteria was their ability to ferment glucose. These isolates were to some extent related to the bacteria in cluster B, phenons 4, 5, 6 and 7.

Three bacterial isolates in phenon 3 were related to the genera *Cytophaga/Flavobacterium* and *Pseudomonas/Alcaligenes* (Palleroni, 1984; Kersters & De Ley, 1984) in their characteristics. A presumed *Cytophaga/Flavobacterium* strain was isolated from Conwy (A75) and the remaining two were Reculver isolates. These isolates were pigmented, oxidative, motile and of variable motility.

Phenon 4 consisted of bacterial isolates from Reculver. Two strains showed 100 % identity in the tests done and were related to *Pseudomonas/Alcaligenes* (A26 and A19). A third isolate A33 was similar to the Enterobacteriaceae and shared approximately 96 % identity with the two tentatively identified *Pseudomonas/Alcaligenes* isolates. These bacteria were motile and did not produce ornithine decarboxylase or elastase but produced lecithinase and amylase. They were resistant to the vibriostatic agent 0/129 (150 µg), one feature that could distinguish these bacteria from *Vibrio* (Baumann & Schubert, 1984; Holt *et al.*, 1994).

Phenon 5 included a mixture of partially identified bacterial isolates from Reculver. These strains were partially identified as *Pseudomonas/Alcaligenes,* Enterobacteriaceae and *Moraxella* (Bøvre, 1984). None of the isolates were identical and the presumptive *Pseudomonas/Alcaligenes* shared approximately 90 % similarity between them. The strains in this group were motile, oxidase and indole positive and resistant to 0/129 (150 µg). They hydrolysed starch and lecithin but not elastin and did not use L-citrulline and L-leucine.

The 7 strains of phenon 6 were from Reculver with the exception of A80, a Conwy isolate. The group was composed of 7 strains tentatively identified as *Micrococcus* (Schleifer, 1984), *Enterobacteriaceae, Pseudomonas/Alcaligenes* and *V. metschnikovii* (A25) (Holt *et al.*, 1994). The oxidase and nitrate negative characteristics shown by strain A25 are unique to *V. metschnikovii.* Most of the isolates in this phenon produced decarboxylase, were nitrate negative, oxidase, indole and ONPG positive and were resistant to 0/129 (150 µg). These isolates were capable of utilising all of the single test carbon sources, hydrolysing gelatin and starch.

Phenon 7 consisted of 4 Reculver isolates. This group of isolates contained a *V. metschnikovii*-like (A35) isolate, that was oxidase and nitrate negative and tentatively identified as belonging to the *Pseudomonas/Alcaligenes* and Enterobacteriaceae groups. These strains were indole positive, oxidase- and ONPG-negative. They hydrolysed gelatin and starch but did not produce lecithinase or elastase. They were able to utilise all the test carbon sources tested. These isolates were very similar in characteristics to phenon 6 but differed from them by their responses to decarboxylase and oxidase tests.

Isolates in phenon 8, all from Reculver, possessed some of the characteristics of *Cytophaga/Flavobacterium.* They were pigmented, non-motile, oxidase, indole and ONPG positive. They utilised all the test carbon sources, produced elastase and acid from sucrose but not gelatinase, lecithinase or amylase. The isolates in this phenon shared characteristics with the bacterial isolates in cluster A, phenon 1 and cluster B, phenon 3.

Phenon 9 contained presumptive *Moraxella* and *Pseudomonas/Alcaligenes* when compared with bibliography references (Bøvre, 1984; Palleroni, 1984; Kersters & De Ley, 1984). The isolate A77, identified as *Moraxella*, was isolated from Conwy, whereas the other isolates were from Reculver. These isolates were non-motile, pigmented, and did not produce decarboxylases. They were oxidase- and indole-positive, and sensitive to the vibriostatic agent, 0/129 (150 µg). All the test carbon sources, with the exception of DL-3-hydroxybutyrate (50 % utilisation), were utilised by these isolates. These isolates also hydrolysed gelatin and starch .

The final cluster (C, Section 3.2.2, Fig. 6) consisted of 7 phenons (10 to 16 inclusive). All of the isolates in this cluster grew on TCBS, were Gram-negative rods and did not luminesce.

Phenon 10 contained strains that were identified as *V. alginolyticus*. They were oxidase and indole positive, ONPG-negative, swarmed and hydrolysed gelatin

but not elastin, lecithin or starch and they did not use D-glucuronate or succinate. These isolates produced acid from salicin, lysine and ornithine decarboxylases. These features closely resembled those described for *V. alginolyticus* (Lee *et al.*, 1979; Baumann & Schubert, 1984; West & Colwell, 1984; Austin & Allen-Austin, 1985a, 1985b; Lee & Donovan, 1985; Bryant *et al.*, 1986; Holmes *et al.*, 1986 and Holt *et al.*, 1994). All of the isolates in this phenon came from Reculver with the exception of A86 which was from Conwy.

Phenon 11 consisted of 3 isolates from Reculver, one possible *Vibrio* and two isolates were presumptively related to the family, Enterobactericeae. Those strains that resembled Enterobacteriaceae were motile, V-P-positive, ONPG-, indole- and oxidase-negative in character; the *Vibrio* was oxidase-positive. They did not hydrolyse xanthine or gelatin or utilise L-arabinose, D-glucuronate, L-citrulline, L-leucine and succinate. The Enterobacteriaceae were resistant to the vibriostatic agent 0/129 (150 µg) but not the *Vibrio* strain. All isolates produced acid from sucrose and secreted elastase and amylase.

The 4 isolates in phenon 12 were presumably related to the Enterobacteriaceae. These isolates were indole-negative, oxidase- and ONPG-positive. They decomposed lecithin and aesculin. They did not reduce nitrate or produce arginine dihydrolase, gelatinase, amylase or utilise L-arabinose, D-cellobiose, D-melibiose. All isolates were resistant to 0/129 (150 µg) and varied in their haemolytic ability.

Phenon 13 consisted of two tentative *Pseudomonas/Alcaligenes* isolates from Reculver. They were oxidase, indole and ONPG positive and V-P negative. They exhibited sensitivity to 0/129 (150 µg) and were non-haemolytic. These isolates did not use the single carbon sources D-cellibiose, D-glucuronate, L-citrulline, D-glucosamine, DL-3-hydroxybutyrate and succinate. Both isolates produced gelatinase and amylase, but not elastase. The *Pseudomonas/Alcaligenes* strain in phenon 13 shared characteristics with those bacterial isolates in cluster B, phenons 6, 7 and 9.

Phenon 14 contained two Reculver isolates which were partially identified as Enterobacteriaceae. They were oxidase- and V-P-negative, indole- and ONPG-positive. Both isolates were sensitive to 0/129 (150 μg). These isolates decomposed aesculin and utilised the single carbon sources, D-cellibiose, D-glucuronate, D-glucosamine, DL-3-hydroxybutyrate and succinate. They were unable to produce arginine dihydrolase and varied in their response to nitrate reduction and production of elastase and amylase. The isolates in this phenon shared characteristics with isolates in cluster A, phenon 2, 4, 5, 6, 7 and cluster B, phenon 11, 12, 14 and 15.

Phenon 15 was split into two partially identified clusters of bacteria. One cluster of isolates resembled *Vibrio* and the other cluster was related to *Moraxella* and Enterobacteriaceae. These isolates were positive for indole, gelatinase and amylase, whereas they were negative for oxidase, ONPG and V-P. They were resistant to 0/129 (150 μg) and were unable to utilise L-citrulline, D-gluconate and succinate or to produce elastase. These *Vibrio* strains were oxidase-negative, nitrate-positive and displayed variable fermentative characteristics, which are semi-characteristic of *V. metschnikovii* and other *Vibrio* species.

There were 9 isolates in the final phenon, 16. This phenon was divided into three small clusters. Strains related to the genus *Vibrio* were found in more than one of these small clusters. The other isolates were presumptively related to Enterobacteriaceae, *Micrococcus* and an unknown. These isolates were characteristically, variable in oxidase and swarming tests, ONPG-positive, indole- and V-P-negative and resistant to 0/129 (150 μg). Phenon 16 isolates were capable of decomposing xanthine and producing gelatinase, elastase and were unable to hydrolyse starch or utilise L-citrulline and L-leucine.

New test methods of identification are continuously being devised, and those reported include: enzyme tests, electrophoretic whole-cell protein patterns, lipid and cell-wall analysis, rapid DNA-DNA hybridization, isolation, purification and enzyme sequencing of 5S and 16S rRNA; therefore, new information is being accumulated

continually (Colwell & Grigorova, 1987). Despite the development of these new methods, classical methods have been used in the identification and characterisation of recent new species of *Vibrio* (Tison & Seidler, 1983; Hada *et al.,* 1984; Pujalte *et al.,* 1993; Onarheim *et al.,* 1994; Ishimaru *et al.,* 1995) and in the studies of Vibrionaceae (Kaznowski *et al.,* 1989; Ortigosa *et al.,* 1989, 1994) and other Gram-negative bacteria (Ortigosa *et al.,* 1994). Many authors recommend the use of classical methods for bacterial identification because of the difficulty in identifying vibrios to species level. Recent work on bacterial characterisation has involved a combination of new techniques along with classical methods of identification; this has been beneficial in our understanding of bacterial identities (Kaznowski *et al.,* 1989; Kita-Tsukamoto *et al.,* 1993; Ortigosa *et al.,* 1994; Ishimaru *et al.,* 1995).

4.2 RESISTANCE / SENSITIVITY OF BACTERIAL ISOLATES TO ANTIBIOTICS

Some bacteria may not cause problems in the wild to the extent they do in hatcheries because concentrations are low (seldom exceeding 1 x 10^3/ml; Utting, 1986) and bivalve larvae exist in low concentrations under conditions of low stress. However, elevated temperatures, high larval density and static larval rearing conditions, as well as the accumulation of waste, are ideal for proliferating bacteria, such that vibrio numbers may reach 1 x 10^6/ml (Austin *et al.,* 1988), a concentration which may be teratogenic and fatal to developing embryos and bivalve larvae (Brown, 1987). When hatcheries have problems various options are available, such as disinfecting the sea water with ultra-violet light. However, this also requires water filtration which removes a valuable supply of algal feed. As a result the farmer prefers to use antibiotics to control diseases in the hatchery. This can result in the spread of bacterial resistance so it is important to know the level of resistance prevalent within locations, and the effectiveness of antibiotics in use.

On this basis the characterisation of the isolates from Conwy, Guernsey and Reculver included a study of antibiotic resistance to 19 broad-activity-spectrum

antibiotics. The objective of this study was to reveal the antibiotic resistance pattern of these isolates which could provide useful information for predicting possible problems associated with bacterial resistance to antibiotics in these shellfish hatcheries. The range of antibiotics selected for this study included those commercially used (e.g. penicillin G, streptomycin, gentamicin, tetracycline, erythromycin, chloramphenicol, nitrofurantoin and sulphonamides) for treating bacterial diseases in aquaculture and some medically important antibiotics (e.g. erythromycin, gentamicin, streptomycin and penicillin G) (Tubiash *et al.*, 1965; Sindermann, 1990; Lambert & O'Grady, 1992; Austin & Austin, 1993). Brown, (1987) suggested that broad spectrum antibiotics should be used in bivalve hatcheries where *Vibrio* and *Pseudomonas* are common pathogens of larval cultures.

Antibiotics such as the β-lactams, tetracyclines and aminoglycosides are active against Enterobacteriaceae, *Moraxella* and Pseudomonaceae. Gentamicin which is normally effective against bacteria belonging to the families Cytophagaceae, Enterobacteriaceae and *Moraxella*, was not active against bacteria from the families Cytophagaceae (*Flavobacterium*) and Pseudomonaceae. Many families of bacteria are generally susceptible to tetracyclines and nalidixic acid; they include Cytophagaceae, Enterobacteriaceae, *Moraxella,* Pseudomonaceae and Vibrionaceae. Bacteria belonging to the family Enterobacteriaceae, are resistant to the macrolide erythromycin, an antibiotic used to treat people with short-term infections and those allergic to penicillin. Some members of the Enterobacteriaceae were also resistant to this antibiotic (Greenwood, 1989; Lambert & O'Grady, 1992; Austin & Austin, 1993).

Resistance profiles of these isolates, regardless of their origin and their taxonomic position, were quite varied (Section 3.1.4, Tables 6 to 9) supporting the findings from a survey of bacterial resistance at different fish farms by Austin (1985), high, intermediate and low resistance being observed in isolates from all three locations; high resistance was observed with the β-lactam group (penicillin G, methicillin and cephaloridine) and macrolide, erythromycin. Mainly medium

resistance was observed with the remaining antibiotic groups except for the quinolone and tetracycline groups, chloramphenicol (aromatic antibiotic) and nitrofurantoin, which were very effective against the majority of the isolates. Certain antibiotics such as ticarcillin and nitrofurantoin produced 100 % killing of all isolates from Conwy in comparison to those from Guernsey and Reculver, which showed different levels of resistance to these antibiotics; cotrimazole, nalidixic acid and chloramphenicol were the only antibiotics which were 100 % effective against all isolates from Guernsey, besides tetracycline. The antibiotic resistance of all the bacterial isolates from these 3 locations revealed a wide distribution and no indication was found of specific resistance patterns of particular bacterial groups in a particular geographical location.

Taxonomic studies of the bacterial isolates from Conwy, Guernsey and Reculver showed that all of the isolates from Guernsey were identified as vibrios and aeromonads. Conwy isolates were identified as being *Vibrio, Pseudomonas, Cytophaga/Flavobacterium,* Enterobacteriaceae or *Moraxella.* Reculver isolates included similar bacterial groups to those of Conwy with an additional group belonging to the genus *Micrococcus.* Similar flora were identified at the fish farms during a study carried out by Austin (1985). He reported that bacterial numbers and resistance patterns varied depending on the use of antimicrobial agents.

When antibiotic resistance profiles of these bacterial groups were compared, the results indicated that most vibrios from these 3 locations showed low resistance to ampicillin. High resistance was observed to erythromycin, gentamicin and streptomycin, whereas most of the presumed vibrios from all locations were sensitive to aromatic antibiotics and tetracycline except for a few isolates obtained from Guernsey. It appears that almost all the presumed vibrios isolated from different geographical locations showed very close antibiotic resistance profiles.

The tentatively-identified aeromonads from Guernsey showed different antibiotic resistance profiles when compared to the presumptive vibrios isolated from the same region, or from Conwy and Reculver. They produced 100 % resistance to

penicillin G, cephaloridine, gentamicin, streptomycin, cotrimoxazole, sulphatriad, fusidic acid and novobiocin; they were completely sensitive to tetracycline, nalidixic acid, erythromycin and chloramphenicol.

Bacterial isolates from Conwy and Reculver tentatively identified as *Pseudomonas* spp., produced high resistance to penicillin G, streptomycin, and trimethoprim and low resistance to chloramphenicol. In general, *Pseudomonas* isolates from Reculver showed resistance to a wider range of antibiotics than those from Conwy. Tentative *Cytophaga/Flavobacterium* isolates from these locations showed high resistance to penicillin G, cephaloridine, gentamicin, streptomycin, sulphamethoxazole and colistin sulphate; however tetracycline, nalidixic acid, erythromycin, ticarcillin, ampicillin, chloramphenicol and fusidic acid were effective in killing these bacteria. Strains of *Cytophaga/Flavobacterium* from Reculver were sensitive to nitrofurantoin and resistant to novobiocin, methicillin and trimethoprim whereas those from Conwy showed opposite profiles for these antibiotics.

Isolates tentatively identified as Enterobacteriaceae and *Moraxella* from Conwy and Reculver were very different when resistance profiles were compared between these groups; both bacterial groups from Reculver showed resistance to more antibiotics than those from Conwy. Isolates partially identified as *Micrococcus,* were found only in Reculver and were resistant to almost all of the test antibiotics used in this study; this characteristic separated this group from all other groups identified from Conwy and Guernsey.

It is common practice to use antimicrobial agents when there is an outbreak of disease at bivalve hatcheries. Therefore it is possible that the larvae at Guernsey and Reculver were pre-treated due to the prevalance of disease at both locations, before the bacteria were isolated. Effectively, sensitive microoganisms may have been inhibited to the advantage of the more resistant ones and this may have influenced the patterns of bacterial resistance observed and the type of flora obtained during this study. Drug resistant plasmids are common in aquatic bacteria, and conceivably,

plasmid-mediated resistance could be transferred from fish/shellfish farm organisms to pathogens of veterinary and human importance. Use of antibiotics has resulted in increased levels of resistance in the past in hatcheries but conclusion of chemotherapy has also reduced the levels of resistance (Austin, 1985; Ledo *et al.*, 1987). This too has been observed in human medicine, where any stoppage in usage has led to a decline in resistance levels (Forfar, *et al.*, 1966).

4.3 DEVELOPMENT OF CYTOTOXICITY ASSAYS.

Molluscs harbour an exceptionally rich microflora (Ortigosa *et al.*, 1989, 1994a; 1994b) and can act as vectors of spread of human vibrio pathogens (Colwell, 1984), such as *V. parahaemolyticus, V. vulnificus* and *V. cholerae*. Unlike mammals, healthy invertebrates may have bacteria in their body fluids and tissues. Disease may result in massive increases in bacterial numbers of otherwise harmless bacteria that proliferate in compromised hosts or from changes in environmental factors. Olafsen *et al.* (1993) found that bacteria exist in haemolymph and tissues of *Crassostrea gigas* kept at low temperatures with viable c.f.u. in the range of 1.4 to 5.6 x 10^2 per ml. Dominant species present were *Pseudomonas, Alteromonas, Vibrio* and *Aeromonas*. *Vibrio* and *Pseudomonas* species also have been responsible for many bacterial diseases affecting marine bivalves (Tubiash *et al.*, 1970; Grischkowski & Liston, 1974). Thus, adult bivalves are normally populated with potential pathogens without contracting the disease, but the molecular basis for this interaction is unknown. Bacterial interactions with haemocytes are inevitable during invasive infections or when bacteria are ingested during the normal filtration and feeding processes. In developing larvae this could be of crucial importance.

Rearing of bacteria-free larvae represents an ideal system for subsequent testing of indigenous bacterial isolates for their interaction with larvae. This has been used by Nicolas *et al.* (1992a) to confirm the virulence of particular isolates from

hatcheries in Brittany and for the culture of a herpes virus pathogenic for larvae (Le Deuff *et al.*, 1994).

Here it was shown that *V. anguillarum* NCIMB 6 (Section 3.3.1), isolates (Section 3.3.2) G1, G13 (Guernsey), A10, A45 (Reculver), V322 and V365 (France) caused mortalities in oyster and scallop larvae whereas the native P1-1-1, considered avirulent by Nottage *et al.* (1989) gave only low mortalities. However, it proved difficult to achieve bacteria-free conditions reproducibly and it was not feasible to assess virulence of a large number of isolates. Therefore a more direct assay was considered.

The use of a cell-based assay can be justified in several ways. Firstly, Elston and Leibovitz (1980) showed that their bacterial isolate caused three different types of pathology in *C. virginica* (invasion from exterior, invasion from the gut, toxin-mediated). Of these the toxin-mediated mechanism has been found to be common (Nottage *et al.*, 1989; Nottage & Birkbeck, 1990) and a wide range of bacterial toxins in other diseases have been shown to have cellular targets (Donelli & Fiorentini, 1992; Sansonetti, 1992; Bajolet-Landinat *et al.*, 1994). Secondly, Elston (1984) also noted that the invasive bacteria also probably require toxins to effect cell damage. Thirdly, the haemocyte represents an essential defence in the control of invasive organisms. In microbial infections the interactions between microorganisms and phagocytic cells are crucial determinants in the disease process.

For vibriosis in bivalve hatcheries a number of possible mechanisms, such as the production of ciliostatic and lethal toxins, have been recognised (Nottage *et al.*, 1989). Bacterial toxins can be classified according to their action on mammalian cells, such that three major classes of toxins were identified by Thelesman & Florin (1994). Class I toxins includes those which act analogously to growth factors by binding to cell surface receptors, mediating a transmembrane signal to the intracellular compartment. Type II toxins act directly on the plasma membrane either by pore formation or by disruption of the lipid bilayer, these cytolysins cause gross

structural damage to the plasma membrane and thereby lyse the cell directly. Type III toxins act intracellularly by modification of cytosolic targets (Thelesman & Florin, 1994) and cause slow cell degeneration due to inhibition of macromolecular synthesis or metabolic disturbances, DNA structure, leading to so called programmed cell death (apoptosis).

Well-established examples of these different types of toxins have been reported in the literature, for instance *Escherichia coli* heat-stable enterotoxin (ST) and *Staphylococcus aureus* toxic shock syndrome toxin belong to the type I toxins; S. aureus α-toxin, *Clostridium perfringens* perfringolysin O and *E. coli* haemolysin are some of those belonging to type II. Examples of type III toxins include cholera, diphtheria and pertussis toxins, and *E. coli* heat-labile enterotoxin (LT). The cytopathogenic effect of these toxins is manifested by cellular morphological changes which involves cell lysis and detachment, change of cell shape and surface characteristics, and visible intracellularly changes.

Cytopathogenic effects can be easily seen by inverted light or phase-contrast microscopy. Thus, in this study cytotoxic effects caused by specific bacteria to the target cells used in the tests, were defined as cell shape changes, rounding of cells and cell disintegration. It should be noted that the term cytotoxicity or toxicity used in this study could cover cell invasion and/or bacterial cell multiplication within target cells.

The initial observation that bacteria of different virulence to larvae differed in their toxicity to bivalve haemocytes led to a study in greater depth of bacterium-haemocyte interactions, since interaction between phagocytic cells and bacteria is of great significance in determining the outcome of many infections. Some with apparent irreversible effect from interaction of a haemocyte with a single bacterial cell. The interaction of vibrios with *Mytilus edulis* at the cellular level was investigated previously by Nottage and Birkbeck (1990), who showed that the differences in virulence of two strains of *V. alginolyticus* for oyster larvae reflected

differences in the toxicity of washed bacterial cells to haemocytes of *M. edulis*. The ratio of bacteria to haemocytes used by Nottage *et al.* (1989) was high (50 to 500:1) and the assay depended on the uptake of the vital stain neutral red. A number of alternatives to the neutral red assay of Nottage *et al.* (1989), were considered here.

Cytotoxicity assays are widely used to process samples and to allow initial screening of large numbers; on the basis of this, larval toxicity tests could then be done. The conclusion from all of these tests (neutral red, XTT, calcein AM, ethidium homodimer) was that cells were not being killed, at least over the period of a few hours. As washed suspensions of bacteria were used one would anticipate direct contact bacterial cell-haemocyte interactions to predominate. Thus, gross effects on haemocytes might take longer to be evident when assessed in biochemical tests. Also these tests were almost all carried out with bacterial cells suspended in filtered sea water; later it was shown to slow down the interaction of bacteria with haemocytes. As haemocytes did not appear to be killed rapidly on interaction with bacteria, microscopy was used to study the interaction. As this indicated changes in haemocyte behaviour in the presence of bacteria, time-lapse microscopy was used to investigate the kinetics of the interaction.

Previous studies (Elston & Leibovitz, 1980b) have indicated that the concentration of bacteria, (opportunistic or pathogenic) could possibly affect the eventual invasion of the haemolymph and tissues. These authors reported that oysters selectively removed vibrios from the medium. However, Tubiash *et al.* (1965) found no harmful effects of ingestion of large numbers of *Vibrio* spp. and *Aeromonas* in adult *C. virginica*, *M. mercenaria*, *M. edulis* and *M. arenaria* exposed for 24 hours, in standing sea water cultures. High larval mortalities have been associated with an abundance of vibrios (Leibovitz, 1979), and diseased larvae were massively infected by bacteria in the viscera and mantle tissues. However, large numbers of bacteria in the water may not always be detrimental to the bivalves. Tubiash *et al.* (1965) reported that the presence of *V. anguillarum*, *V. alginolyticus* and *Vibrio* spp. in the

surrounding sea water did not have any effect on *Mya arenaria* at 20°C but when inoculated into the heart and/or siphon tissues they caused vibriosis in clams.

Attachment is a prerequisite of bacterial colonisation of epithelial surfaces and prokaryotes express surface associated molecules such as adhesins (Sharon, 1986), often with high specificities for eukaryotic cell surface proteins or carbohydrate structures. Many pathogenic bacteria can be internalised by normally non-phagocytic host cells (Moulder, 1985) e.g. *Chlamydia trachomatis*. Other pathogens, such as *Salmonella* and *Yersina* spp., probably utilise the process of internalisation by host cells as a prerequisite to establishment of infection and thus enables bacteria to colonize various parts of the body (Une, 1977).

Molluscan haemolymph and body parts contain lectins that agglutinate erythrocytes and various species of bacteria and may also act in defense by facilitating opsonisation and phagocytosis (Cheng *et al.*, 1984; Renwrantz, 1983; Olafsen, 1986, 1988). *C. gigas* haemolymph agglutinates marine vibrios and other bacteria (Hardy *et al.*, 1977; Olafsen *et al.*, 1992). A preference for vibrios was observed in *C. virginica* since its haemolymph specifically agglutinated *V. cholerae* but not 79 other environmental bacterial isolates (Tamplin *et al.*, 1989). Olafsen & Hansen (1992) investigated the bacterial uptake by epithelial cells and found that these cells took up *V. fischeri* and *V. salmonicida* preferentially as compared to *Flavobacterium* spp. Oyster haemolymph contained agglutinins for *V. anguillarum* which did vary after long term challenge; however, none were found for *V. salmonicida*. *M. modiolus* contained no agglutinins for either of these bacteria, whereas horse agglutinins and erythrocyte agglutinins were found in both species (Olafsen *et al.*, 1993).

The requirement for filtered haemolymph to facilitate interaction of bacteria with haemocytes is characteristic of an opsonin. A calcium-dependent opsonin of *Mytilus edulis* was purified by Renwrantz and Stahmer (1983) and a cell-surface location on haemocytes was suggested from work with antiserum to the purified

opsonin. The factor was necessary for effective phagocytosis of yeast cells, in contrast to earlier findings by Bayne *et al.* (1979). From *C. gigas* two haemoagglutinins, gigalins E and H, were described by Olafsen *et al.* (1992). These were part of large molecular weight complexes in the haemolymph and the opsonic activity towards *Vibrio anguillarum* NCIMB 6 was demonstrated in vivo (Hardy *et al.*, 1977). Olafsen *et al.* (1992) absorbed the gigalins from oyster haemolymph at different temperatures by living, heat-killed, and freeze-dried *V. anguillarum*. Challenge with live or heat-killed *V. anguillarum* showed an increase in E activity whereas H activity was very variable. The augmented levels of lectin activity in oyster haemolymph following in vivo exposure to increased bacteria in the sea water suggests their involvement in enhancing bacterial clearance (Olafsen *et al.*, 1992).

Molluscs appear to have effective systems for clearing invading bacteria from their tissues and haemolymph following intracardial injection or natural ingestion of bacteria (Hartland & Timoney, 1979). The role of haemocytes in such clearances has been well documented in bivalves, but the role of humoral factors like lectins remains unresolved. Lectins have been isolated from haemolymph of most invertebrates and their possible role in defense has been reviewed extensively (Cheng *et al.*, 1984; Renwrantz, 1986; Olafsen, 1986, 1988). How bacteria may conquer the defense system remains unresolved plus their role in larval diseases.

Even though most of these lectins were identified by their ability to agglutinate erythrocytes, natural agglutinins for bacteria have been identified in hard clams (Arimoto & Tripp, 1977) and oysters (Tamplin & Fisher, 1989). It is generally inferred that invertebrate lectins take part in the recognition and clearance of bacteria by haemocytes. Agglutinins may increase phagocytosis in invertebrates by acting as opsonins, but the interactions between purified haemolymph lectins and bacterial pathogens have only been rarely investigated. Hardy *et al.* (1977) reported that the affinity purified haemolymph lectins from *C. gigas* were opsonic, increasing the uptake of bacteria (*V. anguillarum* NCIMB 6 and *E. coli* K325) up to 5 times by

oyster haemocytes *in vitro* compared to untreated. Purified *M. edulis* lectins have also been found to stimulate in vitro phagocytosis of yeast cells and erythrocytes by *Mytilus* haemocyte (Renwrantz, 1983).

Humoral factors (like lectins) in marine invertebrates are generally believed to be innate or noninducible. Exposure to bacteria *in vitro* may induce activities of serum and haemocyte bound enzymes (Chu, 1988) or increase humoral antibacterial activity. In the current assay, the opsonic factor in haemolymph may be similar to that described by Renwrantz and Stahmer (1983) but cross absorption of haemolymph with human erythrocytes or yeast cells was not done. However, the activity was reduced by filtered haemolymph absorption with bacterial lipopolysaccharide (24 hours) and this is likely to be the target for recognition of foreign material such as bacteria. The haemolymph of Pacific oysters and eastern oysters (*C. virginica*) have been found to contain lectins that agglutinate all kinds of erythrocytes and bacteria including various laboratory and environmental strains of marine vibrios (Tamplin & Fisher, 1989; Fisher, 1992). The possible role of lectins in invertebrate defense has been well documented (Cheng *et al.*, 1984; Renwrantz, 1986; Olafsen, 1986, 1988).

Rounding of haemocytes can be taken to indicate a cessation of phagocytic activity (Stendahl *et al.*, 1980; Sheterline *et al.*, 1984). The observed effect occurred very slowly, requiring 90 minutes for 50 % of the cells to become rounded at a bacterium/haemocyte ratio of 500:1. Since the dose response curve corresponded to 1-hit curve as shown by Poisson distribution, it was concluded that interaction of a single "virulent" bacterium with a haemocyte is sufficient to inhibit further phagocytic activity by the cell. Such inhibition could occur via interaction of a surface component of the bacterium or by a product excreted by the bacterium after phagocytosis. Sterile bacterial culture supernatants of *V. anguillarum* 2981 also caused rounding but it is not possible from currently available data to differentiate between a secreted bacterial product and a cell surface component as the latter are

often released in association with lipopolysaccharide vesicles (Hammond *et al.*, 1984).

Several different modes of action of cytotoxins have now been established. Diphtheria toxin, an ADP-ribosylating toxin which inhibits cellular protein synthesis, has become the model for many such toxins (Dorner & Drew, 1986). Other ADP-ribosylating toxins affect quite different targets. Cholera and pertussis toxin, by virtue of their modification of G-proteins elevate intracellular cAMP; in the case of cholera leading to an outflow of fluid and in the case of pertussis toxin to modification of the activity of leukocytes, impaired phagocytic activity (Stephens & Pietrowski, 1986). Toxins active against the eukaryotic cytoskeleton have been described for many bacteria. They are capable of inducing cell rounding by disorganizing the cytoskeleton. This can occur without swelling and in the absence of plasma membrane damage, i.e. on exposure to high molecular weight toxins. These toxins include the ADP-ribosylating toxins secreted by several clostridia (Aktories & Wegner, 1992) which cause disruption of the cytoskeleton by modification of G-actin leading to depolymerisation of F-actin, a crucial component of the cytoskeleton. Bacterial toxins which act directly on the cytoskeleton, for example *Clostridium botulinum* C2 which affects actin, cause characteristic cytoplasmic retraction and an actinomorphic effect in fibroblasts, which is later followed by complete rounding (Aktories & Wegner, 1992). Also, the cytotoxic necrotising factor (CNF-1) of certain strains of *Escherichia coli* induces reorganisation of the actin cytoskeleton into "stress fibres' and induces "invasion" of cells (Falzono *et al.*, 1993). Development of plasma membrane blebs are common when the cytoskeleton is affected; such blebs can just be seen by light microscopy in some cells; closer inspection and characterisation requires electron microscopy (Thelesman & Florin, 1994). The cytotoxic heat-labile enterotoxins of (LT) of *E. coli* and *V. cholerae* cause a rounding of certain cells (mouse adrenocortex Y1 cells) and a characteristic elongation of other cells (Chinese hamster ovary, CHO, cells) owing to an increase in cytosolic cyclic AMP. This well known example underlines the finding that different types of cells may respond to the

same toxin with widely differing alterations in shape. Vacuolation has been described in cells treated with aerolysin from *Aeromonas hydrophila* and with the vacuolating toxin produced by *Helicobacter pylori* (Leunk *et al.*, 1988). In this study a similar effect was observed with strain NT17 (Fig 35). This effect has not been reported for other type II toxins and the molecular basis for this effect is unclear. Vacuolation of culture cells, similar in appearance to the above, has long been known to have been induced by weak bases which accumulate in acidic cellular compartments (endosomes, lysosomes). Chloroquine or ammonium chloride could be used as reference substances in comparative studies in the future. Enzymatically active toxins bind to the cell surface, enter into the cytosol by phagocytosis or endocytosis, and block an essential cell function. These toxins have separate domains and fragments involved in the cell binding, translocation and enzyme activity.

The colicins, yeast killer toxin and *Staphylococcus aureus* α- toxin directly form toxic channels in the cell membrane. The channel causes cytotoxicity directly either by leakage of vital intracellular ions (potassium and magnesium), influx of toxic ions or disruption of toxic gradients required for transport or energy production. With indirect toxins, such as diphtheria, tetanus, botulinum, *Pseudomonas* exotoxin A and anthrax toxins, the channel plays a role in facilitating entry of another part of the molecule (ADP-ribosylase) perhaps by forming a protein tunnel through which the toxic molecule can enter the cell. Most of the channel forming experiments have not found specific receptors on the lipid bilayer although reports exist that cholera toxin requires a ganglioside (GM1), these toxins are at home in various lipid environments although some lipids markedly enhance channel forming activity, such as in the case of phosphatidylinositol and diphtheria toxin (Kagan & Sokolov, 1994).

Among the toxins secreted by gram-positive and -negative bacteria, pertussis toxin has been the subject of intensive research. Its toxin is a complex, multisubunit protein composed of six subunits. The toxin acts, after being secreted by *Bordetella pertussis*, by binding to glycoprotein receptors on eukaryotic cells; the toxin gains

access to these cells and ADP-ribosylates a family of GTP-binding regulatory proteins (G proteins) involved in signal transduction (Witvliet *et al.*, 1989; Gilman, 1987). Normally, G proteins transmit signals initiated by hormone binding to receptors on the surface of the eukaryotic cells to effector proteins located within the cell. When the G proteins are ADP-ribosylated, they are inactivated and signal transduction is inhibited resulting in a variety of biological effects (Kaslow & Burns, 1992).

Legionella pneumophila, the aetiologic agent of Legionnaires' Disease, represents a major environmental pathogen. It is a gram-negative facultative intracellular pathogen which may cause severe atypical pneumonia in humans. The capacity of this organism to cause disease is dependent on its ability to invade and to multiply within host phagocytic cells (Winn, 1988), in the absence of adequate cell-mediated immune response. The rapid intracellular replication of *Legionella* and the release of tissue-destructive substances from either the bacteria, the host, or both, result in acute bronchopneumonia. This is characterized by an evasion of the bactericidal oxidative burst, an inhibition of phagosome-lysosome fusion and lysis of the host cell (Horwitz, 1989). The bacterial factors that enable this intracellular pathogen to adapt to intracellular survival and to alter the normal phagocytic response of the macrophage are not fully known. However, several studies indicated that a number of factors may be involved in the pathogenesis of the organism; the important factors included the major outer membrane protein (24 kD to 29 kD) which functioned similarly to the porins of *E. coli* (Gabay *et al.*, 1985), also hemolysin (Baine, 1985) and protease (Dreyfus & Iglewski, 1986); recently, studies indicated that a surface protein, a prokaryotic homologue to the FK506-binding proteins, called Mip (Macrophage Infectivity Potentiator) was required for optimal infection of macrophages and a variety of amoebae by *L. pneumophila* (Cianciotto & Fields, 1992). Moreover, other species of *Legionella* were shown to block a variety of cell functions similar to those inhibited by pertussis toxin which resulted in the disruption of signal transduction in neutrophils (Beker, 1985; Bokoch & Gilman, 1984).

The mechanism of induction of the cell-rounding process is, as yet, unclear; however Aktories and Wagner (1992) showed that ADP-ribosylation of actin in the cytoskeleton leads to cell rounding and loss of cell motility. It is unlikely that an actin-specific toxin is involved as these are, to date, only known to be produced by clostridia. However, elevation of intracellular cAMP is a possible mode of action as production of such toxins occurs among the Vibrionaceae and Enterobacteriaceae (Dorner & Drew, 1986). A further candidate toxin, the ciliostatic toxin identified by Nottage *et al.* (1989), was not produced by all vibrios which induced cell rounding and it is, therefore, unlikely to be responsible for this effect.

Ingestion of Gram-negative bacteria by bivalves normally results in degradation of the bacteria by a mechanism involving lysozyme of the digestive tract of the bivalve (Birkbeck & McHenery, 1982). However, there is evidence (Maginot *et al.*, 1989) that lysozyme is not detectable in the early stages of larval development and this, coupled with the toxicity of certain groups of bacteria to haemocytes, may be related to the high susceptibility of larvae to bacterial infection. Further investigation of these mechanisms are required.

In this study results indicated that host specificity must be considered when investigating haemocyte-bacteria interactions. Thus, bacteria causing disease in clams and scallops, respectively, showed highest activity against haemocytes of the species from which they were isolated. In terms of the mechanisms of virulence of these bacteria this merits further investigation and may reveal the reasons for host specificity in such disease.

4.4 INFLUENCE OF OTHER FACTORS ON THE CYTOTOXICITY OF BACTERIA

Exogenous factors such as nutrients and incubation temperature play a major role in expression of bacterial toxicity. In this study increasing cytotoxic effects of these isolates were correlated with increasing peptone content in their culture media, indicating a possible relationship with nutrients in the media; possibly essential amino

acids contained in the peptone were involved in the expression of bacterial cytotoxicity. Expression of the cytopathogenicity of the isolates was directly related to the number of bacterial cells and/or the presence of nutrients such as peptone in the cocultures

Bacterial lipopolysaccharide (LPS) or endotoxins have been extensively studied and shown to cause cytotoxic effects on mammalian cells (Fouz *et al.*, 1993). Further treatments of haemolymph of *M. edulis* were carried out to determine the nature of the factor (s) involved in the increase of bacterial cytotoxicity; these treatments included filtration of the haemolymph freshly extracted from *M. edulis* using nominal molecular weight limit microcentrifuge filters, and indicated that molecules of unknown nature of molecular weights of less than 10, 000 were probably involved in the induction of the cytotoxic effect of V2981 on *Mytilus* haemocytes.

4.5 ANALYSIS OF CYTOTOXICITY OF TENTATIVELY IDENTIFIED BACTERIA.

Tests using bacteria isolated from Guernsey, Reculver and Conwy revealed varied toxicity profiles. Isolates from Guernsey were of medium to low toxicity to *Mytilus* haemocytes, and the presumptive *V. anguillarum* were considered the most toxic of this group of bacteria. Isolates from Guernsey showed a weakly clustered bacterial group, those isolates from shellfish in Reculver represented a more diverse group in relation to their toxicity to *Mytilus* haemocytes, possibly reflecting the episodic nature of disease at this hatchery. Higher toxicity to *Mytilus* haemocytes was observed with presumptive *Vibrio* and *Cytophaga/Flavobacterium* whereas low activities were observed with tentative Enterobacteriaceae, *Pseudomonas/Alcaligenes* and Moraxellaceae. In contrast with isolates from Guernsey, proteolytic activity was rarely detected in the supernatants of latter bacterial groups from Reculver. Toxic isolates from Conwy were attributed to presumptive vibrios and *Pseudomonas* species, whereas isolates partially identified as *Cytophaga/Flavobacterium* produced

low levels of toxicity to the *Mytilus* haemocytes. From the data obtained one would assume that isolates which were closely related, but from the different locations, produced different effects on the haemocytes. That was the case for presumptive vibrios from Guernsey and Conwy, and activities in the culture supernatants of isolates from Conwy were similar to those observed in Guernsey suggesting a similar bacterial population.

4.6 FURTHER WORK

Parallel studies in France were more successful in identifying specific pathogens causing disease in larval rearing. This was done either by passage of bacteria through fresh larval cultures and identification of those bacteria consistently associated with disease, or by testing the virulence of representaive bacteria from particular phenons revealed by taxonomic studies. Here, it would be of interest to return to the larval axenic culture system to test the virulence of particular isolates from Guernsey and Reculver. Also, for the haemocyte toxicity test it would be of importance to determine whether any correlation exists with virulence of the organisms. Bacteria isolated from larvae from turbot hatcheries were subjected to the haemocyte assay to determine whether the assay might have wider applicability in assessing whether cytotoxicity of these isolates was related to success in larval rearing trials. Good correlation was observed between survival rate of larval turbot and the absence of highly toxic bacteria. This has been pursued and preliminary tests with larval turbot reared in the presence of bacteria screened for haemocyte toxicity indicate that the test may have predictive value in differentiation of harmless and potentially harmful organisms. Thus, significantly greater survival of turbot larvae was recorded after exposure to bacteria of low haemocyte toxicity compared with larvae exposed to bacteria of high haemocyte toxicity (McLean, Munro and Birkbeck, personal communication). Further trials on both turbot and bivalve larvae are required to explore whether incorporation of such bacteria can have consistent benefits in larval rearing.

Further interesting avenues could be explored in the study of bacteria-host interations. These include the use of molecular biological approaches, such as 16S RNA gene sequencing using the Polymerase Chain Reaction for more rapid identification of bacteria. If combined with classical taxonomical techniques this would achieve better characterisation of the bacteria involved. Also, proper analysis of shellfish haemolymph factors (opsonins) would be worthwhile, especially when gene sequences can be elucidated to determine their relationship to other molecules of both invertebrate and vertebrate host defence.

Bacteria were isolated from bivalve hatcheries at Guernsey and Reculver (Kent), where the species *Crassostrea gigas, Ostrea edulis, Pecten maximus* and *Tapes decussatus/semidecussatus* are commercially produced, and from an experimental hatchery at Conwy (North of Wales). The bacteria were isolated during rearing of *Ostrea edulis* or *Crassostrea gigas* larvae, from hatchery water and algae samples, from Reculver (February to April 1991), Guernsey (January 1992) and Conwy (March 1991); during the time when vibriosis was suspected in both the Guernsey and Reculver hatcheries, but not at Conwy.

Identification of bacteria associated with bivalves was carried out using standard biochemical identification tests and cluster analysis (numerical taxonomy). Results of this study revealed the presence of bacteria principally related to three major bacterial groups (Vibrionaceae, Enterobacteriaccae and Cytophagaccae); the geographical distribution of these bacteria was found to be connected with their origin of isolation, vibrios being mainly found in Guernsey, whereas Reculver and Conwy shared diverse bacterial groups including Vibrionaceae, Enterobacteriaceae, Cytophagaceae, *Pseudomonas* spp., *Moraxella* spp. and *Micrococcus* spp.

Initial virulence of bacterial isolates was assessed using bivalve larval viability assays. *Pseudomonas* 1-1-1, isolated from seawater, had little or no effect on the shell growth and survival of oyster (*C. gigas*) larvae over a period of 3 days Whereas 90 % of the oyster larvae survived up to 48 hours in the presence of 1 or 10 c.f.u. of the potential bivalve pathogen *Vibrio anguillarum* NCIMB 6, there was a significant decrease thereafter in larval survival. Reduced shell growth was observed in larvae exposed to 10 c.f.u. (ratio bacteria/larvae) of NCIMB6. Experimental infections were performed with oyster (*C. gigas*) or scallop (*P. maximus*) larvae and a variety of French, Spanish and U.K. isolates. Certain isolates from Guernsey (G13) and Reculver (A10, A45) appeared to be virulent towards to 12 day old oyster larvae, whereas French (V322) and Spanish (GR86, GR82, BZ162, BZ168) isolates were

non-virulent to 13 day *C. gigas* larvae. Moderate virulence was observed in scallop larvae when exposed to A1 (Reculver) and G1 (Guernsey) after day 5 and 6 respectively, whereas V365 provoked mass mortalities after day 3. Control scallop larvae without antibiotics also began to die by this time.

Experimental infections were too time consuming for routine screening of large numbers of bacteria for potential virulence to bivalve larvae. Therefore, a cytotoxicity bioassay was developed based on the use of bivalve haemocytes, and this proved to be a rapid and reproducible laboratory test. Several approaches to assess the cytotoxicity of bacteria, using neutral red or molecular probes, were compared by spectrophotometry or direct visual analysis (direct microscopic observation) of the haemocytes in the presence of bacteria. Neutral red viability stain, XTT, calcein-Am and ethidium homodimer were used to detemine cell viability/death after haemocyte incubation with different bacteria suspended in filtered sea water. Although cells became rounded after interaction with many of the bacteria tested, the use of XTT, calcein-AM and ethidium homodimer indicated that these were still viable.

Direct microscopic observation of *Mytilus edulis* haemocytes in the presence of bacteria suspended in filtered haemolymph, showed an effect which was more pronounced and readily detected. Haemocytes were exposed to non-toxic bacteria (*Pseudomonas* 1-1-1) suspended in filtered seawater and filtered haemolymph behaved similarly to haemocytes not exposed to bacteria in that they spread, rounded up and re-spread continuously. However, haemocytes incubated with a potential pathogen, *Vibrio* 2981 soon ceased normal activity and remained rounded up for a prolonged time. The rate of cell rounding was dependent on bacterial concentration, the presence of filtered haemolymph and incubation time. Thus, when suspended in neat filtered haemolymph, 500, 50, 10, c.f.u. of V2981 induced 50 % haemocyte rounding after 90, 120, 150 minutes respectively. When the haemolymph was diluted up to 1/16 with filtered seawater the same effect was produced as with neat filtered

haemolymph but further dilutions caused a reduced effect towards the haemocytes. The kinetics of the reaction were observed and it was found that the number of V2981 bacteria per haemocyte required to cause an irreversible reaction, after data was normalised to take account of rounded haemocytes in the control, closely resembled those expected for a one-hit interaction from a Poisson Distribution. Similar results were obtained for Reculver isolate A7. Using this assay, the toxicity of standard pathogenic vibrios was assessed after 150 minutes incubation with *Mytilus* haemocytes. Greater than 70 % haemocyte rounding was observed with vibrios (V1337, V1338, V1339, V1340, V2164, V2165, V2166, V2981, V4679, V5679, V91079, VB1), approximately 50 % cytotoxicity with V1336 and VB2 but less than 30 % with V1197 and P1-1-1.

Haemocytes from different bivalve species (*Mytilus edulis, Mercenaria mercenaria, Pecten maximus, Tapes decussatus, Tapes semidecussatus, Ostrea edulis, Crassostrea gigas*) were tested and compared for their sensitivity to 9 selected different cytopathogenic bacteria. Haemocytes responded differently to the different bacteria tested, *M. edulis* haemocytes being the most readily affected by all bacteria. Assays using different haemocyte types also showed that isolates, V2981 and A7, obtained from *Ostrea edulis* were extremely toxic to all cell types, where as V110 although the least toxic of those bacteria tested showed specificity towards haemocytes of *O. edulis* to which it is a pathogen. *Pecten maximus* haemocytes were the most resistant to the test bacteria with the exception of one *Pecten maximus* isolate, V365. Isolates Vp1 (isolated from *Tapes decussatus*), V1339 (from *Mercenaria mercenaria*) and V365 (from *Pecten maximus*) were relatively virulent to the majority of the test hamocytes.

Screening for bacterial haemocyte toxicity among the hatchery isolates (Guernsey, Reculver and Conwy) revealed that a large number of isolates, mainly belonging to Vibrionaceae and *Cytophaga/Flavobacterium* caused highest levels of

rounding of *M. edulis* haemocytes. From Guernsey, 90 % of the isolates were vibrios and 35 % of all the strains tested were moderately toxic to *Mytilus* haemocytes. Presumptive *V. anguillarum* (G3, G5, G8, G20, G25) and *V. alginolyticus* (G7, G15) produced highest toxicity to *Mytilus* haemocytes whereas tentative-identified *V. tubiashii* produced different levels of toxicity. From Reculver, presumptive *Cytophaga/Flavobacterium* spp. and *Vibrio* spp. were highly toxic to the haemocytes when compared to other bacterial groups such as Enterobacteriaceae, *Vibrio* spp., *Micrococcus* spp., *Moraxella* spp. and *Pseudomonas* spp. Vibrionaceae were the most dominant bacterial group isolated from Reculver. Few bacterial isolates from Conwy showed toxicity to the haemocytes; these bacteria were tentatively identified as *Vibrio* spp. and *Cytophaga/Flavobacterium* spp.

Several experiments were carried out to determine the effect of temperature, peptone concentration and lipopolysaccharide on the toxicity of *V. anguillarum* 2981 to *Mytilus* haemocytes. Maximum cytotoxicity was observed during an incubation temperature between 10 and 20 ^{o}C, whereas poor haemocyte viability resulted at 37 ^{o}C, and the bacterial cytotoxic effect was reduced at lower temperatures. Culture in nutrient-deficient medium or low peptone concentration also produced a reduced toxic effect caused by V2981. Filtered haemolymph plus partially purified lipopolysaccharide induced a high degree of haemocyte rounding. SDS-polyacrylamide gel analysis of filtered haemolymph revealed two protein bands 41 and 70 kDa approximately. Trypsin treatment of the filtered haemolymph decreased the toxicity of V2981 to *Mytilus* haemocytes, and the effect was dose related. Pre-absorption of filtered haemolymph with V2981 for 24h reduced the toxicity towards *Mytilus* haemocytes by 40 %.

The cytotoxicity of *V. anguillarum* (V2981) was related to bacterial numbers and was possibly due to production of a secreted toxin (s) of molecular weight < 10,000 Da.

Further analysis of the supernates from a selected group of Reculver and Conwy isolates, showed different degrees of toxicity to the haemocytes; A65 and V2981 were highly toxic at neat and 1/2 dilution where as A79, A61, A63 and A64 were only toxic when tested neat. Determination of ciliostatic toxin (CT) and proteases in bacterial culture supernates revealed that both were produced by many of the bacteria isolated from all three locations. A high percentage of Guernsey isolates, *Vibrio alginolyticus,Vibrio anguillarum,* and *V. tubiashii* to a lesser extent produced CT and proteases.

A comparative study was carried out using bacterial isolates from turbot larval rearing trial. Of the various bacterial isolates tested (Vibrionaceae, Aeromonas, Enterobacteriaceae and *Pseudomonas/Alcaligenes),* those identified as *Vibrio* spp. produced higher cytotoxic effects to *Mytilus* haemocytes. Among those isolates were *V. alginolyticus* and *V. splendidus.* It was also observed that the most toxic bacterial isolates were associated with turbot larvae which had the lowest survival rate. Bacteria from other larval trial isolates from Scotland, Spain and Norway were tested for toxicity to *Mytilus* haemocytes to determine the relative toxicity of these bacteria and the most toxic isolates were those isolated from incidences of disease.

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Appendices

Appendix I : Tables of identification and origin of bacterial isolates.

APPENDIX I.1: Identification and origin of bacterial isolates from Guernsey.

Isolate code	Identification	Percent identified	Origin
G1	*V. tubiashii*	97	Quarry water
G3	*V. anguillarum*	77	Quarry water
G3a	*A. salmonicida*	52	Quarry water
G4	*A. sobria*	41	Quarry water
G5	*V. anguillarum*	99	Quarry water
G7	*V. alginolyticus*	57	Sea water
G8	*V. anguillarum*	99	Sea water
G9	*V. alginolyticus*	99	Quarry water
G10	*V. tubiashii*	99	Sea water
G12	*V. alginolyticus*	99	Untreated larvae*
G13	*V. alginolyticus*	94	Untreated larvae
G15	*V. alginolyticus*	98	Treated larvae
G16	*V. alginolyticus*	75	Treated larvae
G17	*V. alginolyticus*	91	Treated larvae
G18	*V. alginolyticus*	92	Treated larvae
G19	*V. alginolyticus*	70	Treated larvae
G20	*V. anguillarum*	99	Quarry water
G22	*V. tubiashii*	94	Quarry water
G23	*V. anguillarum*	99	Quarry water
G24	*V. alginolyticus*	86	Sea water
G25	*V. furnissii*	77	Sea water
G26	*V. alginolyticus*	98	Sea water

* Larvae : *Crassostrea gigas*
Isolation : 1/92

APPENDIX I.2 : Identification and origin of bacterial isolates from Reculver.

Isolate code	Identification	Percent identified	Origin
A1	*V. anguillarum*	100	Untreated larvae 26-2-91
A2	*Moraxellaceae*		Untreated larvae 26-2-91
A3	*Pseudomonas*		Untreated larvae 26-2-91
A5	*Pseudomonas*		Hatchery water 26-2-91
A6	*Pseudomonas*		Hatchery water 26-2-91
A7	*V. anguillarum*	100	Hatchery water 26-2-91
A8	*Enterobacteriaceae*		Treated-larvae * 26-2-91
A9	*V. neresis*	99	Treated-larvae * 26-2-91
A10	*V. neresis*	93	Treated-larvae * 26-2-92
A11	*V. neresis*	94	Untreated larvae 26-2-91
A12	*Phenon 36*	100	Treated-larvae 26-2-91
A13	*V. neresis*	94	Hatchery water 26-2-91
A14	*V. anguillarum*	97	Untreated-larvae 26-2-91
A15	*Enterobacteriaceae*		Treated-larvae 26-2-91
A16	*Pseudomonas*		Hatchery water 10-3-91
A17	*Cytophaga/Flavobacterium*		Hatchery water 10-3-91
A18	*Cytophaga/Flavobacterium*		Hatchery water 10-3-91
A19	*Pseudomonas*		Hatchery water 10-3-91
A20	*Pseudomonas*		Hatchery water 10-3-91
A22	*Cytophaga/Flavobacterium*		Untreated larvae 10-3-91
A23	*Pseudomonas*		Untreated larvae 10-3-91
A24	*Moraxellaceae*		Untreated larvae 10-3-91
A25	*V. parahaemolyticus*	100	Untreated larvae 10-3-91
A26	*Pseudomonas*		Treated-larvae 10-3-91
A27	*Cytophaga/Flavobacterium*		Treated-larvae 10-3-91
A28	*Cytophaga/Flavobacterium*		Treated-larvae 10-3-91
A30	*Pseudomonas*		Treated-larvae 10-3-91
A32	*Cytophaga/Flavobacterium*		Treated-larvae 10-3-91
A33	*Enterobacteriaceae*		Hatchery water 10-3-91
A34	*Pseudomonas*		Hatchery water 10-3-91
A35	*V. parahaemolyticus*	100	Hatchery water 10-3-91
A36	*Cytophaga/Flavobacterium*		Hatchery water 10-3-91
A37	*V. parahaemolyticus*	44	Untreated larvae 10-3-91
A39	*Cytophaga/Flavobacterium*		Untreated larvae 10-3-91
A40	*Micrococcus*		Untreated larvae 10-3-91

**Treated larvae were exposed to benzalkonium chloride before bacterial analysis.
Larvae: *Ostrea edulis*

APPENDIX I.3 : Identification and origin of bacterial isolates from Reculver.

Isolate code	Identification	Percent identified	Origin
A41	*Micrococcus*		Untreated larvae 19-3-91
A42	*Cytophaga/Flavobacterium*		Treated-larvae * 19-3-91
A43	*Cytophaga/Flavobacterium*		Treated-larvae 19-3-91
A44	*Pseudomonas*		Treated-larvae 19-3-91
A45	*V. fluvialis*	98	Treated-larvae 19-3-91
A46	*Cytophaga/Flavobacterium*	82	Untreated larvae 19-3-91
A48	*Cytophaga/Flavobacterium*		Untreated larvae 19-3-91
A49	*V. harveyii*	65	Untreated larvae 19-3-91
A50	*V. fluvialis*	99	Untreated larvae 19-3-91
A51	*Acinetobacter*		Untreated larvae 19-3-91
A53	*Pseudomonas*		Hatchery water 19-3-91
A54	*V. alginolyticus*	99	Hatchery water 19-3-91
A56	*Enterobacteriaceae*		Hatchery water 19-3-91
A57	*Cytophaga/Flavobacterium*		Hatchery water 19-3-91
A59	*V. neresis*	100	Hatchery water 19-3-91
A60	*Micrococcus*		Hatchery water 23-4-91
A61	*Cytophaga/Flavobacterium*		Hatchery water 23-4-91
A63	*V. tubiashii*	94	Hatchery water 23-4-91
A64	*V. neresis*	99	Untreated larvae 23-4-91
A65	*Cytophaga/Flavobacterium*		Untreated larvae 23-4-91
A66	*Enterobacteriaceae*		Untreated larvae 23-4-91
A68	*V. pelagius*	98	Treated-larvae 23-4-91
A71	*V. alginolyticus*	86	Treated-larvae 23-4-91
A72	*Cytophaga/Flavobacterium*		Treated-larvae 23-4-91
A73	*Cytophaga/Flavobacterium*		Treated-larvae 23-4-91
A74	*Moraxellaceae*		Treated-larvae 23-4-91
A87	*Enterobacteriaceae*		Algae culture
A88	*Cytophaga/Flavobacterium*		Algae culture
A89	*Moraxellaceae*		Algae culture
A90	*Moraxellaceae*		Algae culture
A91	*Pseudomonas*		Algae culture
A93	*Pseudomonas*		Algae culture
A94	*Pseudomonas*		Algae culture
A96	*V. anguillarum*	75	Algae culture

* Larvae were pre-treated with benzalkonium chloride before bacterial analysis.
Larvae: *Ostrea edulis*

APPENDIX I.4: Identification and origin of bacterial isolates from Conwy.

Isolate code	Identification	Percent identified	Origin
A75	*Cytophaga/Flavobacterium*		*Ostrea edulis*
A76	*Cytophaga/Flavobacterium*		*Ostrea edulis*
A77	*Moraxellaceae*		*Ostrea edulis*
A78	*Cytophaga/Flavobacterium*		*Ostrea edulis*
A79	*Cytophaga/Flavobacterium*		*Crassostrea gigas*
A80	*Pseudomonas*		*Crassostrea gigas*
A81	*Cytophaga/Flavobacterium*		*Crassostrea gigas*
A82	*Vibrio sp.*		*Crassostrea gigas*
A83	*Vibrio sp.*		*Crassostrea gigas*
A85	*Enterobacteriaceae*		*Crassostrea gigas*
A86	*V. alginolyticus*	100	*Ostrea edulis*

Isolated :- 23/4/91

APPENDIX I.5 : Identification and toxicity of group R bacterial isolates from turbot.

Isolate code	Identification	Percent identified	Toxicity
R1	*V. parahaemolyticus*	65	low
R2	*V. parahaemolyticus*	65	low
R3	*V. parahaemolyticus*	65	low
R4	*A. sal. achr. mal*	65	medium
R5	*V. campbellii*	96	medium
R6	*V. campbellii*	96	medium
R8	*V. ordallii*	99	medium
R11	*V. campbellii*	71	low
R12	*V. campbellii*	100	medium
R13	*V. parahaemolyticus*	65	low
R14	*Enterobacteriaceae*	-	low
R15	*Enterobacteriaceae*	-	low
R16	*Enterobacteriaceae*	-	low
R18	*Phenon 6 (35)*	32	low
R19	*A. salmonicida*	100	low
R20	*Phenon 6 (35)*	92	low
R21	*V. campbellii*	55	low
R23	*V. splendidus 11*	99	low
R24	*V. splendidus 11*	66	low
R25	*A. salmonicida*	34	low
R27	*A. salmonicida*	48	low
R29	*A. salmonicida*	48	low
R32	*V. campbellii*	57	low
R35	*V. costicola*	43	low
R36	*V. splendidus 1*	63	low
R37	*V. splendidus 1*	63	low
R38	*V. anguillarum*	99	low
R39	*A. salmonicida*	99	low
R40	*A. sal. achr. mal.*	33	medium
R42	*V. campbellii*	40	low
R44	*A. salmonicida*	43	low
R45	*A. salmonicida*	43	low
R46	*V. campbellii*	40	low
R47	*A. salmonicida*	43	low
V2981	*V. anguillarum*	-	high
Control	Pseudomonas 1-1-1	-	low

** Many of the haemocytes displaying low toxicity were highly vesiculated and these vesicles contained bacteria

APPENDIX I.6 : Identification and toxicity of group ER bacterial isolates from turbot.

Isolate code	Identification	Percent identified	Toxicity
ER1	*V. campbellii*	59	low
ER3	*V. splendidus 11*	97	low
ER4	*V. splendidus 11*	99	low
ER5 (L)*	*A. salmonicida*	73	medium
ER5 (S)*	*A. salmonicida*	73	medium
ER6	*V. alginolyticus*	98	medium
ER8 (L)*	*V. alginolyticus*	96	medium
ER8 (S)*	*V. alginolyticus*	96	medium
ER9	*V. alginolyticus*	96	medium
ER10	*V. parahaemolyticus*	65	medium
ER12	*V. campbellii*	61	medium
ER13	*V. parahaemolyticus*	65	low
ER14	*V. parahaemolyticus*	76	low
ER15	*V. alginolyticus*	66	low
ER16	*V. pelagius*	54	low
ER17 (L)*	*A. sobria*	80	medium
ER17 (S)*	*A. sobria*	80	medium
ER18	*V. alginolyticus*	39	medium
ER21	*A. sobria*	33	high
ER23	*V. alginolyticus*	96	high
ER24	*V. alginolyticus*	96	high
ER25	*V. anguillarum*	53	medium
ER26	*A. sobria*	33	medium
ER27	*V. alginolyticus*	39	medium
ER28	*A. sobria*	33	medium
ER31	*V. parahaemolyticus*	76	medium
ER32	*V. alginolyticus*	96	high
ER33	*V. tubiashii*	37	medium
ER34	*V. campbellii*	36	medium
ER36	*A. salmonicida*	76	medium
ER39	*V. parahaemolyticus*	88	medium
ER40	*V. parahaemolyticus*	88	medium
ER41	*A. salmonicida*	43	medium
ER43	*A. salmonicida*	86	medium
ER44	*A. sobria*	61	low
ER45	*V. campbellii*	96	medium
ER47	*V. parahaemolyticus*	76	low
ER49	*V. parahaemolyticus*	76	medium
ER50	*A. salmonicida*	86	medium
ER51	*A. salmonicida*	86	medium
V2981	V. anguillarum	-	high
Control	Pseudomonas 1-1-1	-	low

* (L):-Large colony, *(S):- Small colony.

APPENDIX I.7 : Identification and toxicity of group IR bacterial isolates from turbot.

Isolate code	Identification	Percent identified	Toxicity
IR1	*V. alginolyticus*	62	high
IR2 (L)*	*V. alginolyticus*	99	medium
IR2 (S)*	*V. alginolyticus*	99	medium
IR3	*V. alginolyticus*	54	medium
IR4	*Phenon 36*	100	medium
IR5	*V. alginolyticus*	95	high
IR6	*V. campbellii*	87	medium
IR7	*V. campbellii*	87	medium
IR8 (R)*	*V. parahaemolyticus*	87	low
IR8 (Sm)*	*V. parahaemolyticus*	87	medium
IR10 (L)*	*V. alginolyticus*	96	low
IR10 (S)*	*V. alginolyticus*	96	low
IR11	*V. campbellii*	87	medium
IR12	*V. parahaemolyticus*	65	low
IR13	*V. campbellii*	87	medium
IR14	*V. parahaemolyticus*	78	low
IR17	*V. parahaemolyticus*	76	medium
IR18	*V. alginolyticus*	96	low
IR19	*V. parahaemolyticus*	80	low
IR20	*V. campbellii*	80	low
IR21	*V. alginolyticus*	61	low
IR22	*V. alginolyticus*	39	medium
IR24	*Suc. Aer*	41	medium
IR26	*Suc. Aer*	63	low
IR27	*A. sobria*	33	medium
IR28	*V. alginolyticus*	54	low
IR30	*A. sobria*	33	medium
IR31	*V. alginolyticus*	39	low
IR32	*V. parahaemolyticus*	88	low
IR33	*V. alginolyticus*	73	medium
IR34	*V. alginolyticus*	96	medium
IR35	*Pseudomonas/Alcaligenes*	-	medium
IR37	*V. anguillarum*	82	medium
IR38	*Suc. Aer*	70	low
IR39 (L)*	*V. alginolyticus*	73	medium
IR39 (S)*	*V. alginolyticus*	73	medium
IR42	*V. pelagius*	64	medium
IR46	*V. alginolyticus*	98	medium
IR46 (b)*	*V. alginolyticus*	98	high
IR47	*V. splendidus 1*	93	medium
IR48	*Phenon 6*	99	medium
IR50	*A. sobria*	40	low
V2981	*V. anguillarum*	-	high
Control	Pseudomonas 1-1-1	-	low

*Colony types (L): Large, (S) small, (R) rough, (Sm) smooth.

Appendix II : Materials and methods supplement.

IDENTIFICATION TESTS.

Acid From Carbohydrates (Cowan and Steel, 1965)

Phenol red broth:

NaCl	10 g
Peptone water (Oxoid)	10 g
Phenol red	0.018 g
Distilled water	1000 ml

The pH was adjusted to pH 7.1 and the medium was autoclaved at 121 ^{o}C for 15 minutes. Filtered sterilised (0.2 µm millipore filtered) carbohydrate was added aseptically to a final concentration of 1 % for arbutin and sucrose, or 0.5 % for salicin. Each medium was dispensed in 1 ml amounts into sterile tubes, inoculated and incubated at 20 ^{o}C for up to 14 days. A yellow colour indicated acid production. Alkali production was indicated by a pink colour. A red orange colour was regarded as a negative.

Aesculin Hydrolysis (Lee and Donovan, 1985)

Aesculin agar:

Aesculin	1 g
Ferric citrate	0.5 g
NaCl	10 g
Technical agar No. 3 (Oxoid)	15 g
Tryptone (Oxoid)	10 g
Distilled water	1000 ml

Agar, tryptone and NaCl were dissolved by heating. Then the aesculin and ferric citrate were added. The medium was autoclaved at 115 ^{o}C for 10 min. and poured into petri dishes. The plates were spot inoculated with no more than 12 isolates per plate and incubated at 20 ^{o}C for 5 days. Blackening indicated aesculin hydrolysis.

Amylase (Cowan and Steel, 1965)

Starch agar:

Potato starch	10 g
NaCl	10 g
Distilled water	50 ml
Nutrient agar	1000 ml

Lugol's iodine solution:

Iodine	5 g
KI	10 g
Distilled water	100 ml

The starch was mixed with water to a smooth cream and added to molten nutrient agar supplemented with NaCl before autoclaving at 115 ^{o}C for 10 min. After cooling, the agar was poured into petri dishes. The plates were spot inoculated and incubated at 20 ^{o}C for 5 days then flooded with Lugol's iodine solution. A clear zone around the colony indicated a positive result.

Dihydrolase / Decarboxylase Test (Furniss *et al.*, 1979)

Decarboxylase medium (Difco) was prepared with the addition of 1 % NaCl, 0.4 % $MgCl_2.H_20$ and 0.4 % KCl. The medium was distributed into 4 x 100 ml amounts amounts and the amino acids L-Arginine, L-Lysine and L-Ornithine were added to final concentrations of 1 % (w/v). The 3 amino acid media and the basal medium were dispensed into 5 ml screw-capped bottles and covered with a layer of liquid paraffin before autoclaving at 121 ^{o}C for 15 min.

The 4 bottles were inoculated through the liquid paraffin, with a heavy inoculum from growth on solid medium and incubated at 20 ^{o}C for 4 days. Vibrios should show acid (yellow colour) in the blank. A positive result was indicated by an alkaline reaction (purple colour).

Elastase (Cowan and Steel, 1965)

Elastin overlay:

Tris	0.363 g
$CaCl_2.2H_2O$	0.558 g
Distilled water	100 ml
Elastin	1 g
Pure agar	2 g

The Tris and $CaCl_2.2H_2O$ were prepared in distilled water and the pH was adjusted to pH 8.0. To this solution, elastin and pure agar were added and sterilised by autoclaving at 121 ^{o}C for 15 min. The overlay was then poured onto marine agar plates. Afterwards the plates were spot inoculated with no more than 12 isolates per plate. The plates were incubated at 20 ^{o}C for approximately 28 days. A clear zone around the colony indicated a positive result.

Gelatinase (Cowan and Steel, 1965)

Gelatin agar:

Distilled water	50 ml
Gelatin	4 g
NaCl	10 g
Nutrient agar (Oxoid)	1000 ml

Acid mercuric chloride solution:

Mercuric chloride	12 g
Distilled water	80 ml
HCl	16 ml

Mercuric chloride was mixed with water before addition of acid. Gelatin was dissolved in distilled water and added to the nutrient agar supplemented with NaCl. The medium was autoclaved at 121 ^{o}C for 15 min. the poured into petri dishes. The

plates were then spot inoculated and incubated at 20 °C for 3 days. Gelatin hydrolysis was evident after the plate surface was flooded with mercuric chloride solution and clear zones appeared around the inoculated spot.

Haemolysis of Sheep Red Blood Cells (Furniss *et al.*, 1979)

Brain heart thioglycolate cysteine agar:

Brain heart infusion broth (Oxoid)	500 ml
10 % L-cysteine	2.5 ml
10 % Sodium thioglycolate solution	2.5 ml
Technical agar No. 3 (Oxoid)	5 g

The medium was autoclaved at 121 °C for 15 min., cooled to 51 °C and 20 ml of fresh 20 % saline suspension of washed sheep red blood cells was added. Plates were spot inoculated (not more than 12 isolates per plate) and incubated at 20 °C for 24 h. The presence of haemolysis was indicated by a clear zone around the colony.

Indole Production (Cowan and Steel, 1965)

Kovac's reagent:

p-Dimethylaminobenzaldehyde	5 g
Isoamyl alcohol	75 ml
HCl	25 ml

The aldehyde was dissolved in isoamyl alcohol by warming to 50 °C, cooled and the acid added. The reagent was stored in the dark at 4 °C. A few drops of Kovac's reagent were added to 1 ml of a 24 h culture in 1 % tryptone (Oxoid) supplemented with 2 % NaCl. A positive result was indicated by a red ring on the surface.

Lecithinase (Furniss *et al.*, 1979)

Lecithin agar:

Egg yolk emulsion	10 ml
NaCl	10 g

Nutrient agar (Oxoid)	1000 ml

The nutrient agar supplemented with NaCl was autoclaved at 121 °C for 15 min., cooled to 51 °C and egg yolk emulsion was added before pouring into petri dishes. The plates were spot inoculated and incubated at 20 °C for 5 days. An opaque zone around the colony was regarded as a positive result.

Nitrate Reduction (Lee *et al.*, 1979)

Nitrate broth:

KNO_3	1 g
NaCl	10 g
Nutrient broth (Oxoid)	1000 ml

Solution A:

Sulphuric acid	0.5 g
Glacial acetic acid	30 ml
Distilled water	120 ml

Solution B:

1-Naphthylamine-7 sulphonic acid	0.2 g
Glacial acetic acid	30 ml
Distilled water	120 ml

Nitrate broth was distributed in 1.0 ml amounts into tubes and autoclaved at 121 °C for 15 min. Inoculated broth was incubated at 20 °C for 4 days. Solution A (1 ml) was added followed by 1 ml of solution B; a red colour was indicative of nitrate reduction to nitrite. Powdered zinc (up to 5 mg per ml) was added to tubes not showing a red colour, if a red colour appeared, then nitrate was present in the medium (i.e. no reduction), whereas no red colour indicated the reduction of nitrate to nitrite.

ONPG (*0*-Nitrophenyl-*β*-D-galactopyranoside) (Cowan and Steel, 1965)

ONPG solution:

ONPG (Koch-Light Laboratories Ltd.)	6 g
0.01 M Na_2HPO_4	1000 ml

Test solution:

ONPG solution	250 ml
Sterile peptone water with 1 % NaCl (Oxoid)	750 ml

ONPG was dissolved in the phosphate solution at pH 7.5 at room temperature, sterilised by filtration and stored in the dark. ONPG solution (250 ml) was added to 750 ml peptone water and distributed into 1ml amounts. The inoculated medium was incubated at 20 ^{o}C for 24 h. A deep yellow colour indicated a positive result (*β*-galactosidase activity).

Oxidase Test (Furniss *et al.*, 1979)

A few crystals of tetramethyl-p-phenylene diamine hydrochloride were dissolved in 10 ml distilled water and used to soak filter paper. A small portion of colony was rubbed onto the paper. The appearance of a violet colour within a few seconds indicated a positive result. (Note: A nichrome wire should not be used for this test, glass, platinum or wood are satisfactory)

Oxidation/Fermentation

Hugh & Leifson (1953) medium with 1.5 % NaCl was prepared in 5 ml amounts and sterilised by autoclaving at 121 ^{o}C for 15 min. To this medium 10 % filtered sterilised Bacto Dextrose (D-glucose) was added. The tubes were prepared in duplicate, one set with liquid paraffin and the other without. The tubes were inoculated and incubated at 20 ^{o}C for 48 h.

Utilisation of Single Carbon Sources (Baumann *et al.*, 1971)

Artificial sea water:

$CaCl_2.2H_2O$	0.02 M
KCl	0.02 M
$MgSO_4.7H_2O$	0.1 M
NaCl	0.4 M
Distilled water	1000 ml

Basal medium:

$FeSO_4.7H_2O$	0.1 mM
$K_2HPO_4.3H_2O$	0.33 mM
NH_4Cl	190 mM
Tris-HCl (pH 7.5)	50 mM
Half strength artificial sea water	1000 ml

Basal medium agar was prepared by separately sterilising, then mixing, equal volumes of double-strength basal medium and 20 gl^{-1} purified agar (Oxoid), then distributed into 200 ml amounts. Each test substance was dissolved in 10 ml distilled water, filtered-sterilised and added to 200 ml of basal medium agar to give a final concentration of 0.1 %. The media was poured into petri dishes and the isolates were replica plated (no more than 12 isolates per plate).

Voges-Proskauer Test (Furniss *et al.*, 1979)

Voges-Proskauer (V-P) semi-solid medium:

Bacteriological peptone (Oxoid)	12 g
Glucose	10 g
NaCl	10 g
Technical agar (Oxoid)	3 g
Yeast extract (Oxoid)	1 g
Distilled water	1000 ml

Solution A:

5 % α-naphthol in absolute alcohol, protected from light and stored at 4 °C.

Solution B:

Creatine	0.3 g
KOH	40 g
Distilled water	100 ml

The pH was adjusted to pH 7.0 and the V-P medium was distributed into 3 ml amounts. the medium was inoculated by stabbing and incubated at 20 °C for 24 h. Then 0.2 ml of solution A and 0.1 ml of solution B were added. Results were read after 15 min. and a red ring at the surface of the agar indicated a positive result.

Xanthine Decomposition

Xanthine	4 g
Marine agar in distilled water	1000 ml

Xanthine was added to the marine agar and autoclaved at 121 °C for 15 min. The plates were spot inoculated with up to 12 isolates and incubated at 20 °C for up to 28 days. A clear zone indicated xanthine decomposition (a positive result).

POLYACRYLAMIDE GEL ELECTROPHORESIS (Laemmli 1970)

Stock Solutions:

Acrylamide / Bis:

Acrylamide (Highly toxic)	30 g
N, N-bis-methylene acrylamide	0.8 g
Distilled water	100 ml

Lower buffer:

Tris	18.1 g
SDS	0.4 g
Distilled water	70 ml

Adjust pH to 8.8 with concentrated HCl and make up the final volume to 100 ml with distilled water.

Upper buffer:

Tris	6.06 g
SDS	0.4 g
Distilled water	70 ml

Adjust pH to 6.8 with concentrated HCl and make up the final volume to 100 ml with distilled water.

Solubilizing buffer for proteins:

Glycerol	10 ml
2-mercaptoethanol	5 ml
SDS	3 g
Bromophenol blue	0.01 g
Upper buffer (1 in 4 dilution)	100 ml

Mix the sample and solubilising buffer in a 1:1 ratio and boil for 5 min. then load or store at - 20 ^{o}C for less than one week or at -70 ^{o}C for less than a month.

Running buffer:

Tris	6.06 g
Glycine	28.8 g
SDS	2 g

Adjust pH to 8.3 with concentrated HCl.

Distilled water	2000 ml

Slab-Gel Preparation:

Lower separating gel:

	12.5 %	10 %
Lower buffer	10 ml	10 ml
Distilled water	13.4 ml	16.6 ml
Acrylamide/bis	16.6 ml	13.4 ml

After degassing for 20 min. the following were added:

Ammonium persulfate	200 µl	200 µl
(10 % freshly prepared in distilled water)		
Temed (undiluted)	20 µl	20 µl

Adjust to pH 8.8 with concentrated HCl.

Upper stacking gel (4.5 %):

Upper buffer	2.5 ml
Distilled water	6 ml
Acrylamide / bis	1.5 ml

After degassing the following were added:

Ammonium persulfate (10 %)	30 µl
Temed	20 µl

Adjust to pH 6.8 with concentrated HCl.

STERILITY AGENTS

Iodine Solution:

KI	3 g
I_2	2 g
Distilled water	50 ml

The KI was dissolved in a little of the distilled water, then the iodine was added and the volume was made up to 50 ml with distilled water. The working solution was diluted 1:100 for use.

ALGAL CULTURE MEDIUM

Filtered sterilised sea water	10 litres
Stock solution A	10 ml
Vitamin solution	1ml

Stock solution A:

$FeCl_3.6H_2O$	2.6 g
$MnCl_2.4H_2O$	0.72 g
H_3BO_3	67.2 g
disodium EDTA	90.0 g
$NaH_2PO_4.2H_2O$	40.0 g
$NaNO_3$	200.0 g
Trace metal solution	2 ml
Distilled water to	2 litres

Trace metal solution:

$ZnCl_2$	21 g
$CoCl_2.6H_2O$	2 g
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.9 g
$CuSO_4.5H_2O$	2 g
Distilled water to 100 ml	

Vitamin solution:

Either		*or*	
Vitamin B_{12}	10 mg	Bacto Yeast extract	1 g
Vitamin B_1	200 mg	Distilled water to 100 ml	
Distilled water to	200 ml		

Vitamin solution should be filtered sterilised whereas the stock A solution and the filtered sea water should be sterilised at 10 psi for 20 minutes.

SEM FIXATIVE AND BUFFER.

Solution A:

HCL	0.2N	15.0 ml	
Na cacodylate		21.4 g	(*Highly toxic, handle with care*).
NaCl		10.0 g	
$CaCl_2.H_2O$		0.147 g	
Glutaraldehyde	50%	10.0 ml	
Distilled water		250 ml	

Buffer was adjusted to pH 7.4.

Solution B: Osmium tetroxide (OSO_4), 0.5 g in 25 ml of distilled water.

Acetone solutions: 100%, 90%, 70%, 50%, 30%.

Fixative: 0.7 ml of Solution A and 0.7 ml of solution B

Buffer: Solution A without the addition of glutaraldehyde.

Appendix III : Tables of biochemical and physiological characteristics of bacterial isolates.

APPENDIX III.1: Biochemical and physiological characteristics of bacterial isolates from Guernsey.

Test number	Characteristic	Isolate code																							
		G1	G2	G3	G3a	G4	G5	G7	G8	G9	G10	G12	G13	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24	G25	G26
1	Growth on TCBS medium	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0
2	Gram Stain	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	Oxidation / Fermentation of Glucose **	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0
4	Pigment	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	Motility	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6	Swarming	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	1	0	1	1	0	1	0	0
7	Growth on CLED medium	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
8	Arginine Dihydrolases	0	1	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0	1	0	0	1	0	1	1
9	Lysine Decarboxylases	0	1	0	1	1	0	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	0	1	1
10	Ornithine	0	1	0	1	1	0	1	1	1	0	1	1	1	1	1	1	1	0	0	0	1	1	0	1
11	Nitrate Reduction	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
12	Oxidase Test	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
13	Indole Production	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
14	ONPG Hydrolysis	1	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	1	1	0	0	0
15	Voges Proskauer Test	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	1	0	1	0	0	1	0	0
16	Resistance to: O/129 10 µg	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	0	1	0	1	1	1
17	O/129 150 µg	0	0	0	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1
18	Ampicillin 10 µg	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0
19	Aesculin Hydrolysis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	Production of enzymes: Gelatinase	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
21	Lecithinase	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
23	Amylase	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
24	Haemolysis of sheep red blood cells	0	0	0	0	1	0	0	0	1	0	1	1	1	1	1	1	1	0	1	0	0	1	0	0
25	Acid from carbohydrates: Arbutin	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0
26	Salicin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
27	Sucrose	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
28	Utilization of single carbon sources: L-arabinose	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1	0	0	1	1	1
29	D-cellobiose	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0
30	D-galactose	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
31	D-melibiose	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0
32	D-gluconate	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1
33	D-glucuronate	0	0	1	0	0	1	0	0	1	0	1	1	0	0	0	0	0	1	0	0	1	0	0	0
34	L-citrulline	0	0	0	0	0	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
35	D-glucosamine	0	0	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	0	0	1	1	0
36	DL-3-hydroxybutyrate	0	0	0	0	1	0	0	0	1	0	1	0	0	1	1	1	1	0	1	1	0	1	1	0
37	Succinate	0	0	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1	0	1	0	0	1	1	1

* Positive: 1 *Negative: 0 **Fermentative: 1 **Oxidative: 0

APPENDIX III.2 : Biochemical and physiological characteristics of bacterial isolates from Reculver.

Test number	Characteristic	Isolate code																										
		A1	A2	A3	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15	A16	A17	A18	A19	A20	A22	A23	A24	A25	A26	A27	A28	A30
1	Growth on TCBS medium	1	0	0	0	1	1	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	Gram Stain	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	Oxidation / Fermentation of Glucose **	1	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0
4	Pigment	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	1	1	1	0
5	Motility	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1
6	Swarming	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Luminescence	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	0
7	Growth on CLED medium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	Arginine Dihydrolases	1	0	0	0	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	0	0	1	1	0	0	0	1
9	Lysine Decarboxylases	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	1	1	0	0	0	1
10	Ornithine "	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	1
11	Nitrate Reduction	1	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0	0	1	0	0	0	0	1	1	0
12	Oxidase Test	1	1	1	0	1	1	0	0	0	0	1	0	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1
13	Indole Production	0	1	1	1	1	0	1	1	1	1	0	1	1	0	1	1	1	0	0	1	0	0	1	0	1	1	1
14	ONPG Hydrolysis	1	1	0	0	0	1	1	1	0	1	1	1	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0
15	Voges Proskauer Test	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	Resistance to: O/129 10 µg	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17	O/129 150 µg	1	1	1	1	1	0	1	0	0	0	1	0	0	1	1	1	0	1	1	0	0	1	1	1	0	0	1
18	Ampicillin 10 µg	1	0	1	0	1	1	1	1	0	0	1	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0	1
	Polymyxin B 50 i.u.	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	0
19	Aesculin Hydrolysis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Production of enzymes: Elastase	1	0	0	1	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	1
20	Gelatinase	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1	0	1	1	0	1	1	1
21	Lecithinase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23	Amylase	1	0	1	1	1	1	1	0	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1
	Xanthine Decomposition	1	0	0	0	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	Haemolysis of sheep red blood cells	1	0	0	0	0	1	1	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25	Acid from carbohydrates: Arbutin	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26	Salicin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
27	Sucrose	0	1	0	0	0	0	1	1	0	0	0	1	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0
28	Utilisation of single carbon sources: L-arabinose	1	1	0	0	1	1	1	0	1	1	0	0	1	1	1	1	0	0	1	0	1	1	1	0	0	0	1
29	D-cellobiose	1	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1	0	1	1	0	1	0	1	1	0	0	1
30	D-galactose	0	1	1	0	1	1	0	0	1	0	1	0	1	1	1	1	0	1	1	0	1	0	1	1	0	0	1
31	D-melibiose	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1	0	1	0	1	0	0	0	1
32	D-gluconate	1	0	1	0	1	1	0	1	0	1	1	1	1	1	1	1	0	0	1	0	1	0	1	0	0	0	0
33	D-glucuronate	0	0	0	0	0	0	1	1	1	1	1	0	1	1	1	1	0	1	1	0	1	0	1	1	0	0	0
34	L-citrulline	0	1	1	0	0	0	0	1	1	1	0	0	0	0	1	1	0	1	1	0	1	1	1	1	0	0	1
	L-leucine	0	1	1	0	1	1	1	1	1	0	0	0	0	0	1	1	0	1	1	0	1	1	1	1	0	0	1
35	D-glucosamine	1	0	0	0	1	1	0	1	1	1	0	1	1	1	1	1	0	1	1	0	1	1	1	1	0	0	0
36	DL-3-hydroxybutyra	1	0	1	0	0	1	1	1	1	1	1	0	1	1	1	1	0	0	1	0	1	1	1	0	0	0	0
37	Succinate	0	1	1	0	0	0	0	0	0	0	1	0	1	1	1	1	0	0	1	0	1	0	0	0	0	0	1

* Positive: 1 *Negative: 0 **Fermentative: 1 **Oxidative: 0

APPENDIX III.3 : Biochemical and physiological characteristics of bacterial isolates from Reculver.

Test number	Characteristic	Isolate code																					
		A31	A32	A33	A34	A35	A36	A37	A39	A40	A41	A42	A43	A45	A46	A48	A49	A50	A53	A54	A55	A57	A59
1	Growth on TCBS medium	1	0	0	0	0	0	0	0	1	1	0	0	1	1	0	1	0	0	1	0	0	1
2	Gram Stain	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
3	Oxidation / Fermentation of Glucose **	0	0	0	0	1	0	1	0	1	1	0	0	0	1	0	1	1	0	1	0	0	1
4	Pigment	1	1	1	1	0	1	0	1	0	0	1	1	0	0	1	1	0	0	0	0	1	0
5	Motility	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
6	Swarming	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
	Luminescence	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
7	Growth on CLED medium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	Arginine Dihydrolases	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0
9	Lysine Decarboxylases	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	1	0	0	1	1	0	0
10	Ornithine "	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
11	Nitrate Reduction	0	1	1	0	0	1	0	0	0	1	1	1	1	1	1	1	1	0	0	0	1	0
12	Oxidase Test	1	1	1	1	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	1	0
13	Indole Production	1	1	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1
14	ONPG Hydrolysis	0	0	0	1	0	0	0	0	0	1	0	0	1	1	0	1	1	1	1	1	0	1
15	Voges Proskauer Test	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	Resistance to: O/129 10 µg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
17	O/129 150 µg	0	0	1	1	1	0	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	0
18	Ampicillin 10 µg	1	0	0	0	1	0	1	0	1	1	0	0	1	1	0	1	1	1	1	0	0	0
	Polymyxin B 50 i.u.	1	1	0	0	1	1	0	1	1	0	1	1	0	0	1	1	0	0	0	1	1	1
19	Aesculin Hydrolysis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Production of enzymes: Elastase	0	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
20	Gelatinase	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0
21	Lecithinase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
23	Amylase	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	1	1	1
	Xanthine Decomposition	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	1
24	Haemolysis of sheep red blood cells	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	1	0	0	1
25	Acid from carbohydrates: Arbutin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
26	Salicin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
27	Sucrose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
28	Utilization of single carbon sources: L-arabinose	1	0	0	0	1	0	1	1	1	1	0	0	1	1	0	0	1	0	0	1	0	1
29	D-cellobiose	0	0	1	1	1	0	1	1	1	1	0	0	1	1	0	1	1	1	0	1	0	1
30	D-galactose	1	0	1	1	1	0	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1
31	D-melibiose	1	0	0	1	1	0	1	1	1	1	0	0	1	1	0	1	1	1	0	1	0	1
32	D-gluconate	1	0	0	0	1	0	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1
33	D-glucuronate	1	0	1	1	1	0	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1
34	L-citrulline	0	0	1	0	1	0	1	0	1	1	0	0	1	1	0	1	1	1	0	1	0	1
	L-leucine	1	0	1	1	1	0	1	0	1	1	0	0	1	1	0	1	1	0	1	1	0	1
35	D-glucosamine	0	0	1	0	1	0	1	0	1	1	0	0	1	1	0	0	1	1	1	1	0	1
36	DL-3-hydroxybutyrate	1	0	0	0	1	0	1	0	1	0	0	0	1	1	0	1	1	1	0	1	0	1
37	Succinate	0	0	0	0	1	0	1	0	1	0	0	0	1	1	0	0	1	1	1	1	0	1

* Positive: 1 *Negative: 0 **Fermentative: 1 **Oxidative: 0

APPENDIX III.4: Biochemical and physiological characteristics of bacterial isolates from Reculver and Conwy.

Test number	Characteristic	Isolate code																														
		A60	A61	A63	A64	A65	A66	A68	A71	A72	A73	A74	A75	A76	A77	A78	A79	A80	A81	A82	A83	A85	A86	A87	A88	A89	A90	A91	A93	A94	A95
1	Growth on TCBS medium	1	0	0	1	0	1	1	1	0	0	0	1	0	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0
2	Gram Stain	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	Oxidation /Fermentation of Glucose **	1	0	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0
4	Pigment	0	1	0	0	1	0	1	0	1	1	0	1	1	0	1	1	0	1	0	0	0	0	1	1	0	0	0	0	0	0
5	Motility	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	1	0	1	1	1	1	0	1	1	0	1	1	1	1
6	Swarming	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	Luminescence	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
7	Growth on CLED medium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
8	Arginine Dihydrolases	1	0	1	1	0	1	0	1	0	0	0	0	0	0	1	0	1	1	1	1	1	0	0	0	1	1	0	0	0	1
9	Lysine Decarboxylases	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	0	1	1	0	0	0	1	0	0	0	1	1	1	1	0
10	Ornithine "	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0
11	Nitrate Reduction	1	1	1	1	1	1	1	1	0	0	1	1	0	1	0	0	0	0	1	1	0	1	0	1	1	0	0	1	0	1
12	Oxidase Test	0	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0	1	0	1	1	1	1	1	1
13	Indole Production	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
14	ONPG Hydrolysis	1	0	1	1	0	1	1	0	1	1	0	0	0	1	1	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0
15	Voges Proskauer Test	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
16	Resistance to: O/129 10 µg	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17	O/129 150 µg	0	0	1	1	0	1	1	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0
18	Ampicillin 10 µg	1	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
	Polymyxin B 50 i.u.	0	1	0	0	1	0	1	0	1	1	1	1	1	1	1	1	0	0	0	0	1	0	1	0	0	1	0	0	0	1
19	Aesculin Hydrolysis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1
	Production of enzymes: Elastase	1	0	1	1	0	1	0	0	0	1	0	1	0	1	0	0	1	1	0	0	1	0	1	1	0	0	0	0	0	0
20	Gelatinase	0	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	0	1	0	0	1	1	1	1	1	1	1
21	Lecithinase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23	Amylase	0	1	1	0	1	0	1	0	0	0	0	0	0	1	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	0
	Xanthine Decomposition	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
24	Haemolysis of sheep red blood cells	1	0	1	1	0	0	0	1	0	0	0	0	0	1	1	0	0	0	1	1	0	1	1	0	1	1	0	0	0	1
25	Acid from carbohydrates: Arbutin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
26	Salicin	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
27	Sucrose	0	0	1	1	0	0	0	0	1	1	0	1	0	1	1	0	0	0	1	0	1	0	0	1	0	1	1	1	0	0
28	Utilization of single carbon sources: L-arabinose	0	0	0	0	0	1	0	0	1	1	1	0	1	1	1	0	1	0	1	1	1	0	1	0	1	1	1	1	1	1
29	D-cellobiose	1	0	1	1	0	1	1	0	1	1	1	1	0	1	1	0	1	0	1	1	1	0	1	0	1	1	1	1	1	1
30	D-galactose	1	0	1	1	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	1	1	1	1	0	1	1	0	0	1	0
31	D-melibiose	0	0	1	1	0	1	1	0	1	1	1	1	0	1	1	0	1	0	1	1	1	0	1	0	1	1	1	1	1	0
32	D-gluconate	1	0	1	1	0	1	1	1	1	1	1	1	0	1	1	0	1	1	0	0	1	1	1	0	1	1	1	1	1	1
33	D-glucuronate	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	0
34	L-citrulline	0	0	0	1	0	0	1	0	1	1	1	0	0	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1
	L-leucine	0	0	1	0	0	0	0	1	1	1	1	1	0	0	1	0	0	0	1	1	0	0	1	0	1	1	1	0	0	1
35	D-glucosamine	1	0	1	1	0	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
36	DL-3-hydroxybutyrate	1	0	1	1	0	1	0	0	1	1	0	1	0	1	1	0	1	1	1	1	1	0	0	0	1	1	1	1	1	1
37	Succinate	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	0	0	1	0	1	0	1	0	0	0	1	1	1	1	0

* Positive: 1 *Negative: 0 **Fermentative: 1 **Oxidative: 0

APPENDIX III.5: Antibiotic sensitivity and resistance of bacterial isolates from Guernsey.

Test number	Antibiotic		Isolate code																								
			G1	G2	G3	G3a	G4	G5	G7	G8	G9	G10	G12	G13	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24	G25	G26	C39*
38	Chloramphenicol	25 µg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
39	Erythromycin	5 µg	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	0	1
40	Fusidic Acid	10 µg	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	0	1	0	1	1	0	1	1	1
41	Methicillin	10 µg	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
42	Novobiocin	5 µg	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
43	Penicillin G	1 Unit	0	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1
44	Streptomycin	10 µg	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
45	Tetracycline	25 µg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
46	Ampicillin	10 µg	0	1	0	0	1	0	0	0	1	0	1	1	1	1	1	1	1	0	1	0	0	1	0	0	0
47	Cephaloridine	5 µg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
48	Colistin Sulphate	25 µg	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
49	Gentamicin	10 µg	0	0	0	1	1	0	1	1	0	0	0	0	0	0	1	1	0	1	0	0	1	0	0	0	0
50	Sulphatriad	200 µg	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	0
	Cotrimoxazole	25 µg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Ampicillin	25 µg	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	0
	Nitrofurantoin	50 µg	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
	Ticarcillin	75 µg	0	1	0	0	1	0	0	0	1	0	1	1	0	1	1	1	1	0	1	0	0	1	0	0	0
	Tetracycline	100 µg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Nalidixic Acid	30 µg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	Trimethoprim	2.5 µg	0	0	0	0	1	1	1	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	1	1	1
	Sulphamethoxazole	50 µg	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0

* 0 : Sensitive, 1 : Resistant

* C39 Standard laboratory isolate from the Microbiology Laboratory, Glasgow University.

APPENDIX III.6 : Antibiotic sensitivity or resistance of bacterial isolates from Reculver

Test number	Antibiotic		Isolate code																						
			A1	A2	A3	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15	A16	A17	A18	A19	A20	A22	A23	A24	A25
38	Chloramphenicol	25 µg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
39	Erythromycin	5 µg	1	0	1	1	1	1	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
40	Fusidic Acid	10 µg	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0	1	1	0
41	Methicillin	10 µg	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1
42	Novobiocin	5 µg	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	0	0
43	Penicillin G	1 Unit	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1
44	Streptomycin	10 µg	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
45	Tetracycline	25 µg	0	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	1	0	1
46	Ampicillin	10 µg	1	1	1	0	1	1	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0
47	Cephaloridine	5 µg	0	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1
48	Colistin Sulphate	25 µg	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0
49	Gentamicin	10 µg	0	0	0	0	1	0	0	0	0	0	1	0	1	1	0	1	1	1	0	1	0	1	1
50	Sulphatriad	200 µg	0	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	1	1	1
	Cotrimoxazole	25 µg	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0
	Ampicillin	25 µg	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	Nitrofurantoin	50 µg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	Ticarcillin	75 µg	1	1	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
	Tetracycline	100 µg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
	Nalidixic Acid	30 µg	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Trimethoprim	2.5 µg	1	1	1	1	0	0	1	1	0	1	1	0	0	1	1	1	0	1	1	0	1	1	1
	Sulphamethoxazole	50 µg	0	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1

* O : Sensitive, 1 : Resistant

APPENDIX III.7: Antibiotic sensitivity and resistance of bacterial isolates from Reculver.

Test Number	Antibiotics		Isolate code																								
			A26	A27	A28	A30	A31	A32	A33	A34	A35	A36	A37	A39	A40	A41	A42	A43	A45	A46	A48	A49	A50	A53	A54	A55	A57
38	Chloramphenicol	25 µg	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
39	Erythromycin	5 µg	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	1	0	0	0	0	1	0	0
40	Fusidic Acid	10 µg	0	0	0	1	0	0	0	0	1	0	1	0	1	1	0	0	0	1	1	1	1	1	1	1	0
41	Methicillin	10 µg	0	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	0	1	1	1	0	1	1	1	1
42	Novobiocin	5 µg	1	1	1	1	0	1	1	0	0	1	0	0	1	0	1	1	0	0	1	1	0	1	0	1	1
43	Penicillin G	1 Unit	0	1	1	1	0	1	0	1	1	1	1	0	1	0	1	1	1	0	1	1	0	1	1	1	1
44	Streptomycin	10 µg	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1
45	Tetracycline	25 µg	0	0	0	1	0	0	0	0	1	0	1	0	1	0	0	0	1	0	0	0	0	1	1	1	0
46	Ampicillin	10 µg	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0
47	Cephaloridine	5 µg	0	1	1	1	0	1	0	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1
48	Colistin Sulphate	25 µg	0	1	1	0	0	1	0	0	0	1	0	1	1	0	1	1	0	0	1	0	0	0	0	0	1
49	Gentamicin	10 µg	1	1	1	0	1	1	1	1	0	1	0	1	1	0	1	1	0	0	1	0	0	0	1	0	1
50	Sulphatriad	200 µg	0	1	1	0	1	1	0	1	0	1	1	0	1	0	1	1	0	0	0	0	0	0	0	0	1
	Cotrimoxazole	25 µg	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	1	0	0	0	0	0	0	0	0	1
	Ampicillin	25 µg	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0
	Nitrofurantoin	50 µg	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0
	Ticarcillin	75 µg	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0
	Tetracycline	100 µg	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	Nalidixic Acid	30 µg	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
	Trimethoprim	2.5 µg	1	0	0	1	0	0	1	1	1	0	1	1	1	0	0	0	1	1	0	1	1	1	1	1	0
	Sulphamethoxazole	50 µg	1	1	1	1	0	1	1	0	1	1	1	0	1	0	1	1	0	0	1	0	1	0	1	1	1

* 0 : Sensitive, 1 : Resistant

APPENDIX III.8 : Antibiotic sensitivity and resistance of bacterial isolates from Reculver, Pseudomonas I-1-1, vibrios V2981 and V6.

Test Number	Antibiotic		Isolate code																					
			A59	A60	A61	A63	A64	A65	A66	A68	A71	A72	A73	A74	A87	A88	A89	A90	A91	A93	A94	V2981	V. NCMB 6	P1-1-1
38	Chloramphenicol	25 µg	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
39	Erythromycin	5 µg	1	1	0	1	1	0	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	0
40	Fusidic Acid	10 µg	0	0	0	1	0	0	1	1	1	1	1	0	1	0	1	0	0	0	0	1	0	1
41	Methicillin	10 µg	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
42	Novobiocin	5 µg	0	1	1	0	0	1	0	0	0	0	0	1	1	1	0	1	1	1	1	0	1	1
43	Penicillin G	1 Unit	0	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
44	Streptomycin	10 µg	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
45	Tetracycline	25 µg	1	1	0	0	1	0	1	1	1	0	0	1	1	0	0	1	1	1	1	1	1	0
46	Ampicillin	10 µg	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	1	1	0	1	0	1
47	Cephaloridine	5 µg	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	1	1	1
48	Colistin Sulphate	25 µg	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	1	1	0
49	Gentamicin	10 µg	0	0	1	0	1	1	0	0	1	1	1	0	1	1	0	0	0	0	0	1	0	1
50	Sulphatriad	200 µg	1	1	1	1	0	1	0	1	0	1	1	0	1	1	0	0	0	0	1	1	1	0
	Cotrimoxazole	25 µg	1	1	0	1	0	1	0	0	0	1	1	0	1	1	0	0	0	0	1	1	1	0
	Ampicillin	25 µg	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	1	1	0	0	1	0
	Nitrofurantoin	50 µg	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1
	Ticarcillin	75 µg	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	1	0
	Tetracycline	100 µg	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
	Nalidixic Acid	30 µg	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	1	0
	Trimethoprim	2.5 µg	1	1	0	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	0
	Sulphamethoxazole	50 µg	1	0	0	1	0	1	0	1	1	1	1	1	1	1	0	0	0	0	0	1	1	0

* 0 : Sensitive, 1 : Resistant.

APPENDIX III.9 : Antibiotic sensitivity and resistance of bacterial isolates from Conwy.

Test number	Antibiotics		Isolate code										
			A75	A76	A77	A78	A79	A80	A81	A82	A83	A85	A86
38	Chloramphenicol	25 µg	0	0	0	0	0	0	0	0	0	0	0
39	Erythromycin	5 µg	1	1	0	1	1	0	0	1	0	1	1
40	Fusidic Acid	10 µg	0	0	0	0	0	1	0	1	1	0	1
41	Methicillin	10 µg	0	1	0	0	0	1	1	1	0	1	1
42	Novobiocin	5 µg	0	0	0	0	1	1	0	1	0	1	0
43	Penicillin G	1 Unit	1	1	0	1	1	1	1	0	0	1	1
44	Streptomycin	10 µg	1	1	1	1	1	1	1	0	1	1	1
45	Tetracycline	25 µg	0	0	0	1	0	0	0	1	0	1	1
46	Ampicillin	10 µg	1	1	0	1	0	0	0	1	0	0	0
47	Cephaloridine	5 µg	1	1	0	0	1	1	1	1	0	1	1
48	Colistin Sulphate	25 µg	0	1	1	0	1	0	1	1	0	0	0
49	Gentamicin	10 µg	1	1	1	0	1	0	1	1	0	0	1
50	Sulphatriad	200 µg	0	0	0	0	0	0	0	1	1	0	0
	Cotrimoxazole	25 µg	0	0	0	0	0	0	1	1	0	0	0
	Ampicillin	25 µg	1	1	0	1	0	0	0	1	0	0	0
	Nitrofurantoin	50 µg	0	0	0	0	0	0	0	0	0	0	0
	Ticarcillin	75 µg	0	0	0	0	0	0	0	0	0	0	0
	Tetracycline	100 µg	0	0	0	0	0	0	0	0	0	0	0
	Nalidixic Acid	30 µg	0	0	1	0	0	0	0	0	0	0	1
	Trimethoprim	2.5 µg	1	1	0	1	1	1	1	1	0	1	1
	Sulphamethoxazole	50 µg	1	1	0	0	1	1	1	1	0	0	1

* 0 : Sensitive, 1 : Resistant.

APPENDIX III.10 : Biochemical test characteristics for reference vibrio species.

Characteristic	Vibrio species											
	V. alginolyticus	*V. anguillarum*	*V. fluvialis* *	*V. parahaemolyticus*	*V. furnissi*	*V. tubiashii*	*V. campbellii*	*V. splendidus**	*V. splendidus***	*V. fischeri*	*V. metschnikovii*	*V. ordalii*
Gram Stain	0	0	0	0	0	0	0	0	0	0	0	0
Fermentation of glucose	1	1	1	1	1	1	1	1	1	1	1	1
Pigment	0	0	0	0	0	0	0	0	0	1	0	0
Motility	1	1	1	1	1	1	1	1	1	1	1	1
Swarming	1	0	0	0	0	0	0	0	0	0	0	0
Luminescence	0	0	0	0	0	0	0	0	0	1	0	0
Growth @ 4 °C	0	1	0	0	0	0	0	0	0	0	1	1
Cled	0	0	0	0	0	0	1	0	0	0	1	0
Arginine Dihydrolases	0	1	1	0	1	1	0	1	1	0	1	0
Lysine decarboxylases	1	0	0	1	0	0	0	0	0	1	1	0
Ornithine " "	1	0	0	1	0	0	0	0	0	0	0	0
Nitrate reduction	1	1	1	1	1	1	1	1	1	1	0	0
Oxidase test	1	1	1	1	1	1	1	1	1	1	0	1
Indole production	1	1	d	1	0	1	1	1	0	0	0	0
ONPG hydrolysis	0	1	1	0	1	1	0	1	1	0	1	0
Voges Proskauer	1	1	0	0	0	0	0	0	0	0	1	0
Resistance to: O/129 10 µg	1	0	1	1	1	0	1	0	0	0	0	0
O/129 150 µg	0	0	0	0	0	0	0	0	0	0	0	0
Ampicillin	1	1	1	1	1	0	1	0	1	1	0	1
Polymyxin	0	0	0	0	0	0	1	0	0	0	0	1
Hydrolysis of: Aesculin	0	0	1	0	0	0	d	d	d	0	0	d
Elastin	0	1	0	0	0	0	0	0	0	0	1	0
Gelatin	1	1	1	1	1	1	1	1	1	0	1	1
Lecithin	1	1	1	1	1	1	1	1	0	1	1	0
Starch (amylase)	1	1	1	1	1	1	1	1	1	0	1	0
Xanthine decomposition	0	0	0	0	0	1	0	0	0	0	0	0
Acid from: Arbutin	0	0	1	0	0	0	0	0	0	0	0	0
Salicin	0	0	1	0	0	0	1	0	0	1	0	0
Sucrose	1	1	1	0	1	1	0	0	0	0	1	1
Utilisation of substrates:												
L-Arabinose	0	1	1	1	1	0	0	0	0	0	0	0
D-Cellobiose	0	1	d	1	0	1	1	1	0	0	0	0
D-Galactose	0	1	1	1	1	1	0	1	0	0	1	0
D-Melibiose	0	0	0	0	0	1	0	0	0	0	0	0
D-Gluconate	1	1	1	1	1	1	0	0	0	0	1	0
D-Glucuronate	1	0	1	1	0	0	0	1	0	0	0	0
L-Citrullize	0	0	1	1	0	0	0	1	0	0	0	0
L-Leucine	1	0	0	1	0	0	0	0	1	0	0	0
D-Glucosamine	1	1	1	1	1	0	0	0	0	0	1	1
DL-3-Hydroxybutyrate	0	0	1	0	1	0	0	0	0	0	0	0
Succinate	1	1	1	1	1	1	1	1	0	0	1	0

* Biovar 1, ** Biovar 2

APPENDIX III.11 : Biochemical test characteristics for group R bacterial isolates from turbot.

Characteristic	Isolate code																																				
	R1	R2	R3	R4	R5	R6	R8	R11	R12	R13	R14	R15	R16	R18	R19	R20	R21	R23	R24	R25	R26	R27	R29	R32	R35	R36	R37	R38	R39	R40	R42	R44	R45	R46	R47
TCBS	1	1	1					1	0	1				1	0	1	1		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Gram Stain	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fermentation of glucose	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Pigment	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Motility	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Swarming	1	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Luminescence	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Growth @ 4 °C	0	0	0	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	1	1	0	0	1	1	0	1	0	1
Cled	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arginine Dihydrolases	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lysine Decarboxylases	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ornithine " "	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nitrate Reduction	1	1	1	1	1	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Oxidase Test	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Indole Production	1	1	1	0	0	0	0	1	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ONPG Hydrolysis	0	0	0	0	0	0	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Voges Proskauer Test	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Resistance to: O/129 10 µg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O/129 150 µg	1	1	1	1	0	0	1	0	0	1	1	1	1	0	1	0	1	0	1	0	1	1	1	1	0	1	1	0	1	0	1	1	1	1	1	1	1
Ampicillin	1	1	1	1	1	1	0	0	1	1	0	1	1	0	0	0	1	1	0	0	0	1	0	1	0	0	0	0	1	1	0	0	0	1	0	0	0
Polymyxin	1	1	1	0	1	1	0	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hydrolysis of: Aesculin	0	0	0	0	0	0	1	1	1	0	0	0	0	1	1	1	1	0	1	1	1	0	0	1	1	1	1	0	0	0	0	1	1	0	1	0	1
Elastin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gelatin	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Lecithin	0	0	0	0	1	1	0	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
Starch (amylase)	1	1	1	1	1	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Xanthine Decomposition	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	1	1	0	0	1	0	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1
Acid from: Arbutin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salicin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sucrose	1	1	1	0	0	0	1	1	0	1	0	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
Utilisation of substrates:																																					
L-Arabinose	1	1	1	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-Cellobiose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-Galactose	1	1	1	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-Melibiose	1	1	1	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-Gluconate	1	1	1	0	0	0	0	1	0	1	1	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-Glucuronate	1	1	1	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
L-Citrulline	1	1	1	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-Leucine	1	1	1	0	0	0	0	1	0	1	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-Glucosamine	1	1	1	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DL-3-Hydroxybutyrate	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Succinate	1	1	1	0	0	0	0	1	0	1	1	1	1	1	0	0	0	0	1	1	1	1	0	1	1	1	1	0	0	1	0	1	1	0	1	0	1

[a] Positive/Fermentative : 1, Negative/Oxidative : 0.

APPENDIX III.12 : Biochemical test characteristics for group ER bacterial isolates from turbot.

Characteristic	Isolate code																																							
	ER1	ER3	ER4	ER5	ER6	ER8	ER9	ER10	ER12	ER13	ER14	ER15	ER15	ER17	ER18	ER21	ER23	ER24	ER25	ER26	ER27	ER28	ER31	ER32	ER33	ER34	ER36	ER39	ER40	ER41	ER42	ER43	ER44	ER45	ER47	ER49	ER50	ER51
TCBS	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Gram Stain	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fermentation of glucose	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Pigment	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Motility	0	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Swarming	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Luminescence	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Growth @ 4 °C	0	0	0	1	0	0	0	0	0	0	0	1	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0	0	1	1	1	1	1	0	0	1	1	1	1
Cled	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arginine decarboxylases	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lysine " "	0	0	0	0	1	1	1	1	0	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0
Ornithine " "	0	0	0	0	1	1	1	1	0	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0
Nitrate reduction	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Oxidase test	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Indole production	0	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ONPG hydrolysis	1	1	1	1	0	0	0	0	1	0	0	1	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0	0	1	1	1	1	1	0	0	1	1	1	1
Voges Proskauer	0	0	0	0	1	1	1	1	0	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0
Resistance to: O/129 10 µg	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O/129 150 µg	0	0	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ampicillin	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	1	0	1	1	0	0	0	0	0	1	1	1	0	0	0
Polymyxin	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0
Hydrolysis of: Aesculin	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	1	1	1
Elastin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gelatin	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Lecithin	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Starch (amylase)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Xanthine decomposition	1	1	1	1	0	0	0	0	1	0	0	0	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0	0	1	1	1	1	1	0	0	1	1	1	1
Acid from: Arbutin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salicin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sucrose	0	0	0	0	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1
Utilisation of substrates:																																								
L-Arabinose	0	0	0	0	1	1	1	1	0	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0
D-Cellobiose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
D-Galactose	0	0	0	0	1	1	1	1	0	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0
D-Melibiose	0	0	0	0	1	1	1	1	0	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0
D-Gluconate	0	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	1	1	0	0	0	0	0	1	1	0	0	0	0
D-Glucuronate	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	0	1	1	0	0	0	0
L-Citrulline	0	0	0	0	1	1	1	1	0	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0
L-Leucine	0	0	0	0	1	1	1	1	0	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0
D-Glucosamine	0	0	0	0	1	1	1	1	0	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0
DL-3-Hydroxybutyrate	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Succinate	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

*Positive/Fermentative : 1, Negative/oxidative : 0.

APPENDIX III.13 : Biochemical test characteristics for group IR bacterial isolates from turbot.

Characteristic	Isolate code																																		
	IR1	IR2	IR3	IR4	IR5	IR6	IR7	IR8	IR10	IR11	IR12	IR13	IR14	IR17	IR18	IR19	IR20	IR21	IR22	IR24	IR26	IR27	IR28	IR30	IR31	IR32	IR33	IR34	IR35	IR37	IR38	IR39	IR42	IR46	IR47	IR50
TCBS	1	1	1		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		1	1	1	1	1	1	1
Gram Stain	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fermentation of glucose	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
Pigment	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Motility	1	1	1	0	1	0	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Swarming	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Luminescence	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Growth @ 4 °C	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	0	1	0	0	1
Cled	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arginine decarboxylases	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lysine " "	1	1	1	0	1	0	0	1	1	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
Ornithine " "	1	1	1	0	1	0	0	1	1	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
Nitrate reduction	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
Oxidase test	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Indole production	0	0	0	0	1	0	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1
ONPG hydrolysis	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	1	0	1	1
Voges Proskauer	1	1	1	0	1	0	0	1	1	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1	0	1	0	0
Resistance to: O/129 10 µg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O/129 150 µg	1	1	1	1	1	0	0	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1
Ampicillin	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	0	1	1	1	0	0	1	1	0	1	1	0
Polymyxin	1	0	0	0	1	0	0	1	0	0	1	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0
Hydrolysis of: Aesculin	0	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	1	0	0	0	1	0	1	0	1	1	0	1
Elastin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gelatin	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Lecithin	1	1	0	0	1	1	1	1	0	1	0	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Starch (amylase)	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Xanthine decomposition	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1
Acid from: Arbutin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salicin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sucrose	1	1	1	1	1	0	0	1	1	0	1	0	1	1	1	1	0	0	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	0	1	
Utilisation of substrates:																																				
L-Arabinose	1	1	1	1	1	0	0	1	1	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	0	0
D-Cellobiose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
D-Galactose	1	1	1	1	1	0	0	1	1	0	1	0	1	1	1	1	1	0	0	1	1	0	0	0	0	1	1	1	1	0	1	1	1	1	0	1
D-Melibiose	1	1	1	1	1	0	0	1	1	0	1	0	1	1	1	1	0	0	0	1	0	0	0	0	0	1	1	1	1	0	0	1	0	1	0	0
D-Gluconate	1	1	1	1	1	0	0	1	1	0	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
D-Glucuronate	1	1	1	0	1	0	0	1	1	0	1	0	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1
L-Citrulline	1	1	1	0	1	0	0	1	1	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0	0
L-Leucine	1	1	1	1	1	0	0	1	1	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	0	0
D-Glucosamine	1	1	1	0	0	0	0	1	1	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	0	0
DL-3-Hydroxybutyrate	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Succinate	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1

* Positive/Fermentative : 1, Negative/Oxidative : 0.

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