Some Aspects of the Ecology of

Listeria monocytogenes

in Salmonid Aquaculture

by

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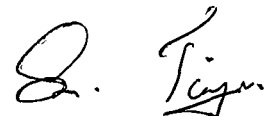Submitted in fulfilment of the requirements for the degree of

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DECLARATION

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ABSTRACT

In this thesis, several related aspects of the ecology of *Listeria* spp. especially *L. monocytogenes* in aquatic environments and foods were studied.

The ecology of the organisms in aquatic habitats was investigated in North West Bay, southern Tasmania, over a 12 month period. *Listeria* spp. most frequently occurred in effluent and river water but less often in receiving estuarine waters. Sediments and shellfish served as a better reservoir for the organisms. Recent rainfall and the level of faecal coliforms and *E. coli* were the most significant variables ($P \leq 0.01$) related to the presence/absence of *Listeria* species and *L. monocytogenes* in estuarine water.

Secondly, the relationship between the occurrence of the human pathogenic species, *L. monocytogenes*, in aquatic environments and in a nearby salmon processing plant and its products was studied. Molecular subtyping methods (multilocus enzyme electrophoresis and repetitive sequence element-PCR) were employed to help trace the distribution of *L. monocytogenes* strains. A high diversity of *L. monocytogenes* was found in the aquatic environment but only a small group was detected in fish and the fish processing environment.

Thirdly, to be able to understand the physiology and growth response of *L. monocytogenes* to temperature, water activity, pH, and lactic acid and which in turn may be used to minimise the consequences of contamination by the pathogen of foods, quantitative microbiology (predictive microbiology) studies were conducted. The results were incorporated into 2 different types of mathematical model. The first type of model, a kinetic model, was developed using a "square root type model" which is useful for predicting the shelf-life of foods. The second type of model, a probability model (a so-called "growth/ no growth interface" model) which is a novel model for *L. monocytogenes* growth limits was developed using a new approach, viz "generalised nonlinear regression method". This type of model is useful for predicting the condition when micro-organisms, especially pathogenic bacteria, might grow or might not grow.

Finally, model predictions were evaluated by comparing them to novel and literature data broadly relevant to the range of conditions in foods for which the models were developed. Limited tests, involving direct addition of different levels of lactic acid onto traditional cold-smoked salmon products were performed as an approach to non-thermal inhibition or inactivation of *L. monocytogenes* and also to test the performance of the models.

ACKNOWLEDGMENTS

CONTENTS

LIST OF ABBREVIATIONS

ACM	Australian Collection of Microorganisms
ATCC	American Type Culture Collection
a_w	water activity
$a_{w\,min}$	notional minimum water activity for growth
BHIA	Brain Heart Infusion Agar
BHIB	Brain Heart Infusion Broth
BOX	the 154 bp interspersed repetitive DNA sequence from *Streptococcus pneumoniae*
CAMP	Christie, R., N.E. Atkins, and E. Munch-Petersen
cfu	Colony Forming Unit
[D]	concentration of dissociated lactic acid
D_{min}	notional minimum concentration of dissociated lactic acid for growth inhibition
DNA	Deoxyribo Nucleic Acid
dNTP	deoxyribonucleoside triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetra acetic acid
ERIC	Enterobacterial Repetitive Intergenic Consensus
ET	Electrophoretic Type
FB	Fraser Broth
GT	Generation Time
HBA	columbia blood agar with 4% horse blood added
L. innocua	*Listeria innocua*
L. ivanovii	*Listeria ivanovii*
L. monocytogenes	*Listeria monocytogenes*
L. murrayi	*Listeria murrayi* including the former *L. grayi*
L. seeligeri	*Listeria seeligeri*
L. welshimeri	*Listeria welshimeri*
[LAC]	concentration of lactic acid
LLO	Listeriolysin O
LTB	Lauryl Tryptose Broth
MCP	minimum convex polyhedron
MEE	Multilocus Enzyme Electrophoresis
MLSA	Membrane Lauryl Sulphate Agar
MR	Methyl Red
OXF	Listeria selective medium; Listeria selective agar base to which is added Listeria Selective Supplement SR 140 (Oxford Formulation),
PCR	Polymerase Chain Reaction
pH_I	pH at inoculation (of a broth culture)

pH_f	final pH (of a broth culture)
pH_{mid}	pH at midpoint of exponential growth
pH_{min}	notional minimum pH for growth
pK_a	dissociation constant for acid
k	growth rate (defined as 1/generation time in hr)
$\sqrt{r}$	square root of growth rate
R. equi	*Rhodococcus equi*
rep-PCR	repetitive sequence element PCR
REP	Repetitive Extragenic Palindrome
RTE	ready-to-eat foods
S. aureus	*Staphylococcus aureus*
SDS	Sodium Dodecyl Sulphate
S. faecalis	*Streptococcus faecalis*
t	Temperature
ΔT	difference of %T at time 0 and %T at time t
%T	percent transmittance
Taq	DNA polymerase enzyme extracted from *Thermus* species
T_{max}	notional maximum temperature for growth
T_{min}	notional minimum temperature for growth
TSA	Tryptic Soy Agar
TSA-YE	Tryptic Soy Agar with 0.6% Yeast Extract
TSB	Tryptic Soy Broth
TSB-YE	Tryptic Soy Broth with 0.6% Yeast Extract
TSI	Triple Sugar Iron agar
TVC	total viable counts
[UD]	Concentration of undissociated lactic acid
U_{min}	notional minimum concentration of undissociated lactic acid for growth inhibition
UVM I	University of Vermont Listeria Enrichment broth
VP	Voges-Proskauer

1 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

During the past 15 years, *Listeria monocytogenes* has emerged as a bacterium of considerable public health significance. Several recent epidemics in North America, Europe and Western Australia were linked to the consumption of commercial food products (Schlech *et al.*, 1983; Watson *et al.*, 1990; Zottola and Smith, 1991; Broome, 1993; Ericsson *et al.*, 1997). These outbreaks have prompted increased interest in understanding the epidemiology of this human pathogen and have stimulated concern over how and when it can be transmitted from the environment and cause human illness. The symptoms of the resulting infection, listeriosis, include severe meningitis, meningo-encephalitis, central nervous system infection, stillbirths, abortions, premature labour and septicemia (Seeliger and Finger, 1983; Lovett, 1989; Miller *et al.*, 1990). The organism mostly affects limited groups within the population, namely pregnant women, foetuses, the elderly and individuals with suppressed immune systems (see review by Ryser and Marth, 1991, pp. 45-65). Listeriosis is considered to be serious because of the high mortality rate: approximately 30% overall, and as high as 55% in foetuses (Watson *et al.*, 1990; Broome, 1993).

The occurrence of *L. monocytogenes* in some environment, foods and foods processing environment have been studied which lead to the improvement of methods for detection, enumeration, identification, and differentiation including subtyping for the study of its ecology and epidemiology purposes. Since food is the major source of listeriosis, the control and prevention of *Listeria* contamination of foods is of interest. The introduction of strategies such as HACCP and predictive microbiology, together with the good education to consumers, could be used as the tools to improve food safety.

1.2 HISTORY OF THE GENUS *LISTERIA*

1.2.1 THE GENUS *LISTERIA* AND RELATED ORGANISMS

Listeria was definitely isolated and described in detail for the first time in England by Murray *et al.* (1926). A small gram positive bacillus was isolated following a spontaneous epidemic infection among laboratory rabbits and guinea-pigs. During the illness, a typical monocytosis was observed in the diseased animals. The authors considered this to be a case of *hitherto* unidentified bacterium, and therefore designated

the organism *Bacterium monocytogenes*. The following year, Pirie (1927) isolated a bacterium from the liver of infected African gerbils (African jumping mice, *Tartera lobengulae*) in South Africa and named it *Listerella hepatolytica*. The generic name was chosen in honour of Lord Lister who discovered antisepsis. Shortly after this, it was established that the organisms from England and Africa were identical, and the name was altered to *Listerella monocytogenes*. However, the generic name *Listerella* had already been used in another branch of biology. Hence, the proposed name change by Pirie (1940) from *Listerella monocytogenes* to *Listeria monocytogenes* was accepted in 1940.

The sixth edition of *Bergey's Manual of Determinative Bacteriology* (Breed *et al.*, 1948), as well as the seventh edition (Breed *et al.*, 1957), ranked the genus *Listeria* with a single species *L. monocytogenes* in the family Corynebacteriaceae. Four species of *Listeria* are described in the eighth edition of *Bergey's Manual* (Buchanan and Gibbons, 1974; Holt, 1977) : *L. monocytogenes*, *L. denitrificans*, *L. grayi* and *L. murrayi*. Of these four species, only *L. monocytogenes* is associated with diseases of man and animals.

The species *monocytogenes* has already been described in detail by Gray and Killinger (1966) and Lovett (1990). In contrast to the *L. monocytogenes* strains isolated from clinical infections, many of *Listeria* strains isolated from healthy individuals and inanimate sources are nonhaemolytic, nonpathogenic for laboratory animals, and incapable of evoking a monocytosis in rabbits. These organisms have been proposed by Seeliger as *L. innocua* (Seeliger, 1981).

Supported by the results of deoxyribonucleic acid relatedness studies, determinations of biochemical characteristics, and studies of pathogenicity for adult mice, Rocourt and Grimont (1983) proposed the species name *L. seeligeri* and *L. welshimeri* which were previously classified as nonpathogenic *L. monocytogenes*. In the following year, Seeliger *et al.* (1984) proposed the name *L. ivanovii* for *L. monocytogenes* serovar 5 strains which are experimentally pathogenic for mice, but the 50% lethal dose of these strains is 10 times higher than that of *L. monocytogenes sensu stricto*.

Subsequently in the ninth edition of *Bergey's Manual of Systemic Bacteriology* (Seeliger and Jones, 1986), the genus *Listeria* was classified among "genera of uncertain affiliation" and comprised of 8 species : *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri, L. grayi, L. murrayi.* and *L. denitrificans*. However, three *Listeria* spp.- namely, *L. grayi, L. murrayi* and *L. denitrificans* have been categorised as species *incertae sedis* (species of uncertain position). This originated from low percentage of DNA homology and phenotypic similarity observed by Stuart and Welshimer (1973, 1974). The authors proposed to transfer *L. grayi* and *L. murrayi* to a new monospecific genus *Murraya* which include *Murraya grayi* subsp. *grayi* (here

Listeria grayi) and *M. grayi* subsp. murrayi (here *Listeria murrayi*). Regarding *L. denitrificans*, Stuart and Welshimer (1973) considered it to be misclassified into the genus *Listeria.*

Consequently, in 1987, the 16S ribosomal ribonucleic acid oligonucleotide catalog of *L. murrayi and L. denitrificans* was determined by Rocourt *et al.* (1987a,b). *L. murrayi* was found to be closely related to that of *L. monocytogenes.* The results provided no support for the exclusion of *L. murrayi* (and the closely related species *L. grayi*) from the genus *Listeria.* Whereas the results from *L. denitrificans* confirmed previous evidence that this organism was not a member of the genus *Listeria*, and was proposed to be transferred to a new genus *Jonesia* as *Jonesia denitrificans* (Rocourt *et al.,* 1987a).

Presently in *Bergey's Manual of Determinative Bacteriology* (Holt *et al.,* 1994), there are 6 species of the genus *Listeria* (Table 1.1) in Group 19. The species *L. denitrificans*, has been transferred to a separate genus *Jonesia* in Group 20. Three species; *L. monocytogenes*, *L. seeligeri* and *L. ivanovii*, produce ß-haemolysis (haemolysin, LLO) on horse and sheep blood agars. Two species; *L. seeligeri* and *L. ivanovii* are significantly pathogenic to animals apart from man and only one; *L. monocytogenes*, is pathogenic to humans and animals (Benedict, 1990).

1.3 CHARACTERISTICS, ISOLATION AND DIFFERENTIATION

1.3.1 CHARACTERISTICS

As described by Seeliger and Jones (1986), *Listeria* are short, regular rods 0.4-0.5 μm by 0.5-2.0 μm with rounded ends. They may be curved, occurring singly or in short chains, often present in a 'V' or 'Y' shape. In old or rough cultures, more filamentous forms, 6-20 μm, may develop. Although older cultures may stain irregularly, young cultures are Gram-positive. They are not acid-fast, not encapsulated, and are non-sporeforming. *Listeria* are facultative intracellular parasites, able to survive and replicate in cells (Racz *et al.*, 1972). They are motile by a few peritrichous flagella, best expressed at 20-22°C. The motility is in a characteristic tumbling or slightly rotating fashion. They are both aerobic and facultatively anaerobic. Grown in nutrient agar, they form colonies 0.5-1.5 mm, round, translucent, dew drop in appearance, low convex with fine texture and entire margin. When exposed to 45° incident transmitted white light, the colony appears bluish. A culture stab in semisolid growth medium (e.g. Bacto motility medium) produces growth along the stab line, spreading horizontally 3-5 mm below the surface in an umbrella pattern. This is probably owing to a combination of motility and a preference for micro-aerophilic conditions (Prentice and Neaves, 1992).

Table 1.1 Differential characteristics of the species of the genus *Listeria* [a,b]

| Characteristics | *Listeria* | | | | | |
	mono-cytogenes	*innocua*	*seeligeri*	*ivanovii*	*welshi-meri*	*murrayi (grayi)*
Dextrose	+	+	+	+	+	+
Esculin	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
MR-VP	+	+	+	+	+	+
Tumbling motility	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Hippurate hydrolysis	+	+	+	+	+	-
Urea hydrolysis	-	-	-	-	-	-
H_2S on TSI	-	-	-	-	-	-
H_2S by lead acetate strip	-	-	-	-	-	+
α-Methyl-D-mannoside	+	+	-	-	+	ND
Mannitol	-	-	-	-	-	+
D-Xylose	-	-	+	+	+	-
L-Rhamnose	+	d	-	-	d	d
ß-Haemolysis	+[c]	-	+	+	-	-
CAMP-*S. aureus*	+	-	+	-	-	-
CAMP-*R. equi*	-	-	-	+	-	-
NO_3 reduction	-	-	-	-	-	+/-

[a] symbols: +, 90% or more of strains are positive; -, 90% or more of strains are negative; d, 11-89% of strains are positive; ND, not determined. [b] The species *Listeria denitrificans*, which was in this genus in Bergey's Manual of Systematic Bacteriology, has been transferred to a separate genus *Jonesia* in Group 20. [c] A few strains negative. (After Holt *et al.*, 1994)

The optimum temperature for growth of *Listeria* is 35.6°C at which the generation time is 33.6 minutes (Ross, 1993). The organism grows at wide range of temperatures between 1 and 45°C (Gray and Killinger, 1966; Junttila *et al.*, 1988). Some strains are capable of growing as low as -0.4°C (Walker *et al.*, 1990). Its ability to grow at low temperatures has led to concern about foods stored at refrigeration temperatures, particularly those consumed without subsequent cooking, ready-to-eat (RTE) foods, are an important source of human infection (Jones, 1990). *L. monocytogenes* demonstrates remarkable tolerance to low water activities (a_w) which are unsuitable for many other bacteria, and can grow at a_w values below 0.93 (Farber *et al.*, 1992). It can grow in 0 to 10% sodium chloride or up to 13-14% providing the pH is ≥5.0 at 15 and 30°C (Farber *et al.*, 1992),

and survive for up to a year in 16-20% sodium chloride (Seeliger, 1961). *L. mono-cytogenes* was also reported to be a fairly acid tolerant. The minimum pH at which *L. monocytogenes* can initiate growth at 30°C was reported to be 4.3 (Farber *et al.*, 1989b). The maximum pH for growth of *L. monocytogenes* at 30°C was 9.2 (Petran and Zottola, 1989). The minimum pH for growth is markedly influenced by incubation temperature and the type of acid added to the medium. For some instances, the minimum pH for growth of *L. monocytogenes* Scott A at 4°C was 5.23 for HCl as acidulant (George *et al.*, 1988), and 5.5 for lactic acid as acidulant (Farber *et al.*, 1989b). In addition, *L. monocytogenes* is claimed to be among the most heat resistant of vegetative bacterial cells. Doyle *et al.* (1987) reported the recovery of *L. monocytogenes* from milk which had been treated at 71.7-73.9°C for 16.4 secs. Fernandez Garayzabal *et al.* (1987) also found *L. monocytogenes* in 71.5% of the milk samples heated at 72°C for 15 secs. Several studies concerning the thermal resistance of listeriae were carried out but conflicting results were obtained by different groups of workers (Ryser and Marth, 1991).

1.3.2 ISOLATION

One of the immediate outcomes of the identification of food as an important epidemiological factor in outbreaks of listeriosis, and identification of environments as an important reservoir of *L. monocytogenes*, has been heightened activity to develop improved methods for the detection and enumeration of *L. monocytogenes*. Much progress has been made since 1985 in developing both conventional and rapid methods for detecting *Listeria* in foods, in particular, *L. monocytogenes*. A variety of conventional or cultural methods have been employed, and intensively evaluated by collaborative studies aiming to provide the standard or reference methods. However, regarding rapid methods, none of the methods proposed has yet obtained universal acceptance to become officially accepted as standard or reference method (WHO Working Group, 1988).

1.3.2.1 Conventional methods

Enrichment

Listeria is known as a nonfastidious organism. Once isolated, the bacteria grow well on the usual bacteriological media (e.g. Tryptose Agar, Nutrient Agar and Blood Agar) (Jones, 1990). However, attempted isolation or reisolation of the organism from artificially or naturally contaminated food and clinical specimens is often unsuccessful. The primary isolation of *L. monocytogenes* from normally sterile sites such as blood, cerebrospinal fluid was often unsuccessful (Murray *et al.*, 1926; Gill, 1937; Gray *et al.*, 1948). More difficulties are encountered when samples such as clinical specimens (tissue

biopsies and autopsy specimens), food or environmental which contain small numbers of *L. monocytogenes* in combination with large populations of other contaminated or indigenous microorganisms.

In 1948, Gray *et al.* (1948) introduced a cold enrichment technique which required storage of the sample in nutrient broth as sole enrichment media at 4°C for several weeks. A portion was plated onto non-selective agar such as blood agar, Tryptose agar and TSA and incubated at 37°C for 18-24 hr and examined with obliquely transmitted illumination as described by Henry (1933) for typical bluish-green, *Listeria*-like colonies. If no *Listeria* is recovered further portions of the refrigerated samples are then plated at intervals for as long as 3 months. In some instances; (e.g. Kampelmacher and van Noorle Jansen, 1961, cited in Gray and Killinger, 1966) 6 months of refrigerated storage was necessary before *L. monocytogenes* could be detected and Weis and Seeliger (1975) also reported prolonged incubations up to 12 months.

The mechanism of the enhancing effect at 4°C is not fully understood. Several theories have been established to explain the success of cold enrichment. For foods samples, some authors (Doyle and Schoeni, 1987; Donnelly, 1988) suggest that the cold enrichment exploits the psychrotrophic nature of *Listeria* and simultaneously suppresses growth of other indigenous non-psychrotrophic micro-organisms. However, at this temperature *Listeria* also multiply slowly with a generation time of 1.5 days (Rosenow and Marth, 1987). Ryser *et al.* (1985) indicated that cold enrichment may play an important role in repairing sublethally injured *Listeria* which may have been present in cottage cheese manufactured from skim milk artificially contaminated with the pathogen.

Enhancement of *Listeria* populations during cold enrichment proved to be successful with such diverse samples as oat silage (Gray, 1960b), vegetation (Welshimer, 1968), and plants and soil (Welshimer and Donker-Voet, 1971). Enumeration of *L. monocytogenes* from various environmental samples such as river water, effluents, sewage, sewage sludge, soil (Watkins and Sleath, 1981; Fenlon, 1985) has been undertaken by Gray's cold-holding method with a most probable number (MPN) system. However, the length of the incubation period involved in cold enrichment makes this procedure impractical for use in routine regulatory analysis of food products.

In an attempt to reduce the period of cold incubation, Gray *et al.* (1950) noted that potassium tellurite gave satisfactory selectivity within 24 hours of incubation at 37°C. However, studies by other investigators (Seeliger, 1961; Kramer and Jones, 1969) have discouraged use of potassium tellurite as a *Listeria*-selective agent. Consequently, several inhibitory substances, including antibiotics, were examined for *Listeria* selectivity. The incorporation of specific selective agents into enrichment media has shortened the time

required to effectively isolate the organism. Ryser and Marth (1991) have extensively discussed the selective agents recommended by various authors. More recently, several enrichment broths have been used commonly for analysis of food products include FDA Enrichment Broth (Lovett, 1988) as well as Fraser Broth and USDA Listeria Enrichment Broth I and II (Dennis and Lee, 1989). As yet, no single protocol has been developed that is sufficiently sensitive to detect *L. monocytogenes* in all types of samples within a reasonable time. However, the FDA and USDA methods which use primary and secondary warm enrichment have recently been unofficially adopted as standard methods for the isolation of *L. monocytogenes* from various food items (Warburton *et al.*, 1991).

Direct Plating

Early attempts to isolate *Listeria* from food and environmental samples relied on clinical laboratory experience based on direct plating procedures and dealing with large numbers of an organism, often growing in almost pure culture under essentially ideal conditions (Albritton *et al.*, 1980). However, direct plating procedures generally have proven to be unsuccessful for isolating *Listeria* from foods and environments due either to the organism occurring in low numbers in the presence of competing microorganisms, or being sublethally injured (Buchanan *et al.*, 1989b) . Therefore, direct-plating does not reliably isolate *Listeria* spp. and typically is used in conjunction with a prior enrichment (Heisick *et al.*, 1995).

1.3.2.2 Rapid detection methods

The FDA and USDA enrichment/plating procedures have been used as standard methods to detect *L. monocytogenes* in dairy and meat products, respectively. Although these methods have drastically shortened the time of analysis as compared to the traditional cold enrichment procedure, the 3- to 6-day period needed to determine that a particular food sample is free of *L. monocytogenes* is unacceptable to large segments of the food industry which deal with highly perishable products such as fluid milk, raw meat, poultry, and seafood. Thus, a need exists for faster methods to detect *L. monocytogenes* and other pathogens in food with a short shelf life.

Recent advances in allied fields of immunology and microbial genetics have led to development of Enzyme Linked Immunosorbent Assays (ELISA), DNA probes, and PCR (Farber and Perterkin, 1991) which can be used to detect *L. monocytogenes* from food samples within several hours following primary and/or secondary warm enrichment (e.g. Oladepo *et al.*, 1992; Fluit *et al.*, 1993; Herman *et al.*, 1995; Avoyne *et al.*, 1997). Several of these assays are available commercially and can be used effectively to screen large numbers of food samples for presence of *Listeria* spp. However, before any of

these rapid methods can be adopted as "standard", scientists first must agree on a standard enrichment/plating procedure that can be used to measure the sensitivity and selectivity of these newly developed assays.

1.3.3 DIFFERENTIATION

1.3.3.1 Species typing methods

Biochemical Tests

The six species of *Listeria* are differentiated by the physical characteristics, biochemical reactions, haemolytic activity and CAMP test given in Table 1.1. Of the biochemical tests, the carbohydrate fermentation patterns are essential for differentiating *Listeria* species, with the exception of *L. monocytogenes* and *L. innocua*, which have identical patterns. These two species are separated by the absence of haemolytic activity in *L. innocua*, which is demonstrated by CAMP test (Christie *et al.*, 1944).

The term "CAMP" test originally applies to the synergistic reaction between *S. aureus* and group B streptococci as defined by Christie *et al.* (1944). Further development of CAMP test for *L. monocytogenes* was constituted by several studies (Fraser, 1964; Groves and Welshimer, 1977; Smola, 1989). Generally, CAMP test is performed on a sheep blood agar plate, with cultures of *Staphylococcus aureus* and *Rhodococcus equi* streaked in parallel in one direction. Test cultures of *Listeria* are streaked at right angles to those streaks, about 2 mm apart from the *S. aureus* and *R. equi* culture lines. After incubation at 37°C for 18 h, the plates are examined for an enhanced zone of haemolysis at either the *S. aureus* or *R. equi* streak line (Fig. 1.1). *L. ivanovii* gives a typical "shovel-shaped" zone of clearing only with *R. equi*. In contrast, *L. monocytogenes* shows smaller, rounder zone with *S. aureus* and negative reaction with *R. equi*. It has been reported recently that some strains of *L. monocytogenes* reacted synergistically with both *S. aureus* and *R. equi* (Skalka *et al.*, 1982; Smola, 1989). Smola (1989) noted the importance of the positive reaction between *L. monocytogenes* and *R. equi* to be related to virulence of *L. monocytogenes*. In support of this, McKellar (1994a), using *L. monocytogenes* mutants, demonstrated that: 1) the synergistic reaction with *S. aureus* involved either a phosphatidylcholine-specific phospholipase C or phosphatidylinositol-specific phospholipase C of *L. monocytogenes*, 2) Listeriolysin O (LLO) which is known to be essential for *L. monocytogenes* virulence (Cossart *et al.*, 1989; Portnoy *et al.*, 1992) is responsible for the CAMP reaction with *R. equi* and 3) *R. equi* cholesterol oxidase may involved in this synergistic reaction. The author suggested the absence of a *R. equi* response with virulent *L. monocytogenes* in some studies was due to failure of *R. equi* to
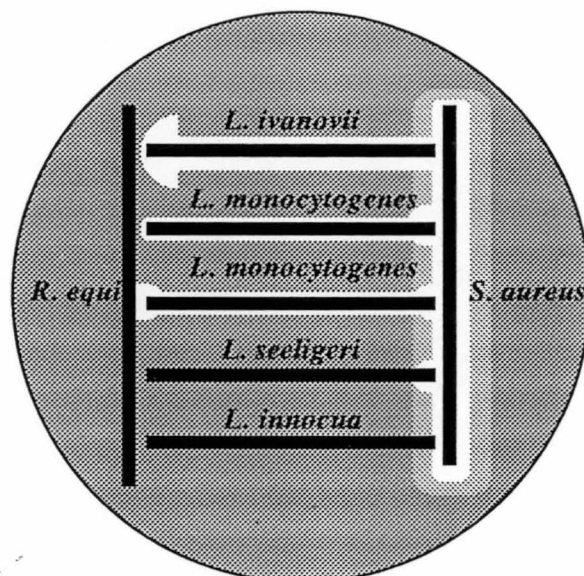
Figure 1.1 The reactions of *Listeria* species in CAMP test. Diagram indicates the locations of haemolytic enhancement regions.

produce sufficient cholesterol oxidase. The need for standardization of *R. equi* to obtain a valid reaction was also emphasized (Smola, 1989; Schuchat *et al.*, 1991b).

In addition, Skalka *et al.* (1982) reported positive hemolysis in *L. innocua* on rabbit erythrocytes which was not enhanced by *R. equi*. This apparent hemolysis was later elucidated by Pongratz and Seeliger (1984), cited in McKellar (1994b) to be attributed to lysis of erythrocytes by acid produced during growth of *L. innocua*.

Rapid Identification Methods

Most of the identification methods to date have only addressed the time consumption problem of the biochemical confirmation step, as they require pure cultures. Miniaturised biochemical tests such as MICRO-ID (Organon Teknika), RAP-ID and Minitek give results within 48 hours, respectively. Analytab Products Incorporated (API 20 STREP and API-ZYM) can identify *Listeria* to genus level after 4 hours of incubation. Vitek-AMS is fully automated and computerised and can provide identification in 4 to 24 hours, but cannot usually be afforded by small laboratories (Ryser and Marth, 1991). By quantitation of cellular fatty acids, gas chromatography can provide precise genera and

species identification within 24 hours, but again the necessary equipment is not available in most laboratories.

Fluorescent Antibodies (FA) provide fast identification and, although only genus specific, can also provide serotypic information and have the potential to be used directly on clinical and food samples or in conjunction with flow cytometry (Donnelly and Baigent, 1986). This technique can detect non-viable organisms or organisms in specimens from which culture is not possible (Vlahovic et al., 1988). Whilst this is an advantage clinically, for example in the diagnosis of a patient already receiving antibiotics, the implication of the presence of non-viable L. monocytogenes in foods is inconclusive in the absence of viable organisms. Furthermore, positive FA results only serve as corroborative evidence in identification of Listeria and confirmation is required by pure culture and biochemical tests (Difco, 1984).

Various systems for detecting L. monocytogenes by using either monoclonal antibodies or nucleic acid hybridisation probes alone or in conjunction with DNA amplification technology have been reported (Bessesen et al., 1990). Monoclonal antibodies developed to cell surface antigens only provide information to genus level (Fitter et al., 1992). Probes can be designed to provide the desired level of identification (genus; for example Gene Trak Listeria colorimetric assays, species or subspecies), but the technology lacks sensitivity and therefore requires large numbers of target cells (10^5-10^6 cfu/ml or colonies on solid agar) in the presence of non-target background (Datta et al., 1987).

1.3.3.2 Intraspecies typing methods

For identification of the source of clinical listeriosis and epidemiological investigations of listeriosis including the source of distribution of L. monocytogenes in food and food processing factories, it is necessary to type isolates beyond the species level. Therefore, potential sources of contamination can be confirmed or excluded and appropriate action taken. Serological and phage typing have been developed. Isolates of Listeria can also be discriminated to strains by several molecular typing methods in some instances by: protein-based method such as multilocus enzyme electrophoresis (MEE), nucleic acid-based methods such as ribotyping, pulse field gel electrophoresis (PFGE), and polymerase chain reaction (PCR)-based fingerprinting etc. Each of these techniques have inherent advantages and drawbacks.

• Serotyping is commonly performed as a means of subtyping L. monocytogenes based on variations in somatic (O) and flagellar (H) antigens. There are at least 16 serovars of Listeria in the current scheme based on the serological grouping of 14 heat-stable somatic

(O) antigens and 4 heat-labile flagellar (H) antigens (Seeliger and Höhne, 1979). The serotyping, however, is limited in application to epidemiological studies of *L. mono-cytogenes* as it lacks sufficient information and discriminatory abilities. There are only three serotypes, 4b, 1/2a and 1/2b, that are responsible for the majority of listeriosis outbreaks (Farber and Peterkin, 1991). However, it may be useful in combination with another typing method.

• Phage typing has also been widely employed for typing of *L. monocytogenes*. The method is based on the lytic properties of different phages. It is highly reproducible and provides an acceptable level of discrimination, however, many strains are untypable with the existing set of phages (McLauchlin *et al.*, 1986; Monfort *et al.*, 1998). In addition, only a small number of laboratories are involved in storing and maintaining phage culture collections against *L. monocytogenes*.

• Multilocus Enzyme Electrophoresis (MEE) is a protein-based method involving the determination of the mobilities, in a starch gel matrix, of a selected set of metabolic enzymes (Selander *et al.*, 1986). MEE is a time consuming method but its results can be directly correlated with the genotype (Swaminathan and Matar, 1993). Therefore, it was used extensively for the study of bacterial populations and evolutionary genetics and for epidemiology of infectious diseases including *L. monocytogenes* (Bibb *et al.*, 1990; Baxter *et al.*, 1993; Nørrung and Skovgaard, 1993).

• Chromosomal DNA restriction analysis or restriction endonuclease analysis (REA) or microrestriction analysis was the first of the chromosomal DNA-based typing schemes (Farber, 1996). The method involves cutting chromosomal DNA with a fragment-cutting restriction enzyme, and separating the DNA fragments by size using electrophoretic techniques. Differences in the fingerprint patterns of two isolates is referred to as a restriction-fragment length polymorphism (RFLP). REA is a rapid, reproducible, inexpensive method and relatively simple to perform. However, the genomic restriction fragments are usually too numerous and too closely spaced (Farber, 1996). Therefore, a number of restriction endonucleases have to be screened before the proper enzyme and conditions can be specified. REA typing has recently been used to demonstrate that *L. monocytogenes* isolates from the 1981 Nova Scotia, 1983 Massachusetts, and 1985 California outbreaks each exhibit a unique restriction enzyme pattern (Wesley and Ashton, 1991).

• Ribosomal DNA RFLP analysis or ribotyping refers to the use of nucleic acid probes to recognize ribosomal RNA (rRNA) genes which are present in all bacteria (Swaminathan and Matar, 1993). Since the genes coding for rRNA are very highly

conserved, a single probe can be used to subtype all eubacteria (Farber, 1996). The method is technically demanding and time consuming. It involves digested bacterial chromosomal DNA, electrophoresed restricted DNA onto agarose gel. The restricted DNA then is transferred onto a solid support for probing which is processed with a labeled probe such as radioactive probes, or nonisotopic cold-labeling systems. Ribotyping provides reproducible patterns which are not too complex in comparison between different strains. Recently, an automated system, the 'Riboprinter™ Microbial Characterisation System', has been developed by E.I. DuPont. An extensive computer database was developed for *Listeria* spp. including *L. monocytogenes* and incorporated computer analysis of a standard so that results which vary in different runs, times and places may be compared (Ryser, 1995).

• Pulse field gel electrophoresis (PFGE) or DNA macrorestriction analysis (Boerlin, 1995) uses restriction endonucleases that cut DNA infrequently which allows the generation of large fragments of chromosomal DNA (Swaminathan and Matar, 1993). Special methodology is needed to avoid shearing the bacterial DNA. The resulting DNA fragments are separated by pulse field gel electrophoresis. PFGE is a time consuming and technically demanding method (Farber, 1996). However, the method is very discriminatory and reproducible and has recently been used in the investigation of foodborne listeriosis in United States (Proctor *et al.*, 1995).

• A major advantage of PCR-based methods, its exquisite sensitivity, is also its main disadvantage since it is extremely sensitive to contamination by template DNA and preamplified PCR product. Preamplified products or amplicons are a highly concentrated source of primer template. Contamination of assays prior to PCR by amplicons may result in false positive results. The PCR-based method may be categorized into two types;

a) Using the restriction fragment length polymorphism (RFLP) method, a large number of fragments released from chromosomal DNA may cause an uneasy comparison of patterns from different isolates. PCR-based RFLP methods overcomes this problem by examining RFLPs within smaller portions of the chromosome (Thomas, 1995). This involves amplifying a known DNA sequence, followed by digestion with restriction enzyme and comparing restriction fragments of the amplified DNA from different strains. The method was reported to be reproducible and provide high discrimination for *L. monocytogenes* strains 1/2a, but less discrimination for strains 1/2b and showed to be identical for strains 4b (McLauchlin, 1996). The method is expensive to establish, but its main drawback in application to *L. monocytogenes* is that the results are frequently too complex for practical use in epidemiological typing, and

b) a PCR-based method that requires no prior knowledge of the target DNA sequence but randomly amplifies segments of the target DNA by using a single primer e.g. random amplification of polymorphic DNA (RAPD) and repetitive sequence element (rep) PCR. The method is probably the simplest DNA-based subtyping method to date with simple, reproducible fingerprints of genomic DNA generated. The RAPD-PCR uses single short oligonucleotides of arbitrary sequence to prime DNA synthesis at low stringency from pairs of sites to which the oligonucleotide almost matches. This generates strain-specific arrays of anonymous amplified DNA fragments (Swaminathan and Matar, 1993). Czajka *et al.* (1993) reported RAPD to be able to discriminate within and between serotypes of *L. monocytogenes*. The rep-PCR, uses consensus primers in the PCR to amplify DNA sequences located between successive repetitive elements. The high homology of repetitive sequences of the primers allows for the use of more stringent PCR conditions compared to RAPD which may reduce experimental variation and increase the reproducibility of the technique (Louws *et al.*, 1994; Jersek *et al.*, 1996). The method has been applied succesfully to *Listeria* spp. especially *L. monocytogenes* (Jersek *et al.*, 1996).

1.4 OCCURRENCE OF *LISTERIA* IN NATURAL ENVIRONMENTS

Listeria is widespread in nature. This organism is frequently isolated from a large variety of environments including plants, soil, silage, animals, sewage, and water and food consumed by humans including vegetable, dairy, red meat, poultry and seafood (Odegard *et al.*, 1952; Welshimer, 1960, 1968; Seeliger, 1961; Gray and Killinger, 1966; Weis and Seeliger, 1975; Watkins and Sleath, 1981; Schlech *et al.*, 1983; Weagant *et al.*, 1988; Colburn *et al.*, 1990; Ryser and Marth, 1991).

1.4.1 PLANT AND SOIL

The epidemiology of listeriosis is perplexing and the habitat of *L. monocytogenes* is obscure. Since *Listeria* have been isolated from many of non-clinical sources e.g. soil, decaying vegetation and silage etc., the concept of *L. monocytogenes* as a "saprophytic pathogen with an opportunistic mode of spread" now becomes increasingly attractive, along with the hypothesis of Seeliger (1961) who commented on the resemblances of the biochemical and cultural characteristic of *L. monocytogenes* to some plant-soil inhabitants. Seeliger (1961) further speculated "that there may well be a primary saprophytic life of *Listeria*", in which event the epidemiology and epizootology of many listeric infections would be more comprehensible. In addition, Weis and Seeliger (1975) found that there was increasing evidence for a high incidence of *Listeria* in plants and soil samples: *Listeria* can be isolated frequently from old faded, or mouldy plants particularly

from the surface soil in uncultivated fields. Many reviews (Brackett, 1988; Sutherland, 1989; Miller *et al.*, 1990; Ryser and Marth, 1991) also described *Listeria* as being isolated from dead and decaying plant matter.

Relatively large numbers of *L. monocytogenes* were isolated from samples of mud which suggests that a moist environment favours growth of the organism (Weis and Seeliger, 1975). Welshimer and Donker-Voet (1971), could not isolate *L. monocytogenes* from soil or dead green vegetation collected in early autumn, however, the organism was detected in almost all samples of soil and decayed vegetation the following spring.

Survival of *L. monocytogenes* in soil depends on type of soil and its moisture content (Welshimer, 1960; Welshimer and Donker-Voet, 1971). Welshimer (1960) demonstrated that *L. monocytogenes* could survive in soil for up to 295 days. Botzler *et al.* (1974) reported that the organism survived at high concentrations in the soil for several weeks despite cold weather during winter at average high and low temperatures of 8° and -15°C respectively, and competition from the microbial flora. Thus, the ability of *Listeria* to multiply at low temperature, its ability to survive for long periods in soil (Welshimer, 1960), and its recovery from decaying vegetation implies a saprophytic existence wherein the plant-soil environment may serve as a reservoir. Accordingly the organism can be contracted by humans and animals via many possible routes from many sources.

1.4.2 ANIMAL FEED (SILAGE)

Several investigators have studied extensively the relationship between listeriosis in ruminants and silage consumption. The possible role of silage in the transmission of listeriosis was suggested in 1922 when results of an investigation in Iceland indicated a disease resembling listeriosis (known in Iceland as votheysveili or silage sickness) which was relatively common in silage fed-animals (Gray, 1960a). Olafson (1940) also observed the close relationship between silage feeding and onset of listeriosis. However, the apparent relationship was not clarified until 1960 when Gray (1960a) demonstrated an epidemiological relationship by isolating the same *Listeria* serotype from the brain of an infected sheep and from the oat silage on which the flock was being fed. In further investigation, Gray (1960a) also reported isolating *L. monocytogenes* from the viscera of a female mouse and the foetuses of a pregnant mouse fed poor-grade silage which was thought to have caused death and abortion in cattle because it was contaminated with *L. monocytogenes*. Identical serotypes of *L. monocytogenes* were isolated postmortem from the mice and cow. Kampelmacher and van Noorle Jansen (1979), cited in Fenlon (1985) that many cases of listeriosis were found in farm animals in The Netherlands during the period 1957-1976 which showed the geographical distribution of the disease

coincided with areas where the silage was significantly inferior in quality: when the standard of silage-making improved the incidence of the disease decreased. In the United Kingdom the number of incidents of listeriosis in sheep increased dramatically from 53 in 1976 to more than 230 in 1983. The pattern of listeriosis is also changing from isolated cases to larger flock outbreaks as highlighted in a recent outbreak in Scotland among a flock of 196 pregnant ewes fed poor quality silage (Low and Renton, 1985); *L. monocytogenes* of the same serotype was isolated from silage and from various organs of the ewes which died, thus supporting the link between silage feeding and listeriosis. Numerous reports exist of listeriosis outbreaks in sheep and cows resulting from consumption of contaminated silage (Grønstøl, 1979, 1980; Fenlon, 1985, 1986; Gitter *et al.*, 1986; Wilesmith and Gitter, 1986).

L. monocytogenes has most frequently been associated with poor-quality silage which had pH >4.5 (Grønstøl, 1979; Fenlon, 1985; Gitter *et al.*, 1986). Grønstøl (1979) isolated *L. monocytogenes* from 22, 37, and 56% of silage samples with pH values <4.0, 4.0-5.0, and >5.0 respectively. Perry and Donnelly (1990) also found 13 and 64% of *Listeria* species in silage samples which had pH below and above 5.0 respectively, and demonstrated that the incidence of *Listeria* increased concomitantly with the increasing of pH of silage. In another survey by Fensterbank *et al.* (1984), cited in Ryser and Marth (1991), *L. monocytogenes* was isolated from 11 of 31 silages of excellent quality which had pH values between 3.6 and 4.0. Gouet *et al.* (1977) showed that *L. monocytogenes* failed to grow at pH <5.0 in gnotobiotic silage manufactured with a defined flora of lactic acid bacteria. Not only did *L. monocytogenes* fail to grow, but the organism was supposedly eliminated from the silage after 30 days of storage at 20°C. In contrast, Dijkstra (1975) demonstrated that *L. monocytogenes* can survive 4-6 years in naturally contaminated silage stored at 5°C.

Fenlon (1985) suggested that low-quality silage with a pH value higher than 6.0 was usually due to aerobic deterioration caused by mould growth. These were the silages most likely to harbour *Listeria* and it is probable that in such bales no fermentation had occurred. It is suggested that fermentation in silages by indigenous lactic acid bacteria which is the dominant micro-organism will result in bacteriocin production and a low pH product which will rapidly inhibit spoilage bacteria (McDonald, 1970). However, in many silage fermentations, the lactic acid levels are too low to reduce the pH to this critical point (approximate pH 4.5), allowing *Listeria* and other organisms to proliferate. In some cases, *Listeria* will not proliferate due to other inhibitory compounds, such as hydrogen peroxide, found in silage (Price and Lee, 1970).

The origin of *Listeria* in silage is still uncertain. Fenlon (1985) suggested birds as possible sources. Gulls and rooks often forage for insects among freshly cut grass

wilting in fields. Gulls may act as a vector, transferring organisms in sewage sludge and untreated sewage outfall from one place to another without becoming overtly infected and, therefore, play a significant role in initial contamination of grasses used for silage. Faecal specimens from seagulls feeding at sewage sites were found to have a higher rate of carriage of *Listeria* spp., with no seasonal difference than those from gulls feeding elsewhere (Fenlon, 1985). The role of other bird species is less certain. Rooks frequently feed on pastures, but do not scavenge on sewage outfalls and this is reflected in their faecal microflora. Faecal specimens from rooks normally presented a low incidence of *Listeria* species (Fenlon, 1985). Other sources of *Listeria* species such as decaying plant material and soil have been suggested (see section 2.3.1).

In addition to traditional silage and less typical varieties prepared from orange peels and artichokes, other types of animal feed were also linked to outbreaks of listeriosis (Vizcaino *et al.*, 1988). For more than 80 years, ranchers in Canada and the North Western United States have recorded numerous cases of listeric-like abortion in cattle that grazed on ponderosa pine needles. Adams *et al.* (1979) isolated *L. monocytogenes* from the blood of mice fed a chow diet consisting of ground ponderosa pine needles. Injection of the *Listeria* isolate into mice caused symptoms that mimicked listeriosis in cattle, which suggests a possible link between the bacterium and "pine needle abortion".

1.4.3 WASTE PRODUCTS

Regarded as a potential risk to human and animal health, waste products i.e. raw sewage, sewage sludge and final discharge are considered to be an important reservoir in the epidemiology of *L monocytogenes*. It has been reported (Watson, 1985) that of the total sludge produced at sewage works in England and Wales, approximately 20% is disposed of at sea, 40% is applied to agricultural land, and 40% is applied to other land or incinerated. *L. monocytogenes* was found to be present in large numbers in sewage and sludge (Watson, 1985). The most popular method for disposal of liquid sludge is application to land (Miller *et al.*, 1990).

Watkin and Sleath (1981) reported finding *L. monocytogenes* at levels between 700 and > 18,000 CFU/L in effluent from primary tanks of sewage treatment plants in England. That investigation has also shown that *L. monocytogenes* is widely distributed in sewage and that the numbers contributed to the environment by sewage and sewage sludge may well be higher and could survive for longer periods than *Salmonella* species. The survival time studies carried out by those authors indicated that for sewage sludge sprayed onto land, these was no detectable reduction in the numbers of *Listeria* eight weeks after spraying. Thus environmental persistence could represent a concern with respect to

public health.   Likewise, Kampelmacher and van Noorle Jansen (1975) detected *L. monocytogenes* from 35 (91.8%) of the 38 effluent samples in the Netherlands.   Watkins and Sleath (1981) and Dijkstra (1982) also isolated *Listeria* from surface waters and suggested that waters receiving effluent may be a route for recycling *Listeria*.   Dijkstra (1982) showed that such waters were contaminated up to a distance of 25 miles (40 kms) from a treatment plant.

Sixty-six samples of waste water and of the effluent after the biological step (via oxidation) in the waste water pretreatment plant of Braunschweig, West Germany, were investigated (Geuenich and Müller, 1984).   The authors detected 697 strains of *Listeria* which 586 (84%) were *L. monocytogenes*.   The concentration of *Listeria* varied between 10^3 and 10^5 cells/L.   In general, there were about 10 times more *Listeria* in the sludge than in the clearly filtered waste water.   Furthermore, a multiplication of *Listeria* in 45% of all cases was also observed.   The authors finally pointed out that the biological oxidation during the waste water treatment does not appear to be highly effective in reducing populations of viable *Listeria* in sewage effluent.

In 1986, Al-Ghazali and Al-Azawi (1986) reported the isolation of *L. monocytogenes* from a sewage treatment plant in Baghdad, Iraq.   *Listeria* was isolated from all test samples at each stage of treatment.   Highest numbers (1,100 counts/g) were recorded in raw sewage sludge, while the lowest (<3 counts/g) were observed in sludge cake. Digested sludge also showed a decrease in the number of *L. monocytogenes*.   Low numbers recorded in sludge cake during the summer period coincided with low moisture content, which was less than 3.7%.   The pH of the sludge cake ranged from 6.1 to 8.6. The same workers continued the study in the same sewage treatment plant together with a newer one in 1988 (Al-Ghazali and Al-Azawi, 1988b).   The results again showed that *L. monocytogenes* survived in all stages of the treatment.   However, a high reduction after the sludge activation and sludge digestion processes in both plants were observed.   It was noted that the numbers of *L. monocytogenes* in incoming raw sewage, which originated mainly from domestic waste, was relatively higher than the combined industrial and domestic sources raw sewage.

The presence of *L. monocytogenes* in domestic sewage can be considered as a primary point-source for spreading it via sewage sludge.   The ability of this organism to survive the treatment process indicates the hazards of applying sewage sludge cake to land.   This can be of epidemiological significance, particularly with respect to the infection of animals (Al-Ghazali and Al-Azawi, 1986).

1.4.4 WATER AND SEDIMENT

Estuarine and coastal environments are continuously subjected to potential contamination with *Listeria* species. Sources include sewage effluents (Al-Ghazali and Al-Azawi, 1986), processing plant effluents (Watkins and Sleath, 1981), and agricultural runoff (Heisick *et al.*, 1989). Faecal coliform bacteria are routinely used to monitor the environmental impact of many of these sources; however, their relationship to *Listeria* species remains undefined. As part of a survey of sewage-impacted environments, Watkins and Sleath (1981) investigated river water in the United Kingdom in 1981. The results indicated that *L. monocytogenes* was present in all samples of river waters (n=7) in considerable numbers (3 to >180 counts/L), often in excess of *Salmonella* species. The authors suggested it was important to determine the presence of *L. monocytogenes* within the water cycle in order to assess better its epidemiological significance.

According to Dijkstra (1982), *L. monocytogenes* occurred in 21% of the surface water samples obtained from canals and lakes in northern Holland. Even though the lakes were frequented by swimmers, no case of human listeriosis was reported. In the same study, *L. monocytogenes* also was detected in 67% of the samples of sewage effluent. Although samples of sea water were negative, the bacterium was still found in a canal 25 miles (40 kms) downstream from the sewage treatment plant at the point where the canal emptied into the sea.

The occurrence of *Listeria* species in sediment, saltwater and freshwater tributaries draining into Humboldt-Arcata Bay, California during winter (January-February) in 1990, was reported by Colburn *et al.* (1990). The results demonstrated *Listeria* spp. were more prevalent in fresh water (81%) than in marine waters (33%) and sediment samples from Elk River, Ryan slough and McDoniel Slough (30.4%). This difference could be due to a variety of reasons such as different levels of available nutrients, presence of toxic compounds, and predation by other organisms (Roszak and Colwell, 1987). The effect of dilution by the large volumes of seawater in the marine environment may also result in lower numbers of *Listeria* spp. in marine habitats compared with fresh water. The incidence of *Listeria* spp. remained high throughout the freshwater tributaries entering Humboldt-Arcata Bay. Furthermore, a given species or *L. monocytogenes* serogroup appeared to predominate in fresh water when domesticated animals (cows, horses) were nearby, whereas greater diversity, and no species predominance was observed in areas with no direct animal influents. Slight variations in salinity due to tidal action did not appear to affect the distribution of *Listeria* spp. in this water system.

Colburn *et al.* (1990) suggested that there was a consistent input of *Listeria* spp. from these fresh water tributaries draining into Humboldt-Arcata Bay. *Listeria* spp. could also

be introduced to the bay via other sources such as by runoff from urban area of Eureka, California. In addition, the influence of a large local seagull population observed there and the presence of other marine birds was also considered to be a consistent source of *Listeria* spp. contaminating the marine environment (Fenlon, 1985).

The water in the US. Gulf Coast was also examined to determine the presence of *Listeria* spp. (Motes, 1991). The highest occurrence (10%) of *Listeria* spp. from water occurred at water temperatures ≤20°C. Salinity of water from ambient environments had little effect on the recovery of *Listeria* spp. These results suggest that the occurrence of *Listeria* spp. in coastal environments is not limited by specific hydrographic parameters and cannot be predicted.

1.5 OCCURRENCE OF *LISTERIA* IN FOOD

1.5.1 DAIRY PRODUCTS

The listeriosis outbreaks associated with milk and its products, including cheese are well known. In 1983, pasteurized milk was incriminated as the vehicle of the outbreak in Massachusetts (Fleming *et al.*, 1985). In Los Angeles Country, California, 142 cases of human listeriosis were reported in 1985. A case-control study implicated Mexican-style soft cheese as the vehicle of infection (Linnan *et al.*, 1988). Mortality rates for both outbreaks were approximately 30%.

Following the outbreak of human listeriosis that occurred in Massachusetts and Connecticut during July and August 1983, Hayes *et al.* (1986) investigated raw milk collected from three different sources; individual farms, the milk cooperative, and the pasteurizing plant in USA. The authors isolated *L. monocytogenes* from 12% of samples with a variety of serotypes, including 1a, 3b, 4b, and 4a,b. In the following year, Lovett *et al.* (1987) investigated the incidence of *L. monocytogenes* in raw milk from three areas of the United States. The incidence varied by area from 0% in California, 3.7% in Tri-State and 7.0% in Massachusetts (Table 1.2). The authors further found a low concentration of the organism (<1 cell/ml) in raw milk which similar to the investigations in UK (Fenlon and Wilson, 1989; Fenlon *et al.*, 1995). Additionally, Liewen and Plautz (1988) determined the incidence of *L. monocytogenes* in raw milk obtained from bulk storage tanks on 100 dairy farms in eastern Nebraska during 1986 (Table 1.2). *L. monocytogenes* was found in 6% and 2% of samples collected in February and July respectively.

A seasonal variation in incidence was noted by Lovett *et al.* (1987), i.e. lowest during hot and highest in cold weather months. On the contrary, Farber *et al.* (1988) reported the

lower incidence in winter when 445 samples of bulk milk in Ontario were examined and an overall incidence of *L. monocytogenes* of 1.3% was found (Table 1.2). The seasonal variation was not apparent in the survey by Fenlon and Wilson (1989) who reported the low contamination of *L. monocytogenes* in milk ranging from 3.8% in summer samples to 1% in autumn.

Table 1.2 Incidence of *L. monocytogenes* in raw milk.

Country	No. of samples analysed	No. positive (%)	Reference
USA	121	(12.0)	Hayes *et al.*, 1986
USA/Tri-state	350	13 (3.7)	Lovett *et al.*, 1987
USA/California	100	0	"
USA/Massachusetts	200	14 (7.0)	"
USA/Nebraska	200	8 (4.0)	Liewen and Plautz, 1988
Canada/Ontario	445	(1.3)	Farber *et al.*, 1988
UK/North-East Scotland	540	14 (2.6)	Fenlon and Wilson, 1989
UK	160 farms[a]	25 farms (16)	Fenlon *et al.*, 1995
Australia/NSW	69	1 (1.45)	Arnold and Coble, 1995

[a] the study was done over one year (4 samplings for each farm).

In Canada in 1988, the Department of National Health and Welfare initiated a project to determine the health risk of food contamination due to *L. monocytogenes* (Farber *et al.*, 1989a). Various retail foods were analyzed including 14 samples of pasteurized milk which were found to be free of *L. monocytogenes*. Of 530 samples of ice cream products obtained at the manufacturing level, only 2 were positive for *L. monocytogenes*.

In addition to identifying specific environmental sources of *Listeria* in dairy plants, Klausner and Donnelly (1991) conducted a survey of 361 environmental samples in 34 Vermont dairy processing plants. By focusing on floors and other nonproduct contact surfaces, the authors indicated that fluid plants had the highest incidence of *Listeria* when compared to cheese plants or other types of dairy manufacturing plants. The overall incidence of *L. monocytogenes* and *L. innocua* were 1.4% and 16.1% respectively.

In Canton de Vaud (western part of Switzerland), the incidence of human listeriosis has been carefully followed since 1970 (Bille, 1990). Only sporadic cases had been observed until 1983: 122 human cases were recorded between 1983 and 1987 in the Canton de Vaud. In 1987 a case control study was initiated and showed that the Vacherin Mont d'Or

soft-ripened cheese was the source of the disease. Thus, in late November 1987, the authorities decided to recall the product and to stop its production. Following these measures, the number of new cases has dropped dramatically in the area.

In Australia, the Microbiology Laboratory of the NSW Health Department Division of Analytical Laboratories reported the investigation of the contamination in foods sold in NSW during January 1986 to November 1993 (Arnold and Coble, 1995). The survey of dairy products revealed the presence of *Listeria* in 9.4% (68 samples) and *L. monocytogenes* in 5.4% (39 samples) of 725 samples. The dairy products positive for *L. monocytogenes* were raw goat milk (1 sample), chocolate coated ice creams (23 samples) and soft cheese (15 samples).

1.5.2 MEAT PRODUCTS

Recognition of *L. monocytogenes* as a foodborne pathogen has raised concerns about the possible role of meat products as vehicles of listeric infections. An outbreak in Western Australia in 1990 has been linked to a contaminated paté (Watson *et al.*, 1990). In the United States, a case-control study involving 82 sporadic cases of listeriosis was undertaken by the Centers of Disease Control, victims were reported to have eaten undercooked chicken or uncooked hot dogs (Schwartz *et al.*, 1988). Following this report, Genigeorgis *et al.* (1989) conducted a study of the skin of poultry wings, legs (drumsticks) and whole livers purchased from supermarkets in Davis, California. The prevalence of *L. monocytogenes* was 10%, 15%, and 14% respectively. The authors also investigated 12 locations and finished poultry products within a slaughterhouse, and isolated *L. monocytogenes* from skins of wings and drumsticks and whole livers at the end of the processing line at 70.0%, 36.7% and 33.3% respectively. After 4 days of storage of the same packages at 4°C *L. monocytogenes* was recovered from 40%, 52% and 72% of the respective products. The prevalence of *L. monocytogenes* on the hands and gloves of the persons hanging birds after chilling, cutting carcasses, and packaging parts was 20%, 45.5% and 59%, respectively.

In January 1987, the Microbiology Division of the Food Safety and Inspection Service (FSIS) initiated national monitoring programs to determine the incidence of *L. monocytogenes* in domestically produced raw meat (Carosella, 1990). There are approximately 1,300 beef slaughter plants operating in the U.S., from which 30% of all the samples were investigated for *L. monocytogenes*. The results from the monitoring program for raw beef showed 41 of 658 samples positives for *L. monocytogenes*. The monitoring program also provided information on seasonal distribution of *L. monocytogenes* which showed a dramatic increase in the incidence of *Listeria* during the spring of 1988. The

author suggested the incidence of *L. monocytogenes* in raw beef may be related to the age of the animals.

During spring 1990, Vorster *et al.* (1993) monitored 134 samples of RTE food, vienna sausages, ham and cervelat, from 17 supermarkets in the Pretoria area (South Africa). Eleven samples (8.2%) contained *Listeria* species, with a higher incidence found in ham (14.0%, n=43) than in cervelat (6.8%, n=44) or vienna sausage samples (4.3%, n=47).

In Beijing, China, the presence of *L. monocytogenes* in retail meats (25 pork, 10 beef, 14 lamb and 21 chicken) were analyzed by Wang *et al.* (1992). Seven pork and one chicken sample contained *L. monocytogenes*, whereas all beef and lamb were free of *L. monocytogenes*. Meanwhile, 15 (60%) pork, 11 (52%) chicken, 7 (70%) beef, and 6 (43%) lamb samples were positive for other *Listeria* spp.

A variety of foods from local markets in Taipei, Taiwan was examined by Wong *et al.* (1990). High incidence of *L. monocytogenes* was found in raw meat samples e.g. 58.8% of pork samples, 50% of chicken carcaseses and 38% of turkey parts, and 34% of frozen semiready foods i.e. various types of dumplings, fish balls and meat balls. However, only 4.4% of frozen cooked foods (frozen dim sum) were positive for *L. monocytogenes*.

1.5.3 FRUITS AND VEGETABLES PRODUCTS

Fruits and vegetables are less often mentioned as sources of *L. monocytogenes* than other foods (Brackett, 1988). However, raw vegetable products, e.g. coleslaw, were implicated in a large outbreak in the Maritime Province of Nova Scotia in 1981. There were 34 cases of perinatal listeriosis (9 cases of abortion or stillbirth, 23 cases of live birth but of a seriously ill infant, 2 cases of live birth of a well infant), 7 cases of nonpregnant adult listeriosis (6 cases of meningitis, and one case of aspiration pneumonia and sepsis). The fatality rate for infants born alive was 27%. The mortality in meningitis cases was 33%. *L. monocytogenes* serotype 4b was isolated from patient's blood, from coleslaw from the refrigerator of the patients, and from unopened packages of coleslaw from the same processing plant.

Schlech *et al.* (1983) reported this outbreak may be a case of indirect transmission of listeriosis from an animal reservoir to human beings. The cabbage used in the implicated coleslaw was grown on a farm fertilizing with sheep manure from flocks with known cases of listeriosis. However, some researchers pointed out that fruits and vegetables could likely become contaminated without manure fertilizing as a causative factor because

of the close relationship of *L. monocytogenes* and plant products and soil (Brackett, 1988; Sizmur and Walker, 1988).

Ho *et al.* (1986) reported other outbreak of *L. monocytogenes* serotype 4b in eight Boston-area hospitals. Fresh celery, tomatoes, and lettuce were linked to listeriosis in hospitalized, immunosuppressed patients.

Several surveillance studies have been conducted on the occurrence of *L. monocytogenes* on fruits and vegetables. An 11-months survey of 1,000 samples of 10 types of fresh produce from Minneapolis area supermarkets (Heisick *et al.*, 1989) revealed the occurrence of *L. monocytogenes* on 28 (21.2%) potato samples, 19 (14.4%) radish samples, 2 (2.2%) of cucumbers, and 1 (1.1%) of cabbage. The researchers indicated the contamination especially on radishes and potatoes were found throughout the year. However, lettuce and mushrooms were only contaminated by *L. innocua*, whereas broccoli, carrots, cauliflower, and tomatoes were free of *Listeria* spp.

Four of 60 samples of refrigerated ready-to-eat salads of ten different varieties including beansprouts alone, mixed vegetable salads, and salads containing nuts and fruit were reported to be contaminated by *L. monocytogenes* (Sizmur and Walker, 1988). Fruits and vegetables included in two types of those contaminated salads were cabbage, celery, onion, carrots, lettuce, cucumber, radish, fennel, watercress, leeks, and sultanas.

A surveillance study of various retail foods in Canada was also conducted (Farber *et al.*, 1989a). No *L. monocytogenes* was found in 110 raw vegetable samples including lettuce, celery, tomatoes, and radishes. However, *L. ivanovii* was isolated from 1 (10%) radish.

1.5.4 SEAFOOD PRODUCTS

Scientists in many countries have surveyed for the occurrence of *L. monocytogenes* in seafood products. Fuchs and Surendran (1989) monitored 35 fish and fishery product samples from local retail outlets in Cochin, India. None of the samples tested was positive for *L. monocytogenes*. However, *L. innocua* was detected in 3 of 10 fresh samples and 5 of 14 frozen samples. No *Listeria* was detected in dried, salted fish.

In the study of Weagant *et al.* (1988) in domestic and imported frozen seafood products from several countries, 35 of 57 samples (61%) tested positive for *Listeria* species and 15 of 57 samples (26%) were positive for *L. monocytogenes*. *Listeria* species were found in samples from 9 different countries of 12 that were examined (Table 1.3).

Table 1.3 Incidence of *Listeria* species in frozen seafoods by country of origin.

Country	No. samples Tested	No. samples (subsamples) positive for[a]:	
		L. innocua	*L. monocytogenes*
Canada	4 (30)[b]	1 (1)	1 (1) {1a (2)}[c]
Chile	7 (70)	4 (14)	3 (14) {4b (6)}
China (PRC)	1 (10)	1 (1)	0
Equador	1 (10)	0	0
Japan	8 (80)	3 (4)	2 (20) {1a (1)}
Korea	18 (152)	10 (32)	3 (4) {1a (1), 1a (2)}
Mexico	1 (10)	0	0
Philippines	6 (60)	4 (13)	1 (1) {4b (6)}
Singapore	1 (10)	1 (3)	0
Taiwan	2 (20)	2 (12)	1 (1) {1a (2)}
Thailand	1 (10)	0	0
USA	7 (58)	0	1 (1) {1a (2)}
Total	57 (520)	26 (80)	15 (54)

[a] No other *Listeria* species found in 57 samples. [b] Number of subsamples tested. [c] Serotype of *L. mono-cytogenes* isolates. (After Weagant *et al.*, 1988)

In the survey of 57 seafood samples, frozen and refrigerated fishes, squids and crabs, from local markets in Taipei, Taiwan, Wong *et al.* (1990) isolated *L. monocytogenes* in 10.5% of the seafood samples. It was noted that the positive results were obtained only in fish and squid samples, in which all of the serotypes were types 1 and 4 with the majority being of type 1.

A quantitative study, using a three-tube MPN method, on the levels of *Listeria* spp. in retail-level food products including seafood was conducted by Buchanan *et al.* (1989b). The levels of *Listeria* spp. detected in the positive seafood samples varied in a great range from 0.36 to >110 MPN(CFU/g). The incidence rate for *Listeria* spp. isolated from both shellfish and finfish was 28%. The positive isolations for *L. monocytogenes*, 11%, were detected only in two finfish samples (flounder and monkfish).

Seventy-one smoked fish samples were surveyed from Newfoundland retail markets and tested for the prevalence of *Listeria* (Dillon *et al.*, 1992). Fifty-one percent of the samples collected were hot smoked including herring, mackerel and caplin and 49% were cold-smoked including salmon, charr and cod. *Listeria* was present in 11.3% of the smoked seafood samples; 4 (50%) smoked cod, 3 (27%) smoked mackerel and 1 (6.7%) smoked caplin were found to harbour the bacterium.

1.6 OUTBREAKS OF LISTERIOSIS

L. monocytogenes has been recognised and studied mainly in industrialised countries. Sporadic cases and occasional outbreaks of human listeriosis with examples contaminated food have been detected. Most of the listeriosis reports have been studied in countries with a temperate climate (e.g. Fleming *et al.*, 1985; Bille, 1990; McLauchlin *et al.*, 1991). While little or no intensive epidemiological investigation being done in tropical or subtropical areas (WHO Working Group, 1988; Nasim and Vahidy, 1993). This may the reason for the non-existent or low prevalence of the organism in other countries such as Asia, Africa, and South America.

1.6.1 THE CYCLE OF *L. MONOCYTOGENES* INFECTION

L. monocytogenes is widespread in nature. The possible routes by which the organism becomes contaminates foods and infects humans have been summarised by Brackett (1988), and shown in Fig. 1.2. The primary means of transmission of *L. monocyto-genes* to humans is considered to be through contaminated food. Some investigators in Europe consider listeriosis to be a direct zoonotic transmission (Owen *et al.*, 1960; Bojsen -Moller, 1972; Hird, 1987) especially to persons in contact with animals, e.g. farm workers handling newborn calves, veterinary surgeons in contact with infected dogs. It
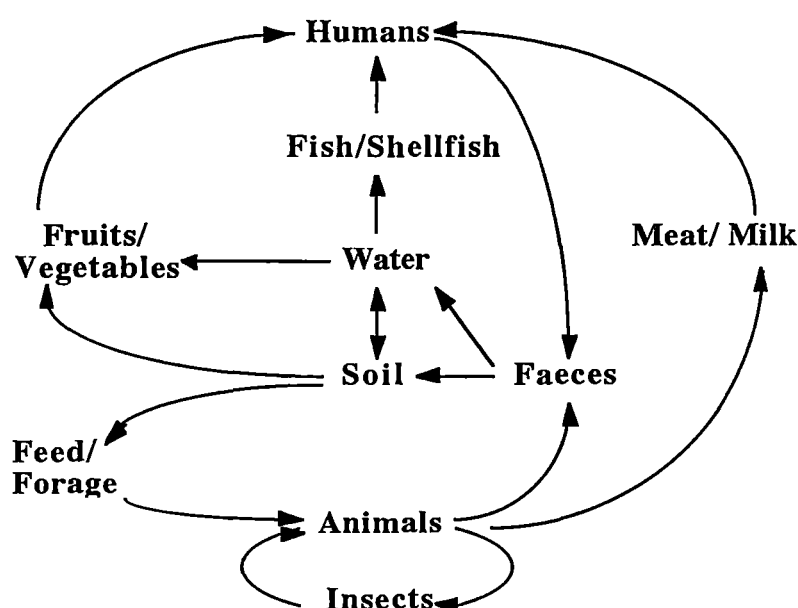
Figure 1.2 Hypothesised cycles of infection for *L. monocytogenes*. (After Brackett, 1988)

can also be transmitted via drinking infected milk. However, in the United States most cases occur among urban residents, with few or no known animal contacts (Schlech *et al.*, 1983).

1.6.2 INFECTIVE DOSE AND INCUBATION PERIOD

The dose of *L. monocytogenes* needed to cause disease in humans has not been defined either in the normal individual or those at increased risk (McLauchlin, 1995). There is no reliable quantitative information of the amount of contaminated foodstuff ingested in relation to the risk of acquiring the disease (WHO Working Group, 1988). Only in a few food associated cases of listeriosis has an estimation of oral dose been possible. McLauchlin (1995) indicated that the infective dose for human foodborne listeriosis is extremely difficult to define and it is probable that the infectious dose is related to host susceptibility.

Since no direct human dose response data is available, Farber *et al.* (1996) suggested a rough approximation for *L. monocytogenes* infective dose (ID), referred to as ID_{10} and ID_{90}, to be 10^7 and 10^9 for normal individuals, and 10^5 and 10^7 for high-risk people. More recently, Buchanan *et al.* (1997) estimated dose-response relationships (Fig. 1.3) on the basis of combining available epidemiologic data with food-survey data for RTE product, i.e. smoked fish, in Germany. This estimation was based on a single dose or multiple doses approach and the assumption that all listeriosis is caused by consuming a single RTE food. The authors proposed this approach for dose-response estimation as a demonstration but not as a definitive value.
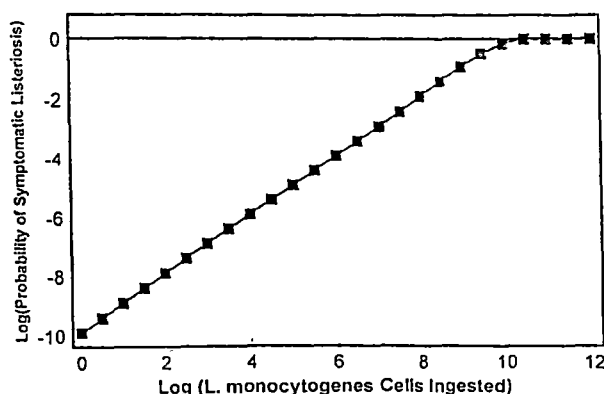
Figure 1.3 The dose-response curve predicted by an exponential model. (After Buchanan *et al.*, 1997).

1.6.3 OUTBREAKS OF LISTERIOSIS IN HUMANS

The first report of listeriosis in humans was by Nyfeldt in 1929, who isolated *L. mono-cytogenes* from the blood of patients with an infectious mononucleosis-like disease (Gray and Killinger, 1966). During 1933 and 1934, *L. monocytogenes* was established as a cause of meningitis and perinatal infections in the United States (Burn, 1936). However, until 1945 the organism was only isolated sporadically from humans and resulted in 36 cases of human listeric infection recorded in the medical literature (Kaplan, 1945). The first recorded massive outbreak of human listeriosis occurred in East Germany between 1949 and 1957 and resulted in a dramatic increase in the number of stillborn infants. This outbreak caused an awareness of listeric infections in humans, which gradually spread from Europe to the United States (Seeliger, 1961; Schuchat *et al.*, 1991a).

Despite increased reports of listeric infection, human listeriosis remains a rare disease compared to other reportable illness. Sonnenwirth (1973) suggested the probability of the disease to be more common but not recognised. The true incidence of human listeriosis is largely unknown because of (a) variable interest in investigating probable cases of listeriosis in different countries, (b) a general inability to detect mild listeriosis cases, and (c) a lack of uniform reporting of the disease in different countries (Kaufmann, 1988).

Heightened awareness of *L. monocytogenes* caused by large-scale outbreaks of food-borne listeriosis in Maritime Provinces, Canada, in 1981 (Schlech *et al.*, 1983); in Boston, Massachusetts, in 1983; in southern California in 1985 (Linnan *et al.*, 1988); in Vaud, Switzerland, in 1984 to 1987; and in the United Kingdom in 1987 has led to development of improved methods to detect the bacterium. Consequently, there have been attempts to reevaluate the incidence of listeriosis in the United States.

Compilation of surveillance studies coordinated by the Centres for Disease Control and Prevention provides the overall rate of listeriosis in the United States as 0.7 case per 100,000 people (Broome, 1993). From this figure, Broome projected that at least 1,850 cases of bacteremia or meningitis due to *L. monocytogenes* occur in the United States each year, resulting in 425 deaths. However, in pregnant women, 12 cases per 100,000 births is a much higher rate. Disease in the mothers was generally not severe, but the illness in the infant could be devastating, resulting in fetal death, stillbirth, or severe neonatal sepsis. In addition, individuals with underlying immunocompromising disease (e.g. chronic renal disease, human immuno-deficiency virus (HIV) infection, or cancer) were at increased risk for listeriosis.

Data concerning prevalence of listeriosis in Canada between 1971 and 1982 reveal a disproportionately high incidence of listeric infections in Nova Scotia (2.68 cases/10^6 people) and Prince Edward Island (2.61 cases/10^6 people) as compared to the remaining Canadian provinces (average of 0.63 case/10^6 people). The higher incidence of listeriosis in Nova Scotia is the direct result of a major foodborne outbreak in 1981 which was linked to consumption of contaminated coleslaw (Schlech *et al.*, 1983).

In the UK, the dramatic increase in the number of listeriosis cases, ca 290 cases/year, during 1987-1989 was reported to be associated with imported paté from a single manufacturer (McLauchlin *et al.*, 1991). However, from 1989 to 1994 the incidence has returned to levels recorded in the early 1980s, ca. 100 cases/year (McLauchlin and Newton, 1995). The authors indicated seasonal distribution particularly in late summer or autumn associated with the marked peak in the numbers of listeriosis cases.

Nasim and Vahidy (1993) reported the incidence of human listerial meningitis in Karachi, Pakistan. Sixty patients who were suffering from symptomatic meningitis or encephalitis were screened for the presence of *L. monocytogenes*. Only one out of 60 CSF samples, but none from blood, was found to harbor *L. monocytogenes*, the incidence being 1.66%. The authors presumed that the consumption of *Listeria* contaminated food was the most probable cause of the infection.

Souëf and Walters (1981) reported the first neonatal listeriosis outbreak in Western Australia between January 1978 and October 1979. Twelve cases of neonatal listeriosis were recorded. The authors indicated that the reduced mortality rate for the cases, 17%, was because the treatment was instituted promptly. A seasonal incidence was observed with 10 of the 12 cases occurring between January and March which are the the the hottest, driest period of the year in the southern half of Western Australia. No other common epidemiological factor was identified. Additionally in 1990, an outbreak of listeriosis in pregnant women occurred in Western Australia (Watson *et al.*, 1990). The King Edward Memorial Hospital for women reported 10 cases in pregnant women and 11 cases in fetuses or infants. Watson *et al.* (1990) demonstrated that in healthy adults (including pregnant women) *Listeria* infection is usually asymptomatic or may cause a minor illness with a mild fever, headache, and aches and pains which are similar to acute febrile illnesses. However, in a case where a baby was infected, the pregnant mother will suffer a significant febrile illness. This epidemic resulted in 6 stillbirths or mid-trimester miscarriages with a case-fatality rate of 55%. Strong circumstantial evidence indicated that the epidemic resulted from a foodborne origin. A number of different types of food, namely cooked diced chicken, paté, pastrami, salami and processed meats in Western Australia were found to be contaminated with *L. monocytogenes*. Furthermore, *L. monocytogenes* was also detected in a sample of paté from patient's refrigerator who had

eaten a certain brand of paté in two weeks prior to delivery of a stillborn child. The authors suggested that the incubation period for listeriosis is not accurately known but probably ranges between two days and three weeks. Recently from May 1990 to July 1993, 73 cases of listeriosis were recorded in Victoria, Australia (Ng and Forsyth, 1993). All cases have been sporadic and isolated. The infection resulted in 33 materno-fetal cases with 13 miscarriages and deaths (39%) and 40 non-pregnant cases with 9 deaths (23%). This series of cases appears typical for those occurring in many countries, and thus listeriosis in humans, although of relatively low incidence, is an extremely serious infection of high mortality.

1.6.4 OUTBREAKS OF LISTERIOSIS IN ANIMALS

L. monocytogenes was first described by Murray (1926) in a colony of laboratory rabbits. The organism has also been recovered from more than 50 species of animals, including both domesticated and feral ruminants and monogastric animals (Seeliger, 1961; Gray and Killinger, 1966; Brackett, 1988; Inoue *et al.*, 1992).

Listeriosis in domestic livestock is being recognized particularly in developed countries with increasing frequency around the world (Ryser and Marth, 1991). However, the exact incidence of listeric infections in domestic livestock remains unknown. In eastern Gippsland, Victoria, Australia, during winter and spring 1978, an outbreak of ovine listerial meningo-encephalitis on sheep farms was reported (Vandegraaff *et al.*, 1981). Sheep of all ages and both sexes were affected, and the highest incidence was observed in lactating ewes and weaners. The morbidity rate in affected flocks ranged from 0.2% to 8.0%, and the case fatality rate was almost 100%. The peak incidence of disease followed a period of continuous heavy rain and folding of grazing pasture, and the majority of affected flocks were located on poorly drained coastal sandy soil.

An outbreak of abortions due to *L. ivanovii* in a flock of 840 five-year-old Merino ewes grazing in the north-west slopes region of New South Wales was reported by Sergeant *et al.* (1991). Approximately 110 lambs were either born dead, or died shortly after birth. The authors suggested the spoiled hay was the source of the organism.

In addition to relatively small numbers of acutely infected sheep, goats, and cattle, Seeliger (1961) suggested that there were substantially larger proportions of animals within a herd which may be asymptomatic carriers of *L. monocytogenes* and shed the organism in faeces and milk. The role of the symptomless carrier was clearly demonstrated in another report in which 30 of 44 listeriosis outbreaks in sheep farms involved introduction of clinically healthy animals from known infected herds (Seeliger,

1961). Thus these results indicate that a substantial pool of asymptomatic carriers exists to disseminate and perpetuate this disease.

The number of cases of animal listeriosis has often been observed to reveal seasonal variation. In the Northern Hemisphere including England, Bulgaria, Hungary, United States, France, and Germany, listeriosis in domestic animals generally occurs from late November to early May and has been most prevalent during February and March (Gray and Killinger, 1966). Climate appears to play a rather important role in listeric encephalitis. Gray and Killinger (1966) observed an increase in the number of outbreaks 2 to 4 days after sudden drops in temperature or heavy snow falls. Gill (1931) reported that, in New Zealand, the greatest incidence occurred during the dry season and disappeared after rains. Numbers of listeriosis cases increased when animals were fed silage during periods of extreme cold, whereas sharp decreases in numbers of reported cases were observed as soon as grass was available. Dijkstra (1971) noted in Ryser and Marth (1991) that most cases of listeric abortion in cattle in The Netherlands occurred between December and May. Approximately 40% of these cases were attributed to consumption of contaminated silage. Recent changes in production methods have reduced levels of *L. monocytogenes* in silage, which in turn had led to a considerable decrease in the incidence of listeriosis in silage-fed animals (Ryser and Marth, 1991).

Listeriosis in aquatic animals had been speculated as early as 1957. In Romania, Stamatin *et al.* (1957), cited in Gray and Killinger (1966) that *L. monocytogenes* was isolated from viscera of pond-reared rainbow trout. The fish had been fed meat from a donkey which died of an undetermined cause. The fish showed listlessness interrupted by brief periods of agitation, loss of appetite, apparent blindness, blackened integument, and bloody discharge from the anus, particularly by the females. The mortality rate was approximately 50%. The disease could be transmitted to trout but not to carp by intra-muscular or intracranial inoculation.

Leung *et al.* (1992) examined channel catfish (*Ictalurus punctatus*) which is the most widely cultured species found in the United State due to the high productivity and low production cost. The catfish, which were grown in aquaculture ponds at Auburn University, Auburn, Alabama had been fed diets containing 26 or 38% protein with restricted and satiety feeding methods for 6 months. The restricted feeding method limits the amount of feed and only one feeding time per day for catfish, while in the satiety feeding method the feeds are available whenever the fish trigger the feeding device. The presumptive enumeration of *Listeria* spp. on the fish surface rinse and visceral samples showed that there was no significant difference (P<0.05) in Listerial concentrations in these samples due to feeding method or feed protein level. Nonetheless, it was noted that there was an approximate 1 log reduction between *Listeria* found on fish skin associated

with the higher protein diet. However, the presumptive Listerial counts were found to be much higher in the fish visceral samples (mean counts 1.99 log cfu/g wet weight of sample) and hence caution should be taking during the evisceration step in fish processing to avoid cross-contamination.

1.7 CONTROL AND PREVENTION OF HUMAN FOODBORNE LISTERIOSIS

Foods are regarded as the major source of human listeriosis, the prevention of the disease should begin at the farm and continue through food processing to selection and handling by consumers. *L. monocytogenes* is ubiquitous and, together with its ability to withstand harsh environments, it has often been detected in a variety of foods as mentioned previously. It is known that the organism cannot always be eradicated from finish products or environment but the risk of infection can be reduced (Farber *et al.*, 1996). The application of HACCP system which was originally developed in 1960's by the Pilsbury Company, the National Aeronautics and Space Administration (NASA), and the U.S. Army Natick Laboratories, has currently been reintroduced (Ray, 1996). The Food Safety and Inspection Service (FSIS) of the USDA, the FDA, the International Commission on Microbiological Specifications for Foods (ICMSF), and the FAO advocate the HACCP system to be used in the food industries in the United States and other countries (FAO, 1994). Generally, HACCP is accepted as a scientific based, food safety management system using the approach of controlling critical points in food handling to prevent food safety problems which is better than end-product testing. HACCP has been recommended to be used from farm to consumer to minimise the risk of listeriosis (Roberts *et al.*, 1996).

1.7.1 FARM

Considering the cycle of *L. monocytogenes* infection (see section 1.6.1), HACCP could be applied at the farm to obtain a good quality i.e. less contamination, of raw material such as vegetables, milk, cattle and fish. For example, animals should be raised in clean environment. Animal feed such as silage (see section 1.4.2) should be controlled to rapidly achieve the pH<4.0 which is the critical point to prevent the proliferation of *L. monocytogenes*. The harvested raw material should be stored at low temperature (e.g. <5°C) until transportation to the processing plant.

1.7.2 PROCESSING

According to WHO Working Group (1988), foods have been placed into four categories.

1. Raw foods e.g. raw vegetables and meats

2. Processed raw foods not treated listeicidally by heating e.g. coleslaw, fermented sausages, raw-milk cheeses, cold-smoked fish

3. Processed foods treated listericidally by heating but subjected to potential recontamination during subsequent handling e.g. certain cheeses and commercially processed meats that are sliced or altered after thermal processing.

4. Processed foods treated listeicidally by heating while in an intact package (e.g. cooked ham) or which are aseptically packaged immediately after listericidal treatment (e.g. certain dairy products).

Adequate cooking of some of these primary food sources and good food handling practices were shown to be sufficient in eliminating and preventing post/cross-contamination. However, emphasis should be placed on high risk foods such as foods associated with outbreaks, ready-to-eat foods (Category 2) that can support growth of *L. monocytogenes* to high populations within the expected product shelf life and are consumed without subsequent cooking.

Regarding the regulatory policy on *L. monocytogenes* contaminated foods, the application of 'zero tolerance' is still employed by several countries e.g. USA, Switzerland and Hungary while the application of food group risk-based policy are accepted by some European countries (Germany, United Kingdom, and Denmark), as well as Australia and Canada (Ben Embarek, 1994; FAO, 1994). There was controversy that the complete exclusion of *L. monocytogenes* from foods and food processing plants is unrealistic, even by the application of the most stringent criteria (Teufel, 1994; Gilbert, 1995). Recently, Canada's updated regulatory policy based on the principles of HACCP and health risk assessment has set a compliance criteria for *L. monocytogenes* in RTE foods (Farber *et al.*, 1996). The highest priority is given to those RTE foods which have been associated with listeriosis and those with a greater than 10 days shelf life (Table 1.4).

Processing plants should implement HACCP principles throughout the processing steps beginning from the reception of raw material to processing, packaging and storage of end-product and distribution to ensure the absence of *L. monocytogenes* in foods. In addition, several strategies can be implemented in an attempt to prevent contamination and further outbreaks of *L. monocytogenes* infection. If possible, foods should be formulated to obtain the condition that are not favourable for the growth or survival of *L. monocytogenes*. Different strategies are applied to different foods aiming to adjust the

intrinsic (e.g. water activity, pH) or extrinsic (e.g. temperature, packaging) property related in control or preventing outgrowth of *L. monocytogenes* in foods. The interactive or synergistic effects of those factors to stabilise and ensure safe foods are well explained by the hurdle technology of Leistner (1994).

Table 1.4 The Canadian compliance criteria for *L. monocytogenes* (LM) in RTE foods

Category	Action level for LM	Immediate action
1. RTE foods causally linked to listeriosis (includes: soft cheese, liver paté, coleslaw mix with shelf-life >10 days, jellied pork tongue)	>0 cfu/50 g	Class I recall to retail level
2. All other RTE foods supporting growth of *L. monocytogenes* with refrigerated shelf-life >10 days	>0 cfu/25 g	Consideration of public alert Appropriate follow-up at plant level Class II recall to retail level
3. RTE foods supporting growth of *L. monocytogenes* with refrigerated shelf-life ≤10 days and all RTE foods not supporting growth	≤100 cfu/g	Allow sale-Class II recall or stop sale depends on the GMP, status

After Farber *et al.*, 1996.

1.7.3 RETAIL

To maintain a good quality of a product and minimise any cross/post contamination, some basic rules recommended by Roberts *et al.*(1996) to be applied at the retail section such as: separate raw food (Category 1) from RTE foods (Categories 2-4), use an effective method of cleaning and disinfecting the surface including equipment that contact RTE foods, maintain proper storage and display temperatures (e.g. <5°C), and sell the products only 'use by' or 'best by' code date.

1.7.4 CONSUMERS

Apart from education and training the food producers, the general public especially the high-risk population also need to be educated about the risks associated with foodborne listeriosis and preventative measures such as foods selection, hygiene and good food handling practices. Educational advisory pamphlets have been published in Australia (Anon., 1994, 1995), for some instances, which deal directly with issues related to pregnant women. The "For pregnant women dietary advice on listeriosis" and "Environmental Health Guide, *Listeria* infection and pregnancy" provide generic information on listeriosis and also more specific information for persons at high risk,

including the avoidance of chilled ready-to-eat foods e.g. paté, smoked seafood, soft cheeses, pre-prepared or stored salads, the hygiene of foods preparation etc. The high-risk individuals have been informed that although listeriosis is a relatively uncommon disease, the mortality rate is relatively high especially in foetuses and newborn babies (up to 30-50%).

2 THE OCCURRENCE OF *LISTERIA* SPP. INCLUDING *L. MONOCYTOGENES* IN NORTH WEST BAY

2.1 INTRODUCTION

L. monocytogenes is widely distributed in the environment and has been isolated from a variety of sources. The organism is frequently found in the intestinal tract of healthy and infected warm-blooded animals and in faeces disposed to soil or septic tanks. Several studies (Seeliger, 1961; Welshimer and Donker-Voet, 1971; Weis and Seeliger, 1975) have suggested that *L. monocytogenes* also lives as a saprophyte in soil and plants. The transmission and distribution of the organism from these reservoirs to aquatic environments such as river water sewage, effluent and estuarine water (Watkins and Sleath, 1981; Al-Ghazali and Al-Azawi, 1988a; Colburn *et al.*, 1990; Motes, 1991) may occur especially due to rainfall or ground water movement. Food is regarded as the major source of human infections (Farber and Peterkin, 1991) and shellfish, as filter feeders growing in such water can accumulate the pathogen (Brackett, 1988). These may, if eaten raw or uncooked, cause listeriosis in the consumer.

Considering that listeriosis has become a major concern in recent years in Australia, there are very few data concerning the occurrence and distribution of *L. monocytogenes* and related species in aquatic environments. This chapter presents an investigation over a 12 months period in North West Bay, which is an area of considerable aquaculture activity in southern Tasmania (see detail in 2.2.2.1). It provides excellent conditions for raising Atlantic salmon (*Salmo salar*), which is currently the most commercially important aquaculture species in Tasmania, and also for blue mussels (*Mytilus edulis*), an emerging industry in this area. The increase in aquaculture activity in this area over the past 10 years has enhanced the potential for public health risks associated with consumption of *L. monocytogenes* contaminated fish or shellfish which are not cooked before ingestion. Furthermore, North West Bay may be served as an example of a small environmental system which received input from rivers, streams, effluent from sewage treatment plant and also from a number of factories established along the bay. Therefore, the emphasis of this chapter is on the following aspects:

- To assess the occurrence and significance of *Listeria* species, especially *L. monocytogenes*, in aquatic environments e.g. inshore water, river water, sewage treatment pond and fish factory effluents, sediments, and in edible shellfish growing in North West Bay, Tasmania. This involved employing an extensive and modified cultural method for the isolation of *Listeria* spp. in these environments.

- Examine the controversial use of faecal coliforms and *E. coli* as the indicators for microbial pathogen occurrence. The study was intended to establish if a relationship exists between the amount of faecal coliforms/ *E. coli* and the occurrence (presence or absence) of *L. monocytogenes* in water.

- The quality of the waterway varies due to a multiplicity of factors. This study was undertaken to investigate the relationship between some physicochemical (i.e. pH, temperature and salinity) and climatological factors (rainfall), and the occurrence of *L. monocytogenes*.

- To use a reliable and sensitive biotyping method, Multilocus Enzyme Electrophoresis (MEE), for intraspecies typing of *L. monocytogenes* and, if possible, to determine the distribution of strains in the environmental system.

2.2 MATERIALS AND METHODS

2.2.1 MATERIALS

Complete details of consumables, reagents and media, equipment and reference cultures used are presented in Appendix A.

2.2.2 METHODS

2.2.2.1 Sampling strategy and site descriptions

North West Bay

North West Bay is a small trapezoidal shaped bay located in southern Tasmania, at longitude 147° 30′ E and latitude 43° S, approximately 22 kilometres south of Hobart (Fig. 2.1). The watershed of the bay is relatively small with 67% of its area still under natural vegetation (Matthews and Volframs, 1978). Small urban and industrial centres are located around the shores of the bay. Due to its close proximity to Hobart, the area offers considerable recreational potential which includes a number of beaches well suited for swimming, diving and fishing. Launching facilities are provided at a number of locations around the bay and the best of these is at Dru Point where there is also a small reserve developed and maintained for picnics and school activities.

The bay is sheltered by the Tasmania mainland and also Bruny Island, approximately 1-2 kms to the east and separated from North West Bay by the D'Entrecasteaux Channel (see Fig. 2.1). The major contributors to inshore water in the bay are the inputs from rivers and creeks on mainland Tasmania which cover a catchment area of approximately 260

square kilometres. Table 2.1 shows the catchment areas of the various rivers and streams which receive agricultural run-off and domestic septic tank effluent which drain into the bay. The bulk of freshwater input to the bay is derived from the catchment of North West Bay River (68.7%) which together with Nierinna Creek and Coffee Creek, drain into the mud flats in the north western corner of the bay. Matthews and Volframs (1978) estimated the total freshwater input to be 118×10^6 cubic metres/year. The other important

Table 2.1 Stream catchments in the North West Bay watershed.

Stream Name	Catchment Area (%) (km^2)	Volume of freshwater discharge (%) (m^3 x 10^6/year)
Coffee Creek	7.5 (2.9)	1.20 (1)
Snug River	23.5 (9.1)	3.61 (3.1)
Nierinna Creek	27.2 (10.5)	7.06 (6)
North West Bay River	178.0 (68.7)	92.97 (78.9)
Other	22.8 (8.8)	12.96 (11)
Total	259.0 (100)	117.8 (100)

After Mathews and Volframs (1978)
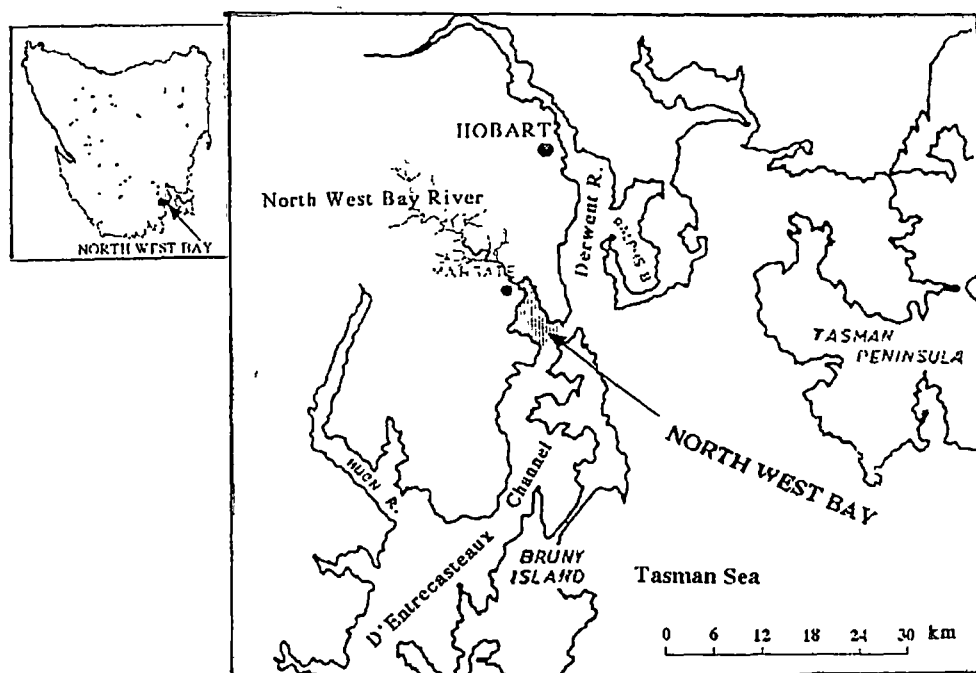
Figure 2.1 Location map of North West Bay.

effects are the input from the municipal sewage treatment pond at Dru Point, operated by Kingborough Council, and effluents from a number of fish processing factories established along the west side of the bay. The treatment systems of some of these factories have been designed to receive both wastewater from the factory and human faeces.

Matthews and Volframs (1978) suggested that the pattern of water circulation within North West Bay was complex. The authors estimated mean velocities of tidal currents in the bay to be less than 2 cm/s and in the directions as shown in Fig. 2.2. The influence of a high energy flow pattern within the D'Entrecasteaux Channel and distinctly opposed flows directed in and out of North West Bay was also reported. In addition, during a period of high rainfall (29 mm in 24 hr), they also observed the discoloured freshwater flow from the North West Bay River as a narrow stream which travelled along the surface down the eastern shore toward the mouth of the bay. During that period, near surface salinities were consistently lower along the eastern shore of the bay, also indicating translocation of freshwater over the surface.
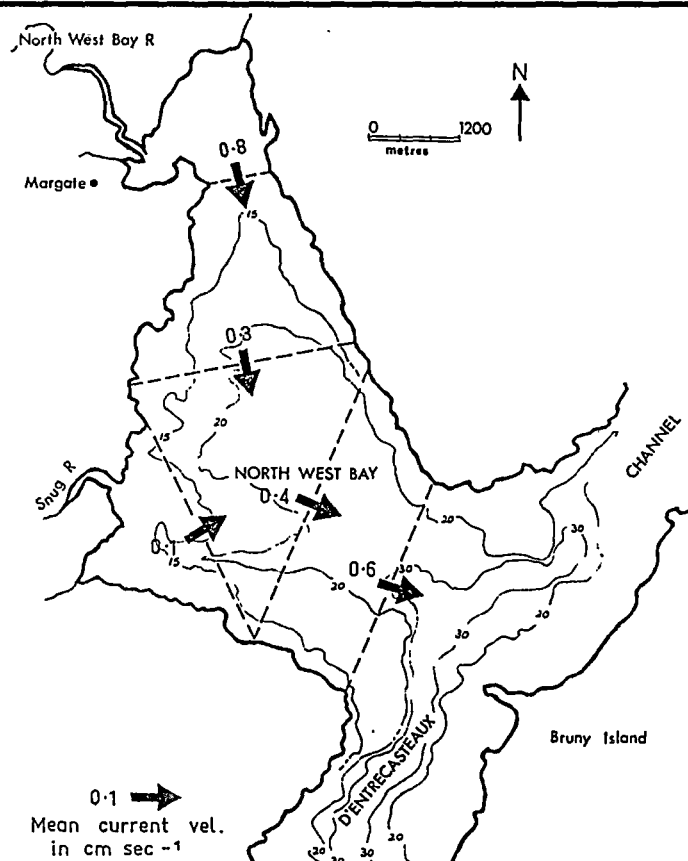
Figure 2.2   Mean ebb velocities estimated from tidal prism (0.5m tide) taken from Matthews and Volframs (1978).

2.2.2.2 Sampling program

Recently, there has been concern raised in regard to the pollution of a number of Tasmanian estuarine and coastal areas, particularly the Derwent estuary and adjacent bays such as North West Bay. Sander *et al.* (1991) reported approximately 91% of effluent from sewage treatment plants (untreated, primary or secondary treatment) in Tasmania is continuously discharged to estuaries, rivers and creeks. Although almost all of those effluents from sewage treatment plants have undergone disinfection process, several contaminated effluents are still being discharged from oxidation ponds which are distributed throughout the municipalities. It has been extensively reported in the literature e.g. ANZECC (1992) that sewage and wastes from domestic sources, animals and animal-processing industries can contain very high numbers of bacteria, viruses and protozoa, some of which may cause illnesses in human and animals. In addition, stormwater and runoff from farmlands, animal feed lots and contaminated soils or vegetation may also distribute potentially pathogenic organisms to the catchment and waterways downstream. These may significantly affect the microbiological quality of the receiving water.

Once in water, pathogens may enter the host by either:

- primary contact, which involves direct exposure of the host to the pathogens through water activities such as swimming or diving, leading to the possibility of ingesting enough water for infections to develop.

- secondary contact, which is limited exposure such as during boating and walking on the beach; in these circumstances contaminated water can spill or spray on some parts of the body, especially on open wounds or be inhaled or swallowed, and may induce an infection to occur.

- consumption of contaminated fish or shellfish

In this study, samples of inshore marine water, river water and effluent were collected at 12 sampling stations every two weeks. Sediment and shellfish samples were collected at 9 and 3 of the 12 sampling stations, respectively on a monthly basis. The investigation was continued for 1 year (May 1994-May 1995). Specific sites sampled including those along various tributaries and foreshore areas of North West Bay are shown in Fig. 2.3. The location and frequency of samples collection is shown in Table 2.2:

- inshore marine waters were sampled at sites 1 to 7.

- river waters were collected at sites 8 and 9.

- sewage treatment pond was sampled at site 10.

- effluents from fish processing factories were collected at sites 11 and 12.

- sediment were sampled at sites 1 to 9.

- Pacific oysters (*Crassostrea gigas*) were collected at sites 3 and 5. Note that these were not approved areas for recreational taking of shellfish.

- Mussels (*Mytilus edulis*) were collected at site 6b.
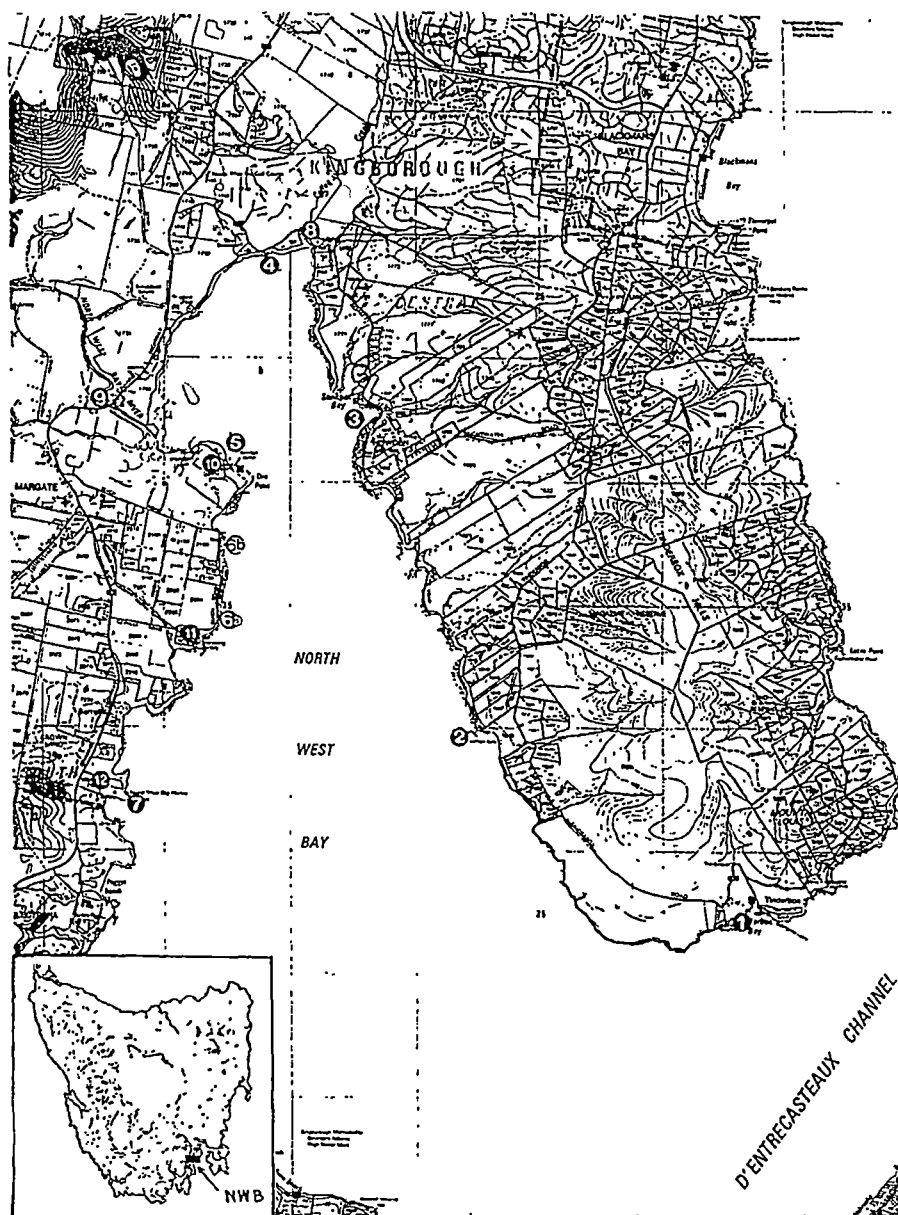
Figure 2.3 The 12 sampling sites around North West Bay, south of Hobart, Tasmania.
No. 1-7 = estuarine water and sediment, No. 8-9 = river water and sediment,
No. 10-12 = waste water, and No. 3, 5 and 6b = shellfish

Table 2.2 Location and frequency of sample collection.

Station No. and Name	Risk area[a,b]/ Input[c,d] /Fish-Shellfish	Sampling Period (wks)		
		Water	Sediment	Oysters/ Mussels
1. Tinderbox	risk area[a]	2	4	–
2. Salmon farm at Powder Jetty	fish (Atlantic salmon)	2	4	–
3. Stinkpot Bay	risk area[b], shellfish (oysters)	2	4	4
4. Sanctuary	risk area[b]	2	4	–
5. Dru point	risk area[b]	2	4	4
6a. North West Bay Commercial Jetty	risk area[b]	2	4	–
6b. Mussels culture at Beach Road Jetty	shellfish (mussels)	–	–	4
7. North West Bay Marina	risk area[b]	2	4	–
8. Coffee Creek	input[c]	2	4	–
9. North West Bay River	input[c]	2	4	–
10. Sewage Treatment Ponds at Dru Point	input[d]	2	–	–
11. Discharge from fish factory 1	input[d]	2	–	–
12. Discharge from fish factory 2	input[d]	2	–	–

[a] Primary contact; [b] Secondary contact; [c] Run-off; [d] Effluent.

2.2.2.3 Methods for detection and identification of Listeria, faecal coliforms and E. coli

Samples collection

- Water : Water samples were collected using a sterile 1-L bottle immersed in water to a depth of approximately 0.5 m to avoid entrapping any air bubbles, then capped under water. The collection was made directly by hand in an upstream movement. A bottle holder (modified golf-ball retriever) with an extension of 3 m was used for collecting the water samples in some stations (sites 2, 10, 11 and 12). These sample bottles were not possible to be capped under water.

- Surface Sediment : At each inshore and river site, approximately 100 g of sediment, consisting of several subsamples was collected with a sterile modified syringe. Regularly at deep water site 2, an Ekman grab (see Appendix A, A.1.5) was used for taking the sediment. Samples were then placed in a sterile polyethylene bottle.

- Shellfish : Naturally grown oysters from shallow water (approx. 0.5 m depth) and commercially grown mussels were collected into a plastic bag.

All samples were maintained at 10°C or lower during transport to the laboratory and were processed within 6 h of collection. Collection of water samples was undertaken every 2 weeks and required approximately 3 hours to complete. Collection of sediment and shellfish was undertaken every 4 weeks and required approximately 4 hours to complete.

Physicochemical parameters

Immediately after the samples at each site were collected, surface water salinity (‰), pH and temperature (°C) were measured at 0.1 to 0.5 m depth with hand-held meters. Between sites the meter probes and hands were cleaned using quaternary ammonium disinfectant (Savlon) and 70% alcohol to prevent cross-contamination.

Meteorological parameters

The annual rainfall records from stations (Margate, Blackmans Bay Treatment Plant station) that were close to the sampling sites were obtained from the Bureau of Meteorology, Hobart, Tasmania.

Microbiological analysis

As there is no standard method for detecting *L. monocytogenes* in environmental samples, currently used methods in the food industry were adapted. The USDA/FSIS method (Dennis and Lee, 1989) was selected and evaluated for use with shellfish, sediment and water samples including the use of filter method as shown in Fig. 2.4.

- Water : A 1 litre volume was filtered through a prefilter and membrane filter 0.45 μm-pore-size, 90 mm diameter. Both the prefilter and 0.45 μm member filter were placed in 100 ml UVM1 (Fig. 2.4) for detection of *Listeria* species.

The enumeration of faecal coliforms and *E. coli* followed the Australian Coliforms-Membrane filtration method for the examination of water and waste water (AS 4276.5) (Australian Standard, 1995): appropriate volumes (0.1, 1, 10, 100 ml) were filtered through 0.45 μm-pore-size, 47 mm diameter membrane filter. The filter was placed on MLSA (Membrane lauryl sulphate agar) and incubated at 30°C for 2-4 hr, then at 44°C for 14-18 hr. Presumptive faecal coliforms were counted on plates with 10 to 100 colonies (yellow) and representative colonies were subcultured into LTB (Lauryl Tryptose Broth) and incubated at 44°C for 24 hr. Confirmed faecal coliforms (gas producers) were then subcultured into tryptone water and incubated at 44°C for 24 hr. Faecal coliforms were confirmed as *E. coli* by a positive indole reaction. Counts of faecal coliforms and *E. coli* were expressed per 100 ml.
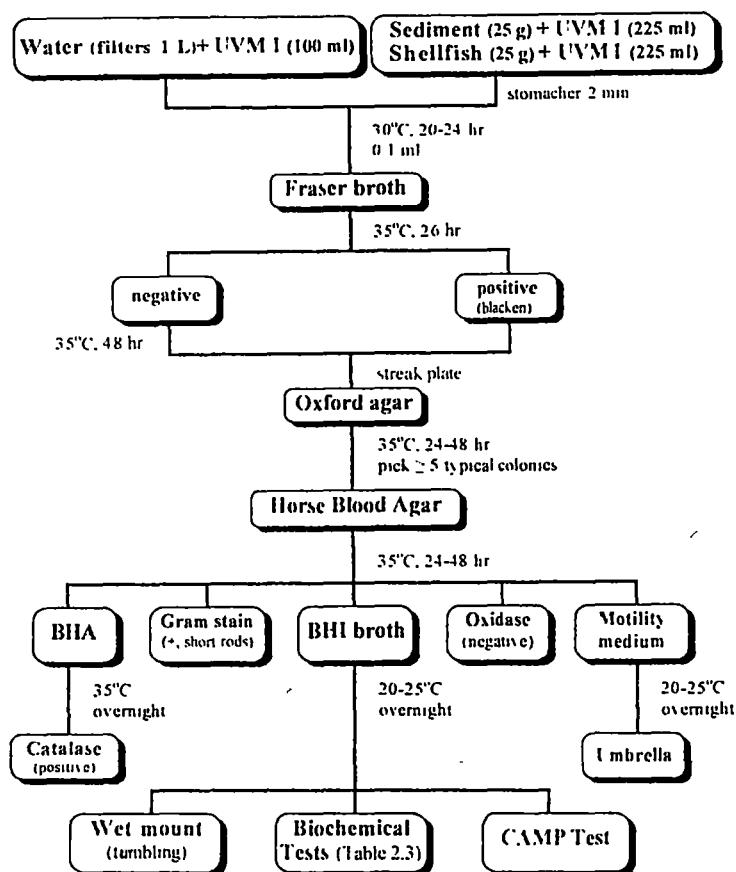
Figure 2.4 Diagram of procedure for the isolation and identification of *Listeria* species, USDA/FSIS (Dennis and Lee, 1989). For abbreviations, see section 1.1.3.

Table 2.3 Abbreviated Scheme for Differentiation of *Listeria* species based on USDA/FSIS (Dennis and Lee, 1989) and Australian Food Standards Code (National Food Authority, 1994).

Characteristic	*mono- cytogenes*	*innocua*	*seeligeri*	*ivanovii*	*welshimeri*	*murrayi (grayi)*
ß-Haemolytic	+	-	+	+	-	-
Tumbling motility	+	+	+	+	+	+
Motility (umbrella)	+	+	+	+	+	+
MR-VP	+	+	+	+	+	+
NO_3 reduction	-	-	-	-	-	+/-
CAMP-*S. aureus*	+	-	+	-	-	-
CAMP-*R. equi*	-	-	-	+	-	-
Mannitol utilisation	-	-	-	-	-	+
Xylose utilization	-	-	+/-	+	+	-
Rhamnose utilization	+	+/-	-	-	+/-	+/-

+, positive; -, negative.

- <u>Surface Sediment</u> : Sediment samples in polyethylene bottles were mixed by stirring with a sterile handheld spoon, and 25 g were added to 225 ml of UVM1 (Fig. 2.4) for detection of *Listeria* species.

- <u>Shellfish</u> : As described in the USDA/FSIS method (Dennis and Lee, 1989) for the microbiological analysis for food, 25 g of each sample was added to 225 ml of UVM1 (Fig. 2.4) and stomached for 2 minutes for detection of *Listeria* species.

Preliminary study of the sensitivity of Listeria detection method (validated recovery)

To demonstrate that the methods described above were adequately sensitive for the purpose of this project and to determine the minimum detection limit, a sensitivity test was set up. The absolute sensitivity of the UVM 1, Fraser broth and Oxford agar warm enrichment was evaluated by inoculation of two sterilized water samples (1 L) with low inoculums of an overnight 37°C BHI broth culture of *L. monocytogenes*. Similar artificial contamination with *L. monocytogenes* was also made with two sterilized sediment samples (25 g) and one oyster sample (25 g). Then the samples were processed according to the methods. A control of each type of sample was processed at the same time. For each sensitivity test, a ten fold dilution series was made in sterile 0.1% Peptone Water from the same original *L. monocytogenes* culture. The number of organisms in each dilution was quantified by a plate count of the dilution series after 24 hr incubation at 37°C on Tryptic Soy Agar (TSA).

2.2.3 METHOD FOR MULTILOCUS ENZYME ELECTROPHOSIS (MEE)

The technique used for MEE was that described by Selander *et al.* (1986). Details of the procedure and reagents used are given in Appendix B. MEE was performed by studying the mobility of the following 12 enzymes: Alanine dehydrogenase (ALA), Catalase (CAT), Fumarate hydratase (FUM), Glucose-6-phosphate dehydrogenase (G6PD), Glyceraldehyde-3-phosphate dehydrogenase (GP), Mannose phosphate isomerase (MPI), Nucleoside phosphorylase (NP), Peptidase-leucyl-leucyl-glycine (PLG), Phosphogluco-mutase (PGM), Phosphoglucose isomerase (PGI), 6-Phospho-gluconate dehydrogenase (6PGD), and Superoxide dismutase (SOD). A brief diagram showing the steps in performing MEE is shown in Fig. 2.5.
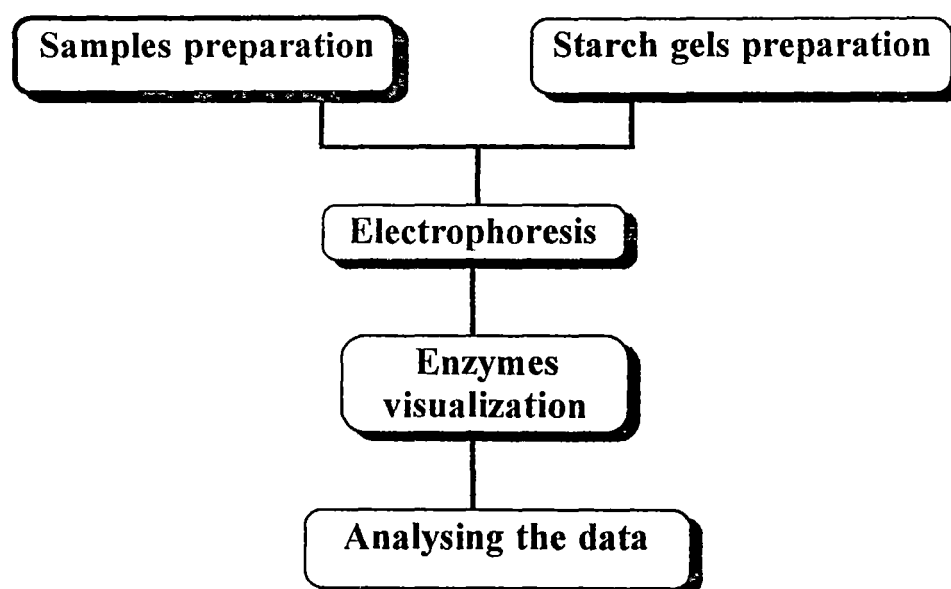
<u>Figure 2.5</u> Brief diagram of multilocus enzyme electrophoresis procedure based on Selander *et al.* (1986). For details see Appendix B.

2.2.3.1 Genetic relationships

The composite genotypes of the strains examined by MEE was used to construct a phylogenic tree indicating the relationships between the strains. Programs "ETDIV" and "ETCLUS", written by Dr. T.S. Whittam, Institute of Molecular Evolutionary Genetics, Pennsylvania State University, USA were used to analyze genetic diversity and relationships among bacterial strains. The "ETDIV" found and listed the electrophoretic types (ETs) in the collection of bacterial isolates with multilocus enzyme profiles. The "ETCLUS" created a dendrogram based on the average linkage algorithm. Distance was measured as the proportion of mismatched loci between pairs of ETs. Null alleles that were scored as "0" were not used in the calculation of pairwise distances.

2.2.4 STATISTICAL ANALYSES

The occurrence of *Listeria* spp. and *L. monocytogenes* in water samples was statistically compared with the physico-chemical variables (pH, temperature and salinity), rainfall

records and the amount of faecal coliforms and *E. coli*/100 ml using the SAS[1] LOGISTIC procedure. A probability model previously developed (Ratkowsky and Ross, 1995), was used to relate growth/no growth to the explanatory variables, the model form being as follows:

$$\text{logit}(P) = b_0 + b_1 \ln(T-T_{min}) + b_2 \ln(pH-pH_{min}) + b_3 \ln(S+1)$$

$$b_4 \ln(Rf24+1) + b_5 \ln(fc+1) + b_6 \ln(ec+1) \qquad (2.1)$$

The logit is a mathematical abbreviation such that logit $(P) = \ln [P/(1-P)]$, where P is the probability that *Listeria* occurs, and ln refers to the natural logarithm. The coefficients b_0, b_1, b_2, b_3, b_4, b_5 and b_6 are the parameters to be estimated by fitting the model to experimental data. The parameters T_{min} and pH_{min} are notional values of minimum temperature and pH respectively, at which the growth rate is predicted to be zero. Both terms were estimated from other modelling of *Listeria* to be -2°C and 4.5 pH unit and were used as constants in model fitting in this study. The measured variables salinity (S) (‰), rainfall recorded during the 24 hr preceding the sampling day (Rf24) (mm), faecal coliforms (fc) and *E. coli* (ec) /100 ml have 1 added to them to avoid having ln(0).

The model performance is assessed by determining the area c under the receiver operating characteristic (ROC) curve which is the proportion of the total number of pairs in which the model resulted in a higher probability for the presence of the interested organism than the absence of it (Lemeshow and Le Gall, 1994). If $c > 0.70$, the model is considered satisfactory (Lemeshow and Le Gall, 1994), whereas $c > 0.8$ is considered excellent discrimination and $c > 0.9$ is deemed outstanding discrimination (Lemeshow, pers. comm.).

2.3 RESULTS AND DISCUSSION

2.3.1 SENSITIVITY OF *LISTERIA* DETECTION METHOD (VALIDATED RECOVERY)

The sensitivity of the *Listeria* culture method used in the study was comparable with published methods (Hayes *et al.*, 1992; Buchanan *et al.*, 1989b), with a minimum level of detection of 2.8 CFU of *L. monocytogenes* in 25 g of sediment and in 1 L of water. In addition, in a validated recovery experiment, artificially introduced *L. monocytogenes*, at a level of 22 CFU in 25 g of oysters sample, was detected by the method (Table 2.4).

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[1] SAS (Statistical Analysis System) (1995). SAS/STAT Guide for Personal Computers, Version 6.10 Edition, SAS Institute Inc., Cary, North Carolina 27512-800, USA.

The levels of *Listeria* spp. detected in the positive seafood samples were reported to varied in a great range from 0.36 to >110 MPN (CFU/g) (Buchanan *et al.*, 1989b). Therefore, the recovery of *L. monocytogenes* at these low levels (Table 2.4) is considered to be sufficient to demonstrate the sensitivity of *Listeria* detection method used here. However, a recovery of *L. monocytogenes* in the presence of background microflora has not been conducted in water and sediment samples.

Table 2.4 The recovery of *L. monocytogenes* from real samples which were artificially inoculated with different amount.

Sample	Amount Tested	Original Culture (CFU/ml)	Inoculum (CFU/sample)
Water(sterilized)	1 L	5.6×10^9	2.8 and 5.6 (0.5 and 1 ml of 10^{-9} diluted original culture)
Sediment (sterilized)	25 g	5.6×10^9	2.8 and 5.6 (0.5 and 1 ml of 10^{-9} diluted original culture)
Oysters	25 g	2.2×10^9	22 (0.1 ml of 10^{-7} diluted original culture)

2.3.2 THE OCCURRENCE OF *LISTERIA*, FAECAL COLIFORMS AND *E. COLI* BY TYPE OF SAMPLES

The results of statistical analysis for values of pH, temperature, salinity and occurrence of faecal coliforms, *E. coli* and *Listeria* are summarized in Table 2.5. Details of the results for individual sites are given in Appendix C. The results are presented in the following order; firstly the input sources i.e. fresh water and sediment (sites 8 and 9) and effluent (sites 10 to 12), then the receiving estuarine water and sediment (sites 1 to 7) and shellfish (sites 6a and 6b):

2.3.2.1 River water and sediment (sites 8 and 9)

The Occurrence

The occurrence of *Listeria* spp. in river water was particularly high, 100% (n=26) in site 8 and 92.3% (n=26) in site 9 (Table 2.5). Fig. 2.6 shows the overall occurrence of *Listeria* spp. and *L. monocytogenes* in river water, 96% and 37% (n=52) respectively. This is similar to the report of Watkin and Sleath (1981) who recovered *Listeria* spp. in all river water (n=7) sampled in the United Kingdom. High occurrence of *L. mono-cytogenes* (47%) in River Don (n=36), Aberdeen, UK was recently reported by Fenlon *et*

Table 2.5 Statistical analysis of physicochemical parameters, occurrence of *Listeria* spp. and *E. coli* in water, and occurrence of *Listeria* spp. in sediments and shellfish in the period of 20 May 1994 - 5 May 1995 (26 rounds).

Station No. and Name	WATER							SEDIMENT		SHELLFISH	
	Mean (Min.-Max.) of Physicochemical parameters			Median (Min.-Max.) of FC / *E. coli*		% Samples Positive with *Listeria*		% Samples Positive with *Listeria*		% Samples Positive with *Listeria*	
	pH	Temp. (°C)	Salinity (‰)	FC / 100 ml	*E. coli* /100 ml	LM	*Listeria* spp.	LM	*Listeria* spp	LM	*Listeria* spp.
Inshore Water 1. Tinderbox	7.88 (7.15-8.22)	13.2 (8.7-18.2)	25.6 (20.1-29.0)	2 (<1-2x10²)	1 (<1-2x10²)	7.7	15.4	0	30.8	NT	NT
2. Salmon Farm at Powder Jetty	7.94 (7.51-8.12)	13.3 (8.9-18.9)	26.0 (20.5-29.0)	<1 (<1-6.9x10¹)	<1 (<1-5.2x10¹)	0	7.7	0	23.1	NT	NT
3. Stinkpot Bay	7.96 (7.62-8.16)	13.8 (7.6-23.6)	25.6 (19.8-28.7)	1.9x10 (<1-3.6x10³)	1.9x10 (<1-3.6x10³)	11.5	26.9	7.7	46.2	15.4	38.5
4. "Sanctuary"	7.89 (7.63-8.17)	14.5 (5.3-27.9)	24.8 (11.4-30.1)	5.8x10 (<1-1.3x10³)	5.8x10 (<1-1.2x10³)	7.7	23.1	15.4	30.8	NT	NT
5. Dru Point	7.82 (7.41-8.14)	14.0 (8.1-24.2)	24.4 (3.14-28.7)	2.1x10² (2.2x10¹-1.7x10⁴)	1.6 x10² (1-1.7x10⁴)	11.5	34.6	23.1	61.5	15.4	61.5
6a. NWB commercial Jetty and 6b. Mussels culture at Beach Road Jetty	8.07 (7.58-8.37)	14.3 (9.4-22.9)	26.6 (22.2-29.0)	8 (<1-1.3x10²)	6 (<1-1.3x10²)	7.7	11.5	7.7	23.1	15.4	23.1
7. NWB Marina	7.98 (7.65-8.34)	14.4 (9.0-24.1)	26.7 (19.5-29.4)	5 (<1-1.5x10²)	5 (<1 -1.5x10²)	0	11.5	30.8	46.2	NT	NT
River Water 8. Coffee Creek	7.39 (7.00-8.20)	11.7 (6.2 - 20.4)	10.1 (0.1-27.4)	7.2 x10² (4.7x10¹-8.2x10⁴)	6.5 x10² (4.7x10¹-7.4x10⁴)	61.5	100	69.2	92.3	NT	NT
9. NWB River	8.23 (7.54-9.06)	11.4 (5.0-22.1)	0.09 (0-0.22)	3.0 x10² (2.0x10¹-3.4x10⁴)	3.0 x10² (2.0x10¹-3.4x10⁴)	11.5	92.3	23.1	76.9	NT	NT
Effluent 10. STP Dru Point	8.51 (7.41-9.78)	13.9 (5.8-24.4)	0.27 (0.02-0.4)	2.7 x10³ (1.4 x10²-4.8 x10⁴)	2.1 x10³ (1.4x10²-4.8x10⁴)	53.8	69.2	NT	NT	NT	NT
11. Discharge from fish factory 1	6.07 (5.33-6.77)	15.4 (7.1-24.6)	9.1 (3.08-13.3)	6.8 x10² (5.0x10¹-1.9x10⁴)	4.8 x10² (5.0x10¹-1.0x10⁴)	34.6	61.5	NT	NT	NT	NT
12. Discharge from fish factory 2	6.51 (4.86-7.18)	15.0 (9.6-22.0)	8.4 (0.8-10.8)	4.1x10³ (5.0x10¹-1.0x10⁶)	2.1 x10³ (5.0x10¹-1.0x10⁶)	100	100	NT	NT	NT	NT

Min., Minimum. Max., Maximum. Temp., Temperature. FC, Faecal coliforms. *LM, L. monocytogenes. Listeria* spp., all *Listeria* species. NT, Not tested
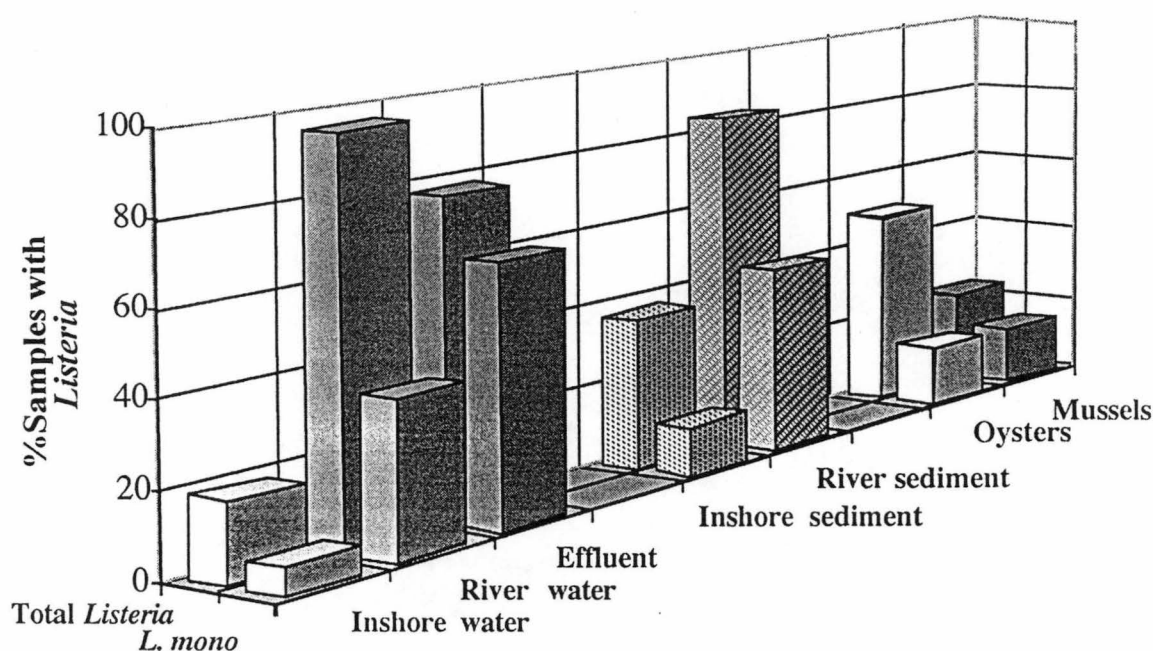
Figure 2.6 Occurrence of *Listeria* spp. and *L. monocytogenes* by sample type. The back column refers to total *Listeria* spp., the front column refers to *L. monocytogenes*.

al. (1996). A recovery rate of 37% of *L. monocytogenes* in surface water (n=180) sampled from canals and lakes in northern Holland was reported by Dijkstra (1982). A high occurrence (81%) of *Listeria* spp. was also detected from 37 fresh water samples collected from various tributaries draining into Humboldt-Arcata Bay, California, during winter (Colburn *et al.*, 1990). *L. monocytogenes* was the most predominant *Listeria* spp. which was isolated from 62% of all water samples. The authors suggested, as discussed in section 1.4.4, the nearby domesticated animals (cows, horses) may influence the distribution of a given species or *L. monocytogenes* serogroup *via* the runoff containing animals faeces. Greater variety, with no species predominance, was observed in areas with no direct animal influence. Dijkstra (1975) reported the detection of *L. monocytogenes* from all 97 faeces samples collected from healthy and *Listeria* infected animals in the Netherlands.

In contrast, Jemmi and Keusch (1994) reported only 11% of *Listeria* spp. and 0% of *L. monocytogenes* from 36 samples of water (ground, spring and river water) collected from three Swiss fish farms for rainbow trout (*Oncorhynchus mykiss*). The authors considered that the difference may arise because two-thirds of the samples were ground or spring water from which no *Listeria* were recovered. However, considering that the

study examined only 10 ml of water sample for *Listeria* analysis, this may be another reason for lower detection of the organisms.

In the present study, *Listeria* spp. were detected in 22 of 26 sediment samples (84.6%) (Fig. 2.6) collected at the same locations as the surface river water samples (sites 8 and 9). *L. monocytogenes* again was frequently recovered, from 12 of 26 sediment samples (46%). This rate was higher than found by Weis and Seeliger (1975) who recovered *L. monocytogenes* in 12 of 38 (31.5%) of mud samples from creeks, rivers and ponds in southern West Germany. A lower incidence of 30.4% and 17.4% of *Listeria* spp. and *L. monocytogenes* respectively, was also observed in 46 samples from tributaries draining into Humboldt-Arcata Bay, California by Colburn *et al.* (1990).

It is noted that the water level in site 8 was normally low and the sediment was mostly vegetative litter, brown to black colour, indicating anoxic conditions with mild sulfur odour occasionally observed. Site 9 has the largest catchment for the bay (Table 2.1) and the sediment consisted of sand, rocks and vegetative litter. Species identification showed that *L. monocytogenes* was the most commonly isolated species of the genus in Coffee Creek; 16 of 26 water samples (61.5%) and 9 of 13 sediment samples (69%) were found to harbour the organism (Appendix C, Table C.8). The presence of *L. monocytogenes* indicates contamination by the organism which probably results from domestic waste, seepage of human faeces from inefficient septic tanks and run-off of animal faeces from grazing land. It has been reported that faeces of clinical healthy human and animals were found to have a *L. monocytogenes* carrier rate of 29.1% and 15.3%, respectively (Kampelmacher and van Noorle Jansen, 1969). However in North West Bay river, *L. seeligeri* was the most predominant species; 46.2% in water samples and 53.8% in sediment samples.

Relationship between Listeria and environmental parameters and faecal coliforms/E. coli

This study indicates that the occurrence of *Listeria* spp. in river water remained high throughout the 12 months of sampling regardless of the temperature, from 5.0°C to 22.1°C (Fig. 2.7). The average occurrence of *Listeria* spp. in river water was the highest of all types of water studied here (Fig. 2.6). However, in the case of *L. mono-cytogenes*, the organism was absent in the period of December 1994 to February 1995 (summer) which was the hottest period of the year, and the driest since 1985 (Bureau of Meteorology, Hobart). The results suggested those conditions were not suitable for *L. monocytogenes* to survive, or that it may become injured and was unrecoverable. Additionally, there was likely to be some competitive effects between species as *L. innocua* and *L. seeligeri* were detected in all those samples (Appendix C, Tables C.8 and C.9). The pH of the river samples varied from 7.00 to 9.06 which did not appear to be

related to the occurrence of *Listeria* spp. The average salinity of the river water (Fig. 2.7) was substantially influenced by the influx of marine water into Coffee Creek which caused the salinity of the water in this site to range from 0.1 to 27.4‰ while the salinity of North West Bay River was in a narrow range from 0.00 to 0.22‰. The levels of faecal coliforms and *E. coli* did not appear to be related to the occurrence of *Listeria* (Fig. 2.7).

Statistical analysis, using the logistic method, confirmed that none of the environmental parameters (temperature, pH, salinity and rainfall) or the level of faecal coliforms and *E. coli* could explain the occurrence of *Listeria* spp. (Appendix E, Table E.1). However, the pH followed by the salinity of the river water appeared to have significantly affected (*P* ≤0.01) the presence/absence of *L. monocytogenes* (Appendix E, Table E.2). Therefore, using pH as the predictor the fitted value for the constant and the values for the parameters of the presence/absence model can be added to the form of the presence/absence model:

$$\text{logit (L. mono)} = \ln \left(\frac{P}{1-P} \right) = 7.3906 - 6.8484 \, (\ln \text{pH}) \tag{2.2}$$

where all the terms were previously defined in Eqn 2.1 and pH is pH - 4.5. The area *c* under the ROC curve obtained from the fitted model (Eqn. 2.2) is 0.779.
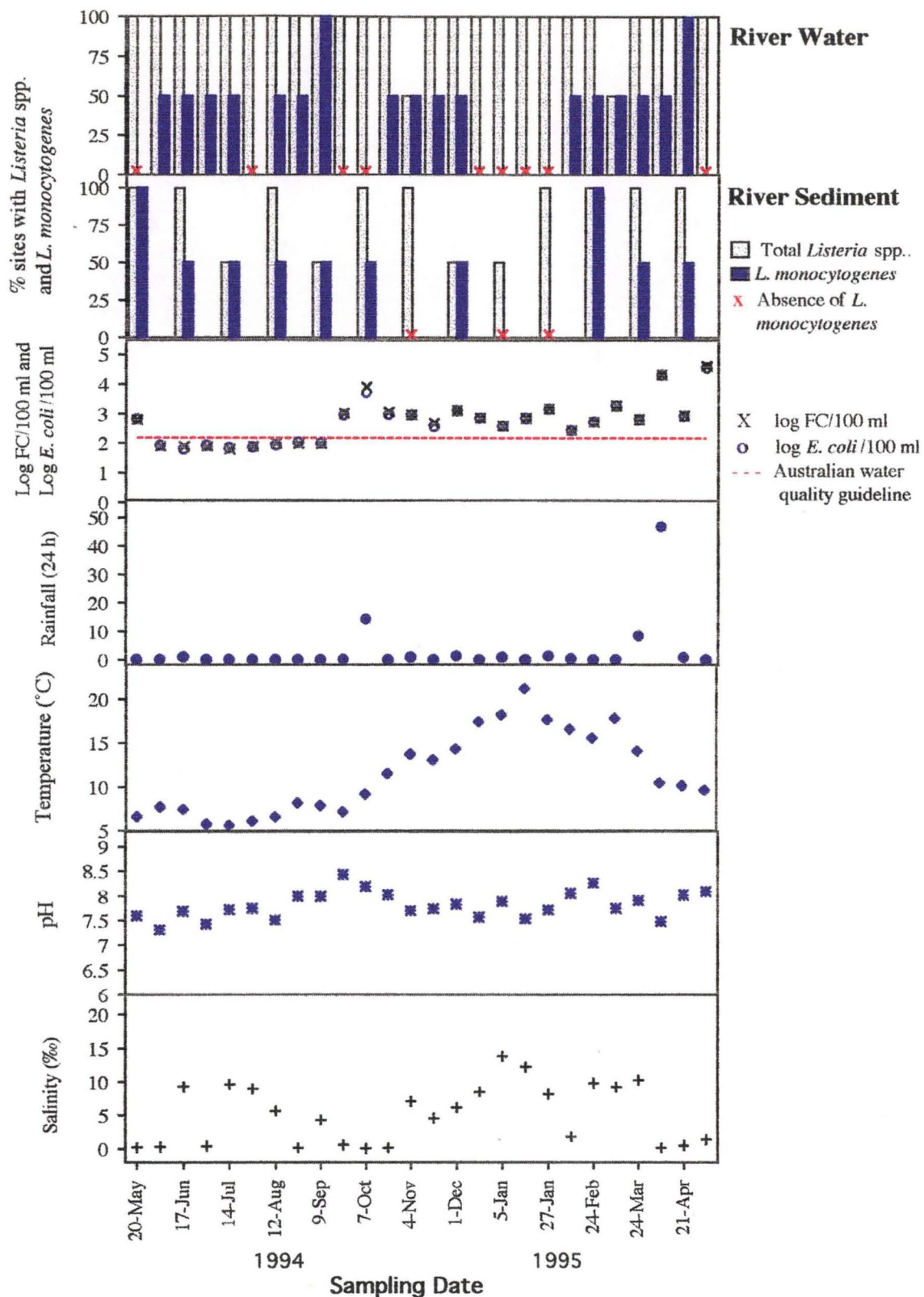
Figure 2.7 Percent of positive sites with *Listeria* spp. and *L. monocytogenes* in river water and sediment samples (sites 8 and 9) compared with the amount of faecal coliforms and *E. coli*, and environmental parameters; the recorded rainfall in the preceding 24 hr, and temperature, pH and salinity in water.

2.3.2.2 Effluent (sites 10 to 12)

The Occurrence

It is well known that raw municipal sewage may contain substantial numbers of various organisms including faecal pathogens.   If sewage influent undergoes mechanical, biological and chemical treatments, most of the microflora present in the sewage should be reduced, if not killed, during the processes.   Despite these treatments however, it has been found by many workers e.g. Gameson (1975), Kawamura and Kaneko (1986), that the final effluents and the sewage end products usually contain many organisms, including pathogens, which vary in number and type depending on the efficiency of treatment and on the ability of each type to survive it.   The pathogenic bacterium, *L. monocytogenes* has also been reported to be recovered frequently from sewage-containing effluents (Al-Ghazali and Al-Azawi, 1986, 1988a).

Listeria spp. in effluent samples were frequently recovered, ranging from 61.5% to 100% (Table 2.5).   Likewise, *L. monocytogenes* was the most frequently detected species in site 10 and 12 while *L. innocua* was commonly found in site 11 (Table 2.5).   Fig. 2.6 shows the overall occurrence of *Listeria* spp., i.e. 77% of effluent samples (n=78). Effluent appeared to be a major contributor of *L. monocytogenes* to the NWB environment as it was found to contain the highest average occurrence of the organism, i.e. 63% (Fig. 2.6).   In particular, the sewage treatment pond receiving municipal sewage was shown to contain *L. monocytogenes* in 54% of samples (Table 2.5).   The effluents from two fish processing factories' treatment plants were found to have *L. monocytogenes* in 100% and 35% of samples (Table 2.5) respectively.   Occasionally, more than one species was isolated from each site.   Less species variation was found in this type of water, presumably due to the limited type of input.

Human faeces are likely to be the major source of the organisms (Kampelmacher and van Noorle Jansen, 1969) in the sewage treatment system.   It is noteworthy that the treatment system of the two factories (sites 11 and 12) are very similar (activated sludge), and both systems receive human faecal waste.   However, *L. monocytogenes* was detected in effluent samples at site 12 approximately 3 times more frequently than at site 11 (Table 2.5).   Furthermore, the amounts of *E. coli* /100 ml in effluent at site 12 were much higher than in effluent at site 11.   The substantial difference may be due to dilution resulting from larger volumes of factory floor wastewater discharged into site 11.

The high occurrence of *Listeria* spp. including *L. monocytogenes* in these sites were similar to the report of Kampelmacher and van Noorle Jansen (1975) who recovered 92% of *L. monocytogenes* from effluent samples (n=38) collected from 8 sewage treatment plants in the Netherlands.   Watkins and Sleath (1981) also reported all effluent samples

(n=39) to be positive for *L. monocytogenes*. Likewise, Al-Ghazali and Al-Azawi (1986, 1988a) isolated *L. monocytogenes* from all stages of the treatment, including final discharge samples, collected from sewage treatment works in Baghdad, Iraq.

The ability of *L. monocytogenes* to survive and even multiply after biological treatment (Geuenich and Müller, 1984) should increase awareness of the potential hazards of such effluent. The discharge of contaminated water results in its distribution to surface receiving water. Consequently the receiving water may become a route for recycling these *Listeria* via irrigation, recreational use or the foodchain. In support of this, Dijkstra (1982) reported a recovery rate of 67% of *L. monocytogenes* on 33 sites along the 5 miles (8 kms) distance from a sewage treatment plant in the Netherlands and emphasised the survival and distribution of the organism which could be detected in fresh water at 25 miles (40 kms) downstream from the sewage treatment plant. Although in the same study no *L. monocytogenes* were recovered from the seawater samples (n=43) into which the canals and lakes emptied, it seems desirable to eliminate, where possible, the potentially pathogenic organism before discharging to the sea and other surface water.

Relationship between Listeria and environmental parameters and faecal coliforms/E. coli

The study indicates effluent samples had the highest occurrence of *L. monocytogenes* especially from the fish factory 2 where all samples gave positive results. From Fig. 2.8, none of the environmental factors, or the level of faecal coliforms or *E. coli* is likely to be related to the occurrence of *Listeria* spp. including *L. monocytogenes* in effluent samples. However, the higher temperature for long periods in spring and summer displays a correlation with survival of *Listeria* in the sewage treatment pond (site 10) as the occurrence of the organism decreased significantly in that period (Appendix C, Table C.10).

The statistical analysis using the logistic method confirmed that none of the environmental parameters (temperature, pH, salinity and rainfall) could explain the occurrence of *Listeria* (Appendix E, Tables E.3 and E.4). However, the level of faecal coliforms appeared to be a significant factor ($P \leq 0.01$) correlated to the presence/ absence of *Listeria* spp. (Table E.3). In addition, both faecal coliforms and *E. coli* displayed a significant correlation with the presence/absence of *L. monocytogenes* (Table 2.9). Adding, the fitted value for the constant and the values for the parameters of the presence/absence model yields:

$$\text{logit (Listeria)} = \ln\left(\frac{P}{1\text{-}P}\right) = 4.0575 - 2.2335 \,(\ln T) + 0.4800 \,(\ln fc) \qquad (2.3)$$

$$\text{logit (L. mono)} = \ln\left(\frac{P}{1\text{-}P}\right) = -6.0792 + 1.4710 \,(\ln pH) + 1.0506 \,(\ln S) + 0.5103 \,(\ln fc) \qquad (2.4)$$
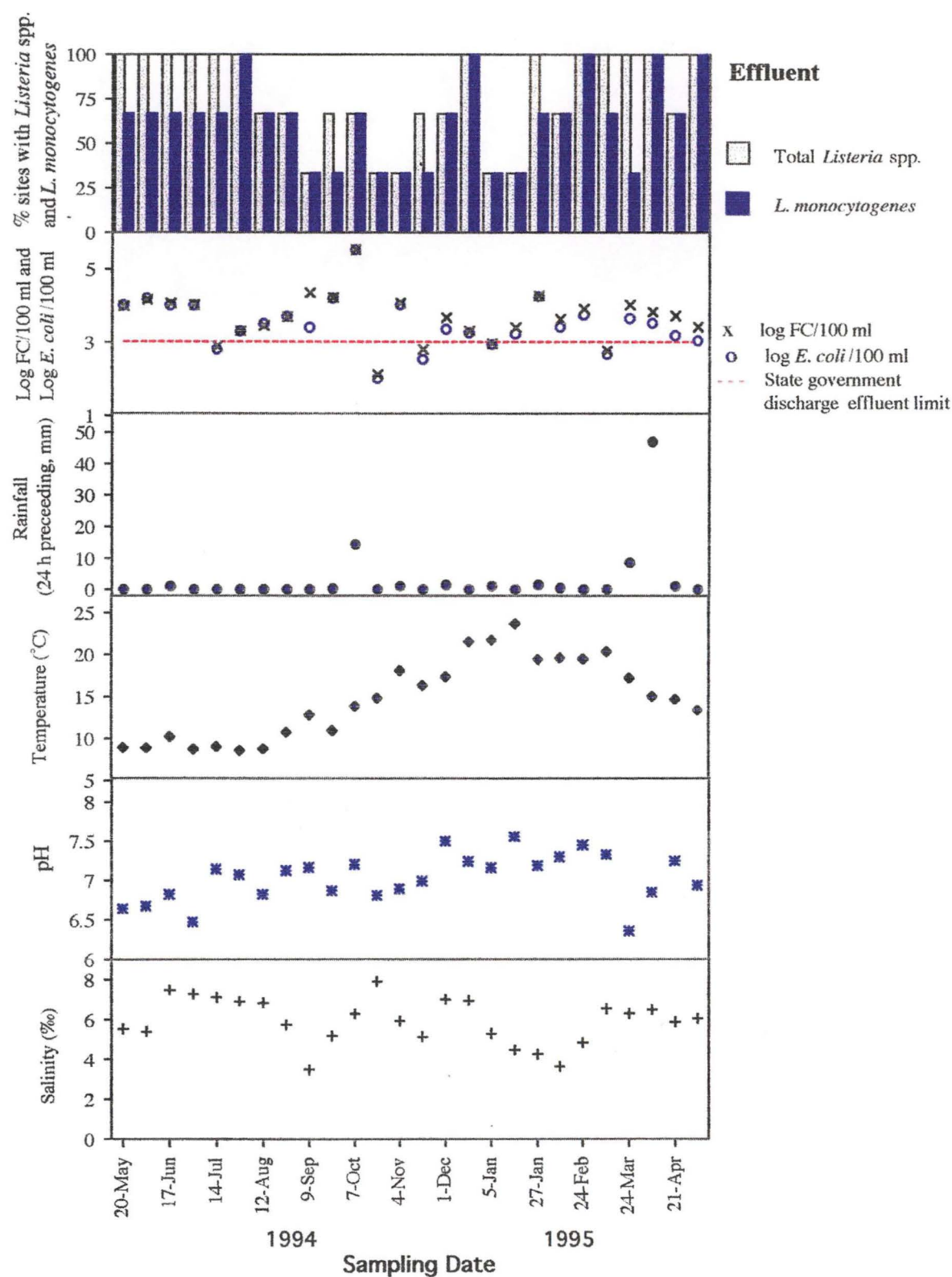
Figure 2.8 Percent of positive sites with *Listeria* spp. and *L. monocytogenes* in effluent samples (sites 10 to 12) compared with the amount of faecal coliforms and *E. coli*, and environmental parameters; the recorded rainfall in the preceding 24 hr, and temperature, pH and salinity in water.

where all the terms were previously defined in Eqn. 2.1. The areas c under the ROC curves obtained from the fitted models, Eqn. 2.3, and Eqn. 2.4, are 0.754 and 0.745 respectively.

2.3.2.3 Inshore marine water and sediment (sites 1 to 7)

The Occurrence

Estuarine environments are continuously subjected to potential contamination with *Listeria* from many natural and anthropogenic sources i.e. sewage effluents, processing effluents, septic tank seepage or overflow, terrestrial run-off etc. From this study however, the average occurrence of *L. monocytogenes* and total *Listeria* spp. in inshore waters around North West Bay (n=182) remained low, 6.6% and 18.7%, respectively (Fig. 2.6). In support of this, a study in the north of the Netherlands (Dijkstra, 1982) showed no *Listeria* spp. in 43 seawater samples although *Listeria* contaminated water was found in the effluent from a sewage treatment plant and along the canals including in the location where this canal drained into the sea. Motes (1991) also reported only 2 (2.9%) positive for *Listeria* spp. from 70 estuarine water samples collected from various shellfish-growing areas along the U.S. Gulf Coast. Likewise, Rørvik *et al.* (1995) reported the recovery of *L. monocytogenes* and other *Listeria* spp. from 3 (9%) and 12 (36%) of 33 environmental seawater samples taken from outside a salmon slaughter house in Norway. Additionally, the authors found no *Listeria* spp. in 6 samples of deep seawater. In a limited survey (n=3), a higher recovery rate of 33% of *Listeria* spp. including *L. monocytogenes* was found in Humboldt-Arcata Bay, California (Colburn *et al.*, 1990).

The lower levels of *Listeria* spp. in estuarine water when compared with other types of water or environment could be due to a variety of reasons such as:

- the effect of dilution by the large volumes of seawater in the marine environment (Colburn *et al.*, 1990);

- organism die-off because of different levels of available nutrients; It has been found that the rate of die-off of a microorganism in the low nutrient level in the sea is approximately proportional to the number of viable cells remaining at any time - or that the logarithm of the number decreases linearly with time (Gameson, 1975).

- organism die-off because of the presence of toxic compounds (Mitchell, 1974);

- organism die-off because of the competition or predation by other organisms (Roszak and Colwell, 1987).

- and other factors including UV damage as it was reported by Gameson (1975) from the Water Research Centre that the radiation damage is one of the most important

mechanisms contributing to the mortality of microorganisms in the sea. The rate of radiation-induced mortality is proportional to the intensity of irradiation. In addition, the type and the physiological state of the microorganisms may also play a role in the lower recovery rate in this estuarine water.

A study with *L. monocytogenes* (Faud *et al.*, 1989) has shown that levels of the organism declined rapidly when it was inoculated into seawater. In addition, the survival of *Listeria* in culturable form in water was reported to be temperature dependent i.e. at 30°C the culturability of cells declined most rapidly within 24 hr when compared to 4°C and 15°C (McKay, 1993). Loss of culturability may be a result of cell death or a transition of cells to a viable but non-culturable form for which resuscitation becomes difficult (McKay, 1993). Therefore, the presence of *L. monocytogenes* in marine water may indicate a recent contamination.

From Fig. 2.6, *Listeria* spp. including *L. monocytogenes* appeared to survive approximately two times as well in surface sediment than in water. The percent positive for these organisms in the sediment samples (n=91), were 37.4% and 12.1%, respectively, while the occurrence in water samples were 18.7% and 6.6%, respectively. The most frequently isolated species in both inshore water and sediment samples.was *L. seeligeri*, 12.8%, followed by 8.1% of *L. monocytogenes* from all samples (n=273). Occasionally, more than one species was isolated from each location (Appendix C, Tables C.1 to C.7). Neither *L. grayi* nor *L. murrayi* was isolated from the inshore water in North West Bay.

Relationship between Listeria and environmental parameters and faecal coliforms/E. coli

The study indicates that the incidence of *Listeria* spp. including *L. monocytogenes* in inshore water throughout the 12 months of the sampling period was not very high when compared with the input from sources (river and effluent). However, there were some peaks of the occurrence of *Listeria* spp. and *L. monocytogenes* in October 1994 and in April 1995 although these did not appear to be related to specific physicochemical parameters (salinity, pH and temperature) (Fig. 2.9). However, rainfall showed some effect on the occurrence of *Listeria* spp. including *L. monocytogenes* (Fig. 2.9). Similarly, the amount of faecal coliforms and *E. coli* / 100 ml in the inshore water also appeared to increase in parallel with the occurrence of *Listeria* spp. and *L. monocytogenes* (Fig. 2.9). Since the effluent were normally discharged directly to the bay, although during the period of high rainfall, the overflow of the diluted sewage may occur. Therefore, the increase in the occurrence of *Listeria* spp. including *L. monocytogenes* and the amount of faecal coliforms may be the result of increasing runoff of animal faeces
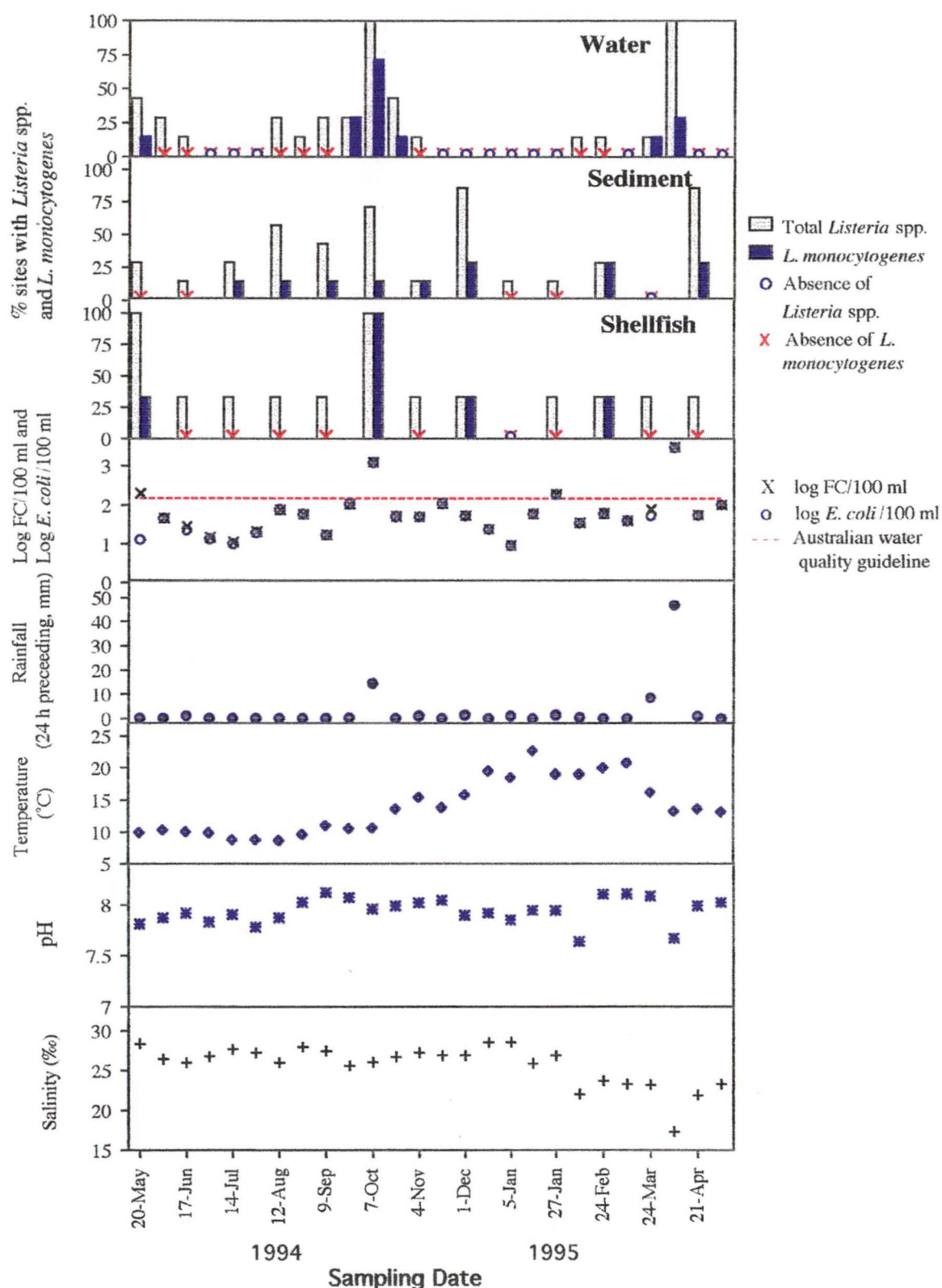
Figure 2.9 Percent of positive sites with *Listeria* spp. and *L. monocytogenes* in estuarine water, sediment and shellfish samples (sites 1 to 7) compared with the amount of faecal coliforms and *E. coli*, and environmental parameters; the recorded rainfall in the preceding 24 hr, and temperature, pH and salinity in water.

from grazing land and increased volume of seepage from septic tanks. An additional influence may be the release of adsorbed faecal coliforms, including *E. coli* (Phillips, 1993), and *Listeria* from sediment particles following dilution of the salt content of the sediment interstitial water by rainwater runoff. The results of MEE typing (see 2.3.3, and Appendix C) support this assumption as some of *L. monocytogenes* strains found in estuarine environment were different from the input sources.

A logistic method was used to determine the effect of the independent variables on the presence or absence of *Listeria* spp. and *L. monocytogenes*. There were 182 observations of which 34 were positive and 148 were negative for *Listeria* spp., while 11 were positive and 171 were negative for *L. monocytogenes*. Summaries of the statistical chi-square distribution including the statistically significant results are shown in Appendix E, Tables E.5 and E.6.

The statistical results (Appendix E, Table E.5) indicated that rainfall recorded during the preceding 72 hr was the most significant environmental parameter ($P \leq 0.01$) for the presence/absence of *Listeria* spp. However, the rainfall at 24 hr also showed significant effect and could also be used to determine the occurrence of *Listeria* spp. Faecal coliforms showed a more significant correlation with the occurrence of *Listeria* spp. than *E. coli*. In addition, the combination of some significant environmental parameters with the amount of faecal coliforms or *E. coli* substantially increased the level of significance.

The rainfall recorded during the preceding 7 days was the most significant environmental parameter for the occurrence of *L. monocytogenes* in estuarine water (Appendix E, Table E.6). However, the rainfall at 48 hr also showed significant correlation and could also be used to determine the occurrence of *L. monocytogenes*. Once more, faecal coliforms showed a more significant correlation with the occurrence of *L. monocytogenes* than *E. coli*. In addition, the combination of some significant environmental parameters with the amount of faecal coliforms or *E. coli* enhanced the level of significance. One should bear in mind that all the independent variables occurred naturally. Furthermore there would be some other variable factors such as the amount and physiology of the organism, the level of available nutrient and the presence of other competitive organisms which also played an important role in determining the occurrence of *Listeria* spp.

The probability models for predicting the presence/absence of *Listeria* spp. including *L. monocytogenes* were fitted from the most significant variable(s). The estimates of the parameters of the model are as follows:

$$\text{logit (Listeria)} = \ln\left(\frac{P}{1-P}\right) = 5.6324 - 2.9703\ (\ln\ T) + 1.1754\ (\ln\ Rf24) \tag{2.5}$$

$$\text{logit (Listeria)} = \ln\left(\frac{P}{1-P}\right) = 4.2206 - 2.9591\ (\ln\ T) + 0.8996\ (\ln\ Rf24) + 0.5022\ (\ln\ fc) \tag{2.6}$$

$$\text{logit (L. mono)} = \ln\left(\frac{P}{1-P}\right) = 7.7671 - 4.5073\ (\ln T) + 1.1960\ (\ln\ Rf24) \tag{2.7}$$

$$\text{logit (L. mono)} = \ln\left(\frac{P}{1-P}\right) = 3.5306 - 3.6274\ (\ln T) + 0.6899\ (\ln\ Rf24) + 0.6303\ (\ln fc) \tag{2.8}$$

where all the terms were previously defined in Eqn 2.1. The areas c under the ROC curves of 0.755 was obtained from Eqn. 2.5 which was derived from two environmental predictors so that the model will be more practical. However when the amount of faecal coliforms was included in the model (Eqn 2.6), c increased to 0.839.

A good agreement between the predicted probabilities given by the fitted model for the presence/ absence of *L. monocytogenes* (Eqn 2.7) and the observed probabilities of the data used to generate the model was shown by $c = 0.892$. Eqn 2.7 was derived from two environmental predictors so that the model will be more practical. Again, by including the amount of faecal coliforms in the model (Eqn 2.8), c increased to 0.948.

Examples of the interface at probabilities $P = 0.10$, 0.50 and 0.90 of the presence of *L. monocytogenes* in estuarine water were calculated from Eqn 2.8 and are graphically shown in Fig. 2.10. The average estuarine water temperature of 13.4°C (Appendix C, Tables C.1 to C.7) was used as a fixed term in Eqn 6 so that the graph can be drawn on a 2-dimensional plane. Similar figures may be drawn for other values of P or for other growth regulating conditions. The dashed line (Fig. 2.10) shows that faecal coliforms at the level of 14 CFU/100 ml which has been used as a shellfish sanitary criteria (ANZECC, 1992) fall in the safe area (i.e. the probability that *L. monocytogenes* would be present is less than 0.50).

2.3.2.4 Shellfish (sites 3, 5 and 6b)

In this study, 26 samples of Pacific oysters (sites 3 and 5) and 13 samples of blue mussels (site 6b) collected throughout the 12 month period were found to be contaminated with *Listeria* spp. on sampling occasions, i.e. 50% and 23.1% (Fig. 2.6) respectively. However, the occurrence of *L. monocytogenes* in these shellfish remained low; i.e. 15.4% in both oysters and blue mussels (Fig. 2.6). Similar findings of a high frequency of *Listeria* spp. (55%) and *L. monocytogenes* (9.2%) in shellfish (n=120) reared in Brittany, western France, were reported by Monfort *et al.* (1998). The authors indicated that there was a significant relationship ($P<0.001$) between the occurrence of *Listeria* and
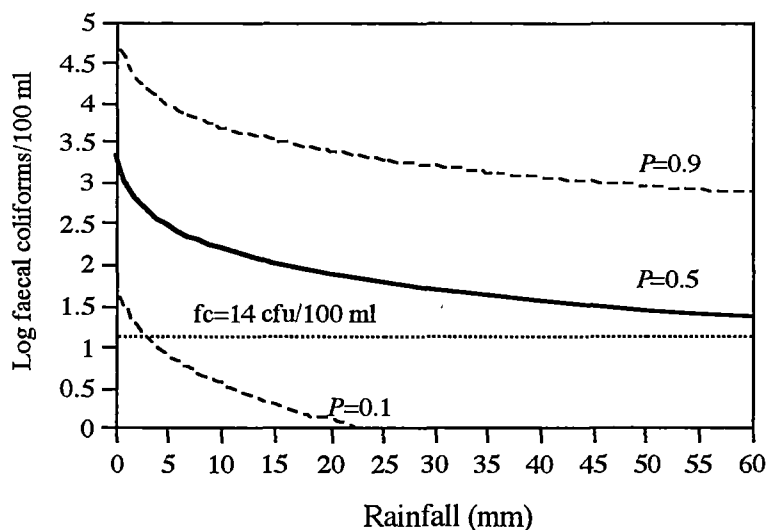
Figure 2.10 Probability of presence of *L. monocytogenes* in 1L of estuarine water using the logistic model when faecal coliforms, rainfall and temperature are predictors (Eqn 2.8). This graph is an example with temperature fixed at 8°C (representative of temperatures in winter), while faecal coliforms (fc) =14 cfu/100 ml, the limitation for shellfish sanitary status (ANZECC, 1992).

the level of faecal coliforms in shellfish. In that study, a higher recovery rate of *Listeria* spp. was reported for winter than in summer. However, in the present study, no seasonal variation could be discerned (Appendix C, Tables C.3, C.5, and C.6).

A low incidence of *Listeria* was reported by Colburn *et al.* (1990), i.e. 0% for *L. mono-cytogenes* and 2.8% for *L. innocua*, in 35 samples of oysters held in Humboldt-Arcata Bay, California, during the winter months. The authors suggested the ability of *Listeria* to survive in marine waters, the degree to which *Listeria* are diluted, and the pumping rate by oysters are all factors that could affect the uptake, retention and depuration of *Listeria* by oysters.

The contamination of oysters and mussels by *Listeria* in the present study may be attributed to water recently contaminated from terrestrial sources. Although other studies have reported the absence of *Listeria* from oysters (Motes, 1991; Buchanan *et al.*, 1989b; Weagant *et al.*, 1988) and mussels (Decastelli *et al.*, 1993), the potential exists for shellfish to contain *Listeria* since the organisms were recovered from the overlying waters, and shellfish, which are filter feeders, can accumulate the organisms from the water column. Fig. 2.11 shows a higher occurrence of *Listeria* including *L. mono-cytogenes* in oysters and mussels when compared to the NWB water column in the same
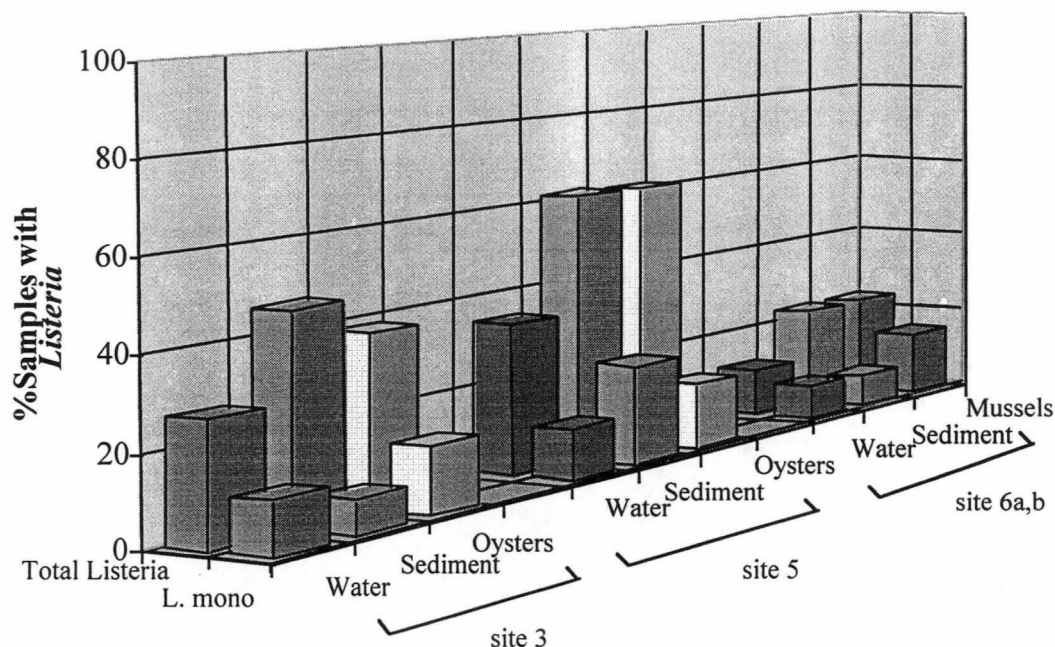
Figure 2.11 Percent of samples positive with *Listeria* spp. and *L. monocytogenes* in inshore water, sediment and shellfish samples in sites 3, 5 and 6a,b.

sampling sites. The acquisition by humans of *L. monocytogenes* can occur by consumption of raw shellfish. It should be emphasised that the shellfish studied in the present study were not taken from areas approved for human consumption.

2.3.3 OCCURRENCE OF *LISTERIA* IN NORTH WEST BAY AS A SYSTEM

Listeria spp. including *L. monocytogenes* are ubiquitous in the environment. However, very few studies have been done on the occurrence of the organisms in aquatic habitats which may relate to the distribution, contamination and epidemiology of listeriosis. In the present study, the inshore marine water of North West Bay was examined in association with the input water; i.e. river and discharged wastewater from factories around North West Bay.

Fig. 2.12 shows percent positive samples of *Listeria* spp. and *L. monocytogenes* in each site during the 12-month study. A relatively high frequency of occurrence was detected from both river and effluent. The highest occurrence of *L. monocytogenes* (100%) was found in effluent samples from site 12 (fish processing factory 2), followed by river
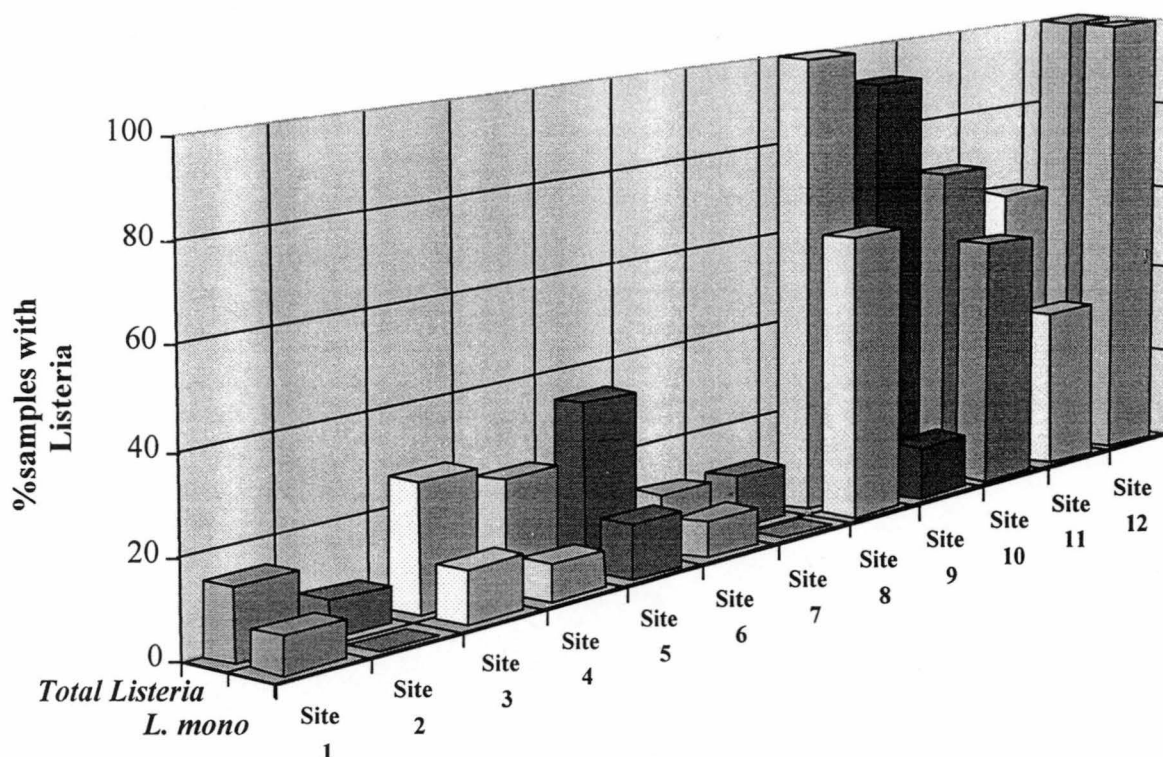
Figure 2.12 Percent of samples positive with *Listeria* spp. and *L. monocytogenes* in water samples. Sites 1 to 7 were inshore marine water, sites 8 and 9 were river water, site 10 was effluent from STP and sites 11 and 12 were effluent from fish processing factories.

water samples from site 8 (69.2%). Despite the input from these contaminated waters, the overall occurrence of *Listeria* in inshore water appeared to be considerably lower. However, the introduction of *Listeria* from these inputs can be clearly observed in site 5 (Dru Point) which received effluent directly from site 10 (STP at Dru Point) and site 9 (NWB river) becoming the most *Listeria* contaminated inshore site (11.5%). The results from both site 5 and site 3 (Stinkpot Bay) which received fresh water from Coffee creek indicated the highest occurrence of *L. monocytogenes* in the Bay. In contrast it was noted that the occurrence of *Listeria* spp. including *L. monocytogenes* in site 7 (North West Bay Marina) was very low, considering that this site received effluent from site 12. This circumstance may be explained by the fact that the water level in this site was relatively deep and more water movement was regularly observed when compared to site 5 and 3. Hence, the discharged organisms may be promptly diluted and dispersed to other parts of the Bay.

The impact of the effluent from site 12, however, can be determined from the highest percentage of the occurrence of *L. monocytogenes* (30.8%) in inshore sediment samples from site 7 (Fig. 2.13). This demonstrates the ability of the organism to survive better in inshore marine sediment than in the water column. In addition, all of the seven sites inshore sediments show higher percentage for *Listeria* spp. than in water although some results of *L. monocytogenes* were lower or equal (Fig. 2.13). The sediment particles may serve as an adsorbent and also sequester some available nutrients for the organism to better survive in the marine environment. According to the current movement in NWB (see section 2.2.2.1), it appeared that the occurrence of *L. monocytogenes* in inshore sediment samples proportionally related to the distance from the sewage outlet of STP at Dru Point (site 10), i.e. site 5 has the high occurrence of *L. monocytogenes* (Fig. 2.13). It is clear that the inshore sites, e.g. site 7, closest to the contaminated input sites, e.g. site 12, consequently presented high occurrence of *L. monocytogenes* especially in sediment. Petran and Swanson (1993) indicated that in the same broth media *L. innocua* outgrows *L. monocytogenes*. However, the overall occurrence observed in this study does not indicate any relationship between the presence or absence of other *Listeria* species and *L. monocytogenes* (results not shown). This is in accord with the findings of Monfort *et al.* (1998).
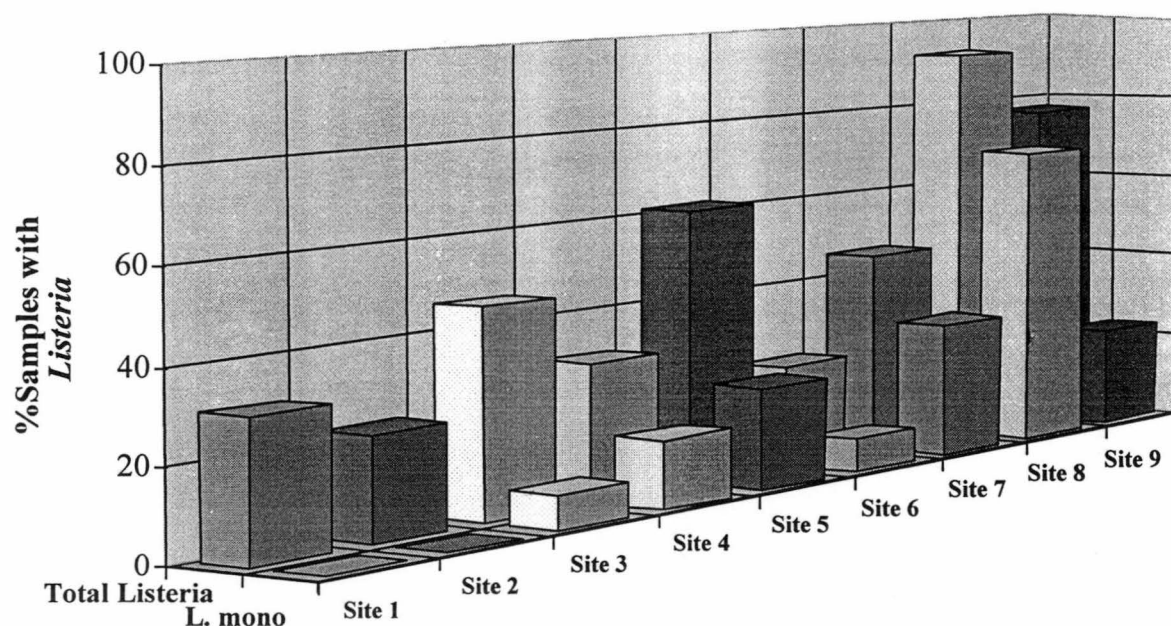
Figure 2.13 Percent of samples positive with *Listeria* spp. and *L. monocytogenes* in sediment samples. Sites 1 to 7 were inshore marine sediment and sites 8 and 9 were river sediment.

The species identification in the present study showed that *L. monocytogenes* occurred in all types of samples i.e. inshore water, river, effluent, sediment and shellfish. In addition, all 26 effluent samples collected over the 12 month period from sewage treatment plant of fish factory 2 were found to contain *L. monocytogenes*. However, it does not indicate that the organism can survive and proliferate in the effluent throughout the year. Occasionally, the isolated organism appeared to have a different pattern of haemolytic activity on CAMP test. Hence, a genetic analysis of all 113 *L. monocytogenes* isolates using multilocus enzyme electrophoresis (MEE) was conducted to determine the genomic relatedness of the organism within the same group of or between the different types of samples and study areas. The 12 enzyme loci analysed were all polymorphic. *L. monocytogenes* has been found (Boerlin *et al.*, 1991) to have no monomorphic loci with specific alleles (alleles not shared with other species).

The results from MEE method verify the variation of the *L. monocytogenes* strains in each sampling time as the 113 isolates represented 85 distinct Electrophoretic Types (ETs) (Table 2.6). The genetic diversity ranged from 0.864 to the highest diversity from effluent isolates, 0.972 (Table 2.6). In particular, 17 ETs were found from the 18 isolates of *L. monocytogenes* collected from the sewage treatment plant at Dru Point (site 10). Of these, two different ETs of *L. monocytogenes* were isolated from the same samples (Appendix C, Table C.10). This indicates a high diversity of *L. monocytogenes* in effluent and suggests that strains of *L. monocytogenes* in the aquatic environment frequently transfer and recombine chromosomal DNA, leading to randomization of alleles. This finding is similar to Nørrung and Skovgaard (1993) who found that the genetic diversity of *L. monocytogenes* in fish, cattle and raw meat ranged from 0.879 to 0.927. In addition, the isolates from seawater in the study of Rørvik *et al.* (1995) showed different genetic diversity. However, other studies (Piffaretti *et al.*, 1989; Bibb *et al.*, 1990; Lawrence and Gilmour, 1995) reported smaller numbers of clonal types in listeriosis patients, foods and industrial environments which can be explained from the fact that only a small fraction (often one or a few) of the existing clones are involved in causing serious disease (Piffaretti *et al.*, 1989) or have adapted and survived in processed foods and industrial environments (Boerlin and Piffaretti, 1991; Fenlon *et al.*, 1996). The following cluster analysis and dendrogram of the 85 ETs (Fig. 2.14) presents genetic distances between ETs.

From the 54 effluent isolates (Table 2.6), 20 ETs were found from the 26 isolates from fish factory 2. Some consecutive isolates from this site, however, showed the same ET (Table 2.7) e.g. W12/14, W12/16, W12/17, and W12/18 were ET-53, W12/20 and W12/22 were ET-67, W12/21 and W12/24 were ET-68, and W12/25 and W12/26 were ET-80.

The enzyme profiles from river water and sediment samples also show the variation of the *L. monocytogenes* strains. The 18 and 10 ETs were found in 24 and 12 isolates from river water and sediment samples, respectively. Some consecutive isolates also showed the same ET (Table 2.7) e.g. W8/13 and W8/14 are ET-50, W8/21, S8/21, W8/22, W8/23, S8/23 and W8/25 are ET-68. The detection of the same ET from water and sediment samples indicates the ability of *L. monocytogenes* to survive in both habitats and exist there for approximately 45 days. On one occasion, while the organism (ET-5) was detected only in sediment (S9/1), the same ET was recovered from the water sample (W9/2) collected from the following round. This result may indicate the survival of *L. monocytogenes* was better in sediment samples.

Table 2.6 The genetic diversity of *L. monocytogenes* in 6 different type of samples.

Population	No. ETs	No. isolates	ET div
Inshore water	10	11	0.882
Inshore sediment	10	11	0.882
River water	15	19	0.904
River sediment	10	12	0.864
Effluent	46	54	0.973
Shellfish	6	6	0.800
Total	85	113	0.974

Table 2.7 The ETs with multiple isolates.

ETs with multiple isolates	ET-Number	Sample type, Station/Round						
ETs with 2 isolates	ET-4	S8/1	O3/1					
	ET-5	S9/1	W9/2					
	ET-7	W10b/2	W10a/3					
	ET-8	W12/2	S8/3					
	ET-32	W8/9	W4/10					
	ET-33	W9/9	S8/9					
	ET-34	W12/9	S7/9					
	ET-38	W3/11	W4/11					
	ET-50	W8/13	W8/14					
	ET-67	W12/20	W12/22					
	ET-74	W1/24	W8/24					
	ET-80	W12/25	W12/26					
ETs with 4 isolates	ET-53	W12/14	W12/16	W12/17	W12/18			
ETs with 14 isolates	ET-68	W8/21	W10/21	W12/21	S4/21	S5/21	S8/21	S9/21
		M/21	W8/22	W6/23	W8/23	S8/23	W12/24	W8/25

W = water, S = sediment, O = oysters, M = mussels

Figure 2.14 (facing page).   Genetic relationships among 85 ETs of 113 *L. mono-cytogenes* isolates. The dendrogram was generated by the average-linkage method of clustering from a matrix of pairwise coefficients of genetic distances, based on electrophoretically demonstrable allelic variation at 12 enzyme loci. Ins, inshore marine water, sediment or shellfish; Riv, river water or sediment; Ef, effluent; W, water; S, sediment; O, oysters; M, mussels; round/site.

SOURCES ET

Ef	16	W10/5
Ef	8	W12/12
Ins	39	W5/11
Riv	44	S8/11
Ef	29	W10v8
Ins	58	S7/15
Ins	52	S7/13
Ef	64	W12/19
Ff	21	W11/6
Ef	63	W11/19
Ef	55	W11/15
Ef	77	W11/24
Ef	70	W11b/21
Ef	75	W10a/24
Ins	57	S5/15
Ef	10	W10b/3
Riv	5	S9/1
Ins	18	S7/5
Ef	71	W10/22
Ef	41	W10/11
Ins	60	O5/15
Riv	15	W8/5
Ef	11	W12/3
Ins	73	W3/24
Ef	36	W12/10
Ef	53	W12/14
Ef	51	W12/13
Ef	67	W12/20
Ef	17	W12/5
Riv	65	W8/20
Ef	31	W12/8
Ef	14	W12/4
Ef	34	W12/9
Ef	3	W12/1
Ef	80	W12/25
Ef	42	W12/11
Ef	49	W12/12
Ef	22	W12/6
Ins	47	M/11
Ef	56	W12/15
Ef	25	W12/7
Riv	9	W8/3
Ef	6	W10a/2
Ef	72	W12/23
Riv	32	W8/9
Ins	46	O5/11
Riv	33	W9/9
Riv	48	W8/12
Riv	74	W8/24
Riv	68	W8/21
Ef	84	W10/26
Riv	4	S8/1
Riv	19	S8/5
Ins	37	W1/11
Riv	12	W8/4
Riv	28	W8/8
Ins	1	W5/1
Ins	38	W3/11
Riv	27	S8/7
Ins	26	S4/7
Riv	23	W8/7
Ins	40	W6/11
Ins	45	O1/11
Ef	30	W10b/8
Riv	54	W8/15
Riv	50	W8/13
Riv	59	S8/15
Ins	43	S3/11
Ef	62	W11/16
Ef	61	W10/16
Ff	69	W11a/21
Ef	66	W11/20
Ff	20	W10/6
Ef	13	W10/4
Ef	76	W10b/24
Ef	7	W10b/2
Ff	2	W10/1
Ef	85	W11/26
Ef	79	W11/25
Ins	81	S5/25
Riv	83	S9/25
Riv	78	W9/25
Ef	24	W10/7
Ins	35	W5/10
Ins	82	S6/25

0   0.1   0.2   0.3   0.4   0.5   0.6   0.7

GENETIC DISTANCE

The distribution of *L. monocytogenes* in North West Bay was shown by the detection of the same ET from different sample types and sampling stations at the same sampling time (Table 2.7). For example on round 1, *L. monocytogenes* in fresh water from Coffee Creek (site 8) appeared to contaminate the oysters in Stinkpot Bay (site 3) as both isolates were ET-4. The same ET (ET-32) from Coffee Creek on round 9, was also found on round 10 in water sample from 'Sanctuary' foreshore (site 4). Moreover on round 24, an isolate from Tinderbox was also found to be the same ET (ET-74) as from Coffee Creek. In addition, the ET-68 isolated from the input sources i.e. Coffee Creek, NWB River showed wide distribution to the Bay i.e. 'Sanctuary' foreshore (site 4), Dru Point (site 5), NWB Commercial Jetty (site 6a) and mussel samples from Beach Road Jetty (site 6b). Several differences of *L. monocytogenes* strains found in estuarine environment and the studied input sources (river and effluent) revealed that other input sources such as runoff of animal faeces from grazing land other creeks and river (see Table 2.1) may also contribute *L. monocytogenes* to the NWB environmental system.

2.3.4 GENERAL DISCUSSION

Environmental samples (Water and sediment): The occurrence of *L. monocytogenes* in the inshore water in North West Bay was relatively low although there were peaks in September and October 1994 and April 1995. The overall marine waters in North West Bay especially at deep water level were generally free of *Listeria*. Although there was no report of *L. monocytogenes* infection from the water sources, caution is needed in areas which are close to discharges of *Listeria* contaminated water i.e. sites 3, 5, 6, and 7. In addition to the high occurrence of *L. monocytogenes* in river and effluent in the present study, any activity involving these contaminated waters should be limited. Furthermore, these waters may be considered as a primary point-source for distribution of this biohazard and other pathogens. It seems desirable to eliminate, where possible, these potentially pathogenic organisms before distribution to the sea and other surface water.

Food (Oysters and Mussels): The naturally growing intertidal oysters in Stinkpot Bay and Dru Point (sites 3 and 5) showed 15.4% (n=26) positive for *L. monocytogenes*. Concurrently, mussels which were specially grown at NWB commercially Jetty (site 6b) for this investigation contained *L. monocytogenes* in 15.4% of samples (n=13). Several studies (Motes, 1991; Chai *et al.*, 1994) indicated that shellfish, being filter feeders, have the ability to concentrate pathogenic micro-organisms from the water column. The agreement was found in this investigation that oysters and mussels bioaccumulated *L. monocytogenes* from NWB water column (see Fig. 2.11). However, as these areas are not approved shellfish-growing area, the oysters and mussels are normally not taken for human consumption. In the present study, it was noted that there was an increased

incidence of *L. monocytogenes* in the environmental samples including shellfish if there was high rainfall in the 24 to 72 hr prior to the sampling time.

The economic importance of smoked Atlantic salmon (*Salmo salar*) industry for local and export purpose for Tasmania, the rate and sources of contamination of *L. monocytogenes* in salmon, surrounding estuarine environment and salmon processing plant will be investigated in the subsequent chapter.

3 THE OCCURRENCE OF *LISTERIA* SPP. INCLUDING *L.* *MONOCYTOGENES* IN A FISH PROCESSING FACTORY

3.1 INTRODUCTION

Knowledge of the ecology of *L. monocytogenes* in the environment is important to be able to understand the prevalence and distribution of the organism. However, how the organism contaminates food and consequently causes the infection is of more interest for control and prevention of sporadic cases or outbreaks of listeriosis. *L. monocytogenes* has regularly been detected in variety of foods including vegetables, dairy products, meat and seafood etc. (Weagant *et al.*, 1988; Ryser and Marth, 1991; Dillon and Patel, 1992; Gibson, 1992; Ben Embarek, 1994). Although the pathogen can withstand a wide range of different treatments, applying adequate heat to foods before consumption is sufficient to eliminate it. However, public health risk has increased partly due to the changes in consumer behaviour, particularly preference for minimally processed, ready-to-eat (RTE), foods which require no further process or heating before consumption (Farber *et al.*, 1996). Among RTE foods, cold-smoked salmon is an economically important product for Tasmania and Australia. Such food is capable of sustaining growth of *L. monocytogenes* (Farber, 1991; Rørvik *et al.*, 1991; Ben Embarek and Huss, 1992; Hudson and Mott, 1993a). Provided that the contaminated fish might undergo merely a cold-smoking process and will be consumed without any further cooking, a small initial inoculum may result in a much larger dose by the time the product is consumed and may, thus, pose a public health risk.

Although there is no evidence that cold-smoked salmon has been associated with any outbreak of listeriosis, two sporadic cases with foetal death were reported in Victoria (Anon., 1993c) and New South Wales, Australia (Arnold and Coble, 1995). In addition, other smoked seafood product e.g. smoked mussels (Baker *et al.*, 1993), and cold-smoked and gravad rainbow trout (Ericsson *et al.*, 1997) were also reported to be associated with sporadic and outbreak listeriosis respectively.

The production of cold-smoked salmon includes no listericidal stage to eliminate *L. monocytogenes* (Truelstrup Hansen, 1995). The products are reported to support growth of *L. monocytogenes* even when stored at 4°C (Farber, 1991). Some earlier studies (e.g. Harvey and Gilmour, 1993; Fuchs and Nicolaides, 1994; Rørvik *et al.*, 1995) have detected *L. monocytogenes* from finished products and fish factory environments. Guyer and Jemmi (1991) found that raw fish was more frequency contaminated than finished

products. Eklund *et al.* (1995) indicated the external surface of fresh and frozen fish to be the primary mode of introducing *L. monocytogenes* into the cold-smoked fish factory. Contamination of cold-smoked fish can occur during or after processing (Rørvik and Yndestad, 1991; Ben Embarek, 1994). There are many possibilities for the pathogen to come into contact with the meat surface e.g. along the processing lines, ice and water used in the process, equipment surfaces, and handling etc. Little information on contamination sources for *L. monocytogenes* within the salmon factory was determined by Truelstrup Hansen (1995).

In terms of epidemiology, infectious micro-organisms responsible for a specific outbreak are clonal; that is, they are the progeny of a single cell and thus are genetically identical or nearly so. Among isolates of the same species collected from different sources and sites and at different times, there is sufficient genetic diversity to allow identification of different clones or clonal groups (Versalovic *et al.*, 1991). Several subtyping methods have been developed to reveal the ecology and epidemiology of *L. monocytogenes* which can help identifying potential sources of contamination and tracing the spread of the pathogen.

It has been reported that only a limited number of strains *L. monocytogenes* were detected in foods and foods processing environment, and a listeriosis patient (Piffaretti *et al.*, 1989; Schuchat *et al.*, 1991a). Serotyping and phage typing were not sufficiently discriminatory and left a significant number of strains untypable (Seeliger and Höhne, 1979; McLauchlin *et al.*, 1986; Boerlin *et al.*, 1997). Several alternative molecular methods which show higher discriminating power have been applied to *L. monocytogenes*: multilocus enzyme electrophoresis (Bibb *et al.*, 1990; Lawrence and Gilmour, 1995), restriction enzyme analysis (Gerner-Smidt *et al.*, 1996), pulsed-field gel electrophoresis (Brosch *et al.*, 1994), restriction fragment length polymorphism (Harvey and Gilmour, 1994). However, most of these methods are complex, time-consuming and labour-intensive (Swaminathan and Matar, 1993). Recently, a PCR-based molecular method, random amplification of polymorphic DNA (RAPD), which requires no knowledge of DNA sequences, and is quick and easy to perform has been applied for the typing of *Listeria* strains (Welsh and McClelland, 1990; Wagner *et al.*, 1996). More recently, the repetitive element sequence-based PCR (rep-PCR) method has been shown to be a powerful tool in subtyping *Listeria* species including *L. monocytogenes* strains (Jersek *et al.*, 1996). The method uses primer sets based on repetitive elements, such as the 35 to 40 bp repetitive extragenic palindromic (REP) sequence, the 124 to 127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence (Jersek *et al.*, 1996) and the 154 bp BOX sequence (Martin *et al.*, 1992) and displays high discriminating power and reproducibility.

In this chapter, an investigation of the occurrence of *Listeria* species, especially *L. monocytogenes*, in the complete process of a batch of cold-smoked salmon from the fish and the marine farm environment, through the process until being vacuum-packed was undertaken. The 20 previous *L. monocytogenes* isolates (Table 3.6) collected from the same factory processing environment and its finished products during May-August 1996 (via a collaborative laboratory) and other isolates, if any, from the recent survey are further identified using the rep-PCR method.

3.1.1 *L. MONOCYTOGENES* AND COLD-SMOKED SALMON

Cold-smoked salmon is a highly appreciated food commodity world wide, but the product is merely lightly preserved and, traditionally, does not undergo a listericidal process (Truelstrup Hansen, 1995). The salting is done by mechanical injection or direct addition of dry salt or brining to obtain an even distribution of salt in the fish in the range of 3-5% water phase salt (Huss *et al.*, 1995). Cold-smoking is performed at ca. 26°C and, currently, has become so mild that it is considered to be a smoke-flavouring rather than smoke-preserving process (Horner, 1992). The smoked salmon is normally vacuum-packed in airtight plastic bags of low oxygen permeability. Storage and distribution of the product is at temperatures ≤5°C (Huss *et al.*, 1995). Some studies (Guyer and Jemmi, 1991; Dillon *et al.*, 1992) have shown that brining and smoking stages do not affect *L. monocytogenes* but support growth of the pathogen even stored at 4°C (Farber, 1991). Cold-smoked salmon is, therefore, considered to be a high risk ready-to-eat food with potential to harbour and allow growth of *L. monocytogenes* (Huss *et al.*, 1995).

The application of the hazard analysis critical control point (HACCP) system to the production of cold-smoked salmon has been introduced, with the final product testing used in the verification programme (Huss *et al.*, 1995). Two types of critical control point (CCP) are identified: CCP1 (ensures full control of the hazard), and CCP2 (minimises but does not ensure full control of the hazard) (Truelstrup Hansen, 1995). It was, however, concluded that there is no CCP1 to control the growth of *L. monocytogenes* in cold-smoked salmon (Huss *et al.*, 1995; Truelstrup Hansen, 1995). Therefore, Huss *et al.* (1995) recommended the use of good manufacturing practices (GMP) to minimise contamination, and to limit shelf life to three weeks at 5°C for cold-smoked vacuum-packed salmon having ≥3% water phase salt (WPS). In addition, the incorporation of additional hurdles into the product is suggested.

Recently, a risk assessment for contamination of smoked salmon with *Listeria monocytogenes* during processing was reported (Rørvik *et al.*, 1997). These authors

indicated that job rotation among departments in the smoked salmon processing facilities was the strongest expressed risk factor (hazard ratio=11) for isolation of *L. monocytogenes* from the smoked salmon.

3.1.1.1 *L. monocytogenes in cold-smoked salmon*

Occurrence and Source of contamination

During the past few years, *L. monocytogenes* has been isolated from cold-smoked salmon produced from several countries (Table 3.1). The contamination rate in finished product ranged from 0% to 79% in a survey of 6 plants which previously had *L. monocytogenes* contamination problems (Table 3.1).

Few studies on sources of *L. monocytogenes* contamination have been discussed in section 3.1. The primary source of contamination may be the external surfaces of frozen and fresh raw fish that came into the processing plants. However, none of typing techniques were applied during their survey. Rørvik *et al.* (1995) investigated a smoked salmon processing plant in Norway and the MEE technique was applied to the *L. monocytogenes* isolates. They found that one strain of *L. monocytogenes* (ET-6) was predominant in the smokehouse and was the only ET (Electrophoretic Type) found in the finished products. In addition, the authors reported that the clone colonized in both environmental and fish samples from smokehouse during the whole eight months investigation period. Since the isolates from sea water and slaughtered fish were different from the strain in finished product, ET-6, the authors concluded that the contamination of *L. monocytogenes* was due to the processing plant contamination. However, the source of contamination of the plant was not determined.

Level of L. monocytogenes contamination

The natural level of *L. monocytogenes* on freshly produced cold-smoked salmon are reported to be low (Table 3.1). However, very high levels of 25,400 cfu/g was reported by Loncarevic *et al.* (1996) who explained that the product might have been temperature abused and that the proliferation of *L. monocytogenes* took place during storage. The other high level of >1,100, and 1,100 MPN/g were found in cold-smoked salmon which have been kept at 2°C and 10°C for 60 and 40 days respectively (Cortesi *et al.*, 1997).

The level of *L. monocytogenes* contamination which should be tolerated in cold-smoked salmon is subject to heated international discussion (Huss *et al.*, 1995). Several researchers questioned the possibility of producing *L. monocytogenes*-free cold-smoked salmon (Huss *et al.*, 1995; Truelstrup Hansen, 1995; Farber *et al.*, 1996). In Australia, smoked salmon products which are intended for export or local consumption must be

Table 3.1 Occurrence, sources and level of *L. monocytogenes* contamination in cold-smoked salmon.

Country produce	No. of samples	% positive for *Listeria* spp.[a]	% positive for *L. mono-cytogenes*	Amount of *L. mono cytogenes*	Source	References
Switzerland	64	12.6	6.3	<1 cfu/g	-	Guyer and Jemmi (1990)
Canada	20	-[b]	25	-	-	Farber (1991)
USA	6	-	50	-	-	"
Chili	2	-	50	-	-	"
Scotland	2	-	0	-	-	"
Norway	2	-	50	-	-	"
Iceland	13	23	0	-	-	Hartemink and Georgsson (1991)
Norway	33	-	9	-	-	Rørvik and Yndestad (1991)
Newfoundland	12	0	0	-	-	Dillon et al. (1992)
New Zealand	12	-	75	-	-	Hudson et al. (1992)
Northern Ireland	16	44	6.3	-	-	Harvey and Gilmour (1993)
Switzerland	388	-	10	-	-	Jemmi (1993)
Canada	`39	3	0	-	-	Dillon et al. (1994)
Australia	56	10.7	17.9	<100 MPN/g	-	Arnold and Coble (1995)
USA	61	-	79	0.3-34.3 cfu/g	surface of frozen/fresh raw fish	Eklund et al. (1995)
Australia	285	-	0.35	-	-	Garland (1995)
Norway	65	11	11	<100 cfu/g[c]	smokehouse	Rørvik et al. (1995)
Sweden	13	0	15.4	400&25,400 cfu/g	-	Loncarevic et al. (1996)
Italy	100	-	20[d]	4 - >1,100 MPN/g	-	Cortesi et al. (1997)
	65	-	18.4[e]	4 - 1,100 MPN/g	-	"

[a] Other *Listeria* spp., [b] Not determined, [c] *L. monocytogenes* was isolated only after selective enrichment, [d] storage at 2°C up to 80 days, [e] storage at 10°C up to 60 days.

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tested to ensure they are free of *L. monocytogenes*. The food standard is nil in 25 g, "zero tolerance", (where is the same as for *Salmonella*, *Campylobacter* and *Vibrio parahaemolyticus*) (National Food Authority, 1994). However, Australia, Canada as well as some European countries (Germany, United Kingdom, and Denmark) have accepted a food group risk-based approach toward the control of *L. monocytogenes*, but the USA still has a 'zero tolerance' policy. The policy required the absence of *L. monocytogenes* in 25 gram of foods which lead to rejection of vast amounts of product (Anon, 1993a; Anon, 1993b) with a resulting severe economic loss for the producers. However, it is known that certain population, the so-called YOPI, are more susceptible to *L. monocytogenes* than the others and since the precise data on minimum infective dose of *L. monocytogenes* is not available in the literature, the subject of 'zero tolerance' has yet to be resolved.

3.1.1.2 *L. monocytogenes in cold-smoked salmon processing factory and related environments.*

Occurrence and sources

The rate of contamination in salmon processing plants and related environments are summarized in Table 3.2. The contamination of fresh fish is most likely related to its ambient water which may be polluted by human and animal faeces (Brackett, 1988; Motes, 1991), and to the sanitation during the subsequent slaughter. Truelstrup Hansen (1995) reported no contamination of fresh and slaughtered fish by *L. monocytogenes*,

Table 3.2 Occurrence of *L. monocytogenes* in smoked salmon processing factory and related environment.

Source of sample	No. of samples	% positive for		References
		Listeria spp.[a]	*L. mono-cytogenes*	
USA[b] : Raw product and processing area	122	33.6	41	Eklund *et al.*
Smoked product and processing area	117	31.6	59.8	(1995)
Norway[c]: Fish farm, water and ice	59	20.3	5.1	Rørvik *et al.*
Slaughterhouse and processing area	133	13.5	4.5	(1995)
Smokehouse and processing area	218	31.2	26.6	
Norway: Sea water from fish farm	8	0	0	Truelstrup
Fresh fish from the net cages	10	0	0	Hansen
Slaughtered fish and processing area	57	0	0	(1995)

[a] other *Listeria* spp., [b] from 5 visits to a cold-smoked salmon processing plant, [c] from a smoked salmon processing plant over 8 months.

harvested from *Listeria*-free sea water, but the occurrence was higher in the study of Rørvik *et al.* (1995) (Table 3.2). The same clone of *L. monocytogenes*, ET-11, isolated from sea water was subsequently found in fish and environmental samples from the smokehouse, although not in the finished product which was reported to be contaminated from the processing plant (Rørvik *et al.*, 1995). Eklund *et al.* (1995) reported a much higher occurrence in both raw and smoked products (Table 3.2). Those authors also reported sanitation and cleanup procedures to be sufficient in eliminating *L. monocytogenes* from the processing line and equipment, but after several hours of reprocessing the contamination recurred (Eklund *et al.*, 1995). The possible sources of *L. monocytogenes* may be raw fish, the personnel and the surrounding environment.

3.1.2 REP-PCR

Families of repetitive DNA sequences are present in a large number of copies and dispersed throughout the genomes of all organisms including eukaryotic and prokaryotic micro-organisms (Britten and Kohne, 1968; Versalovic *et al.*, 1991; Lupski and Weinstock, 1992; Louws *et al.*, 1994). These repetitive sequences are located in non-coding regions and their primary structure is highly conserved (Newbury *et al.*, 1987; Lupski and Weinstock, 1992). Their precise function has not been determined but there is evidence which suggests their presence to be important to the structure and evolution of genomes (Britten and Kohne, 1968; Stern *et al.*, 1984).

The first described and most intensively studied repeated sequences is the 35 to 40 bp repetitive extragenic palindrome (REP), or palindromic unit (PU) sequence (Higgins *et al.*, 1982; Gilson *et al.*, 1984) which was identified in *S. typhimurium* and *E. coli*. An additional 124 to 127 bp repetitive intergenic consensus (ERIC), or intergenic repeat units (IRUs) sequences was further identified in *S. typhimurium* and *E. coli* and other enterobacterial species (Sharples and Lloyd, 1990; Hulton *et al.*, 1991). More recently, the 154 bp BOX elements was identified in *Streptococcus pneumoniae* (Martin *et al.*, 1992).

Recently, Versalovic *et al.* (1991) synthesized REP- and ERIC-specific oligo-nucleotide primers and used them for PCR with chromosomal DNA of different bacterial strains as templates. They found that REP- and ERIC-like sequences could be detected in a large variety of bacterial genomes. Likewise, an additional BOX-like sequence was synthesized and used as an additional primer in PCR (Martin *et al.*, 1992). In this technique, collectively known as repetitive sequence element PCR (rep-PCR), the primers bind to the repetitive sequences which are located in different positions in the prokaryotic genome. These repeated sequences are separated by various distances depending on the

individual bacterial species or strain. The amplification products can be obtained if those primer binding sites are in the proper orientation and within a distance that can be spanned by *Taq* polymerase extension. The PCR products can then be size-fractionated by agarose gel electrophoresis to reveal a specific pattern or genomic DNA fingerprint. These fingerprints were reported to be species and strain specific in several bacterial genera (Versalovic *et al.*, 1991; Louws *et al.*, 1994) including *L. monocytogenes* (Jersek *et al.*, 1996). Regarding the high homology to repetitive sequences of the primers, more stringent PCR conditions can be used which in turn may reduce experimental variation and PCR artefacts (Louws *et al.*, 1994). In addition, the rep-PCR technique is very reproducible and has good discriminatory power when compared to MEE (de Bruijn, 1992) and RAPD techniques (Jersek *et al.*, 1996). The technique has been further developed as 'whole cell rep-PCR' which is useful for rapid and routine diagnostic analysis (Woods *et al.*, 1993).

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

Details of consumables, reagents and media, and equipment used are presented in Appendix A.

3.2.2 METHODS

3.2.2.1 Sample collection

The 87 samples, i.e. 78 samples of factory products and processing sample sites and 9 environmental samples outside the factory, were collected from the fish processing factory. The samples and sites are discussed in Table 3.3.

Swab : A large area (30x30 cm^2) of food processing equipment and environmental surfaces was sampled using sterile gauze (5 layers of 5 cm x 5 cm). Sterile forceps were used to hold gauze aseptically and swab the surface by vigorously rubbing the gauze over the designated area. Approx. 5 ml of sterile 0.1% peptone water was applied directly to the flat dry surfaces and then taken up into the gauze by the rubbing action. Each swab sample was kept in a sterile polyethylene bottle or small stomacher bag. Six layers of 10 cm x10 cm sterile gauze were prepared for drain swabs. The gauze was placed at the drain inlet for approx. 1 hr before being collected into a sterile polyethylene bottle.

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Table 3.3 Sites and type of samples collected at fish factory in February 1997.

Site	Type of samples collection					
	Lab No.	Swab	Lab No.	Fish	Lab No.	Others
Harvesting	S1 S2	Fish skin Bins	F1 F2 F3	Fish Gut Belly flap	W1 W2 W3	bleeding water Ice Ice (used)
Cleaning	S3 S4 S5 S6 S7	Fish skin Bins Processing area Waste collection pipe Drain	F4	Fish after cleaning	W4 W5	Processing water (treated sea water) Gloves
Filleting	S/SS[a]8 S/SS9 S/SS10 S/SS11 S/SS12	Fish skin Racks Filleting table 1 Filleting table 2 Drain	F/SF[a]5 SF 5/1	Fish after filleting Fish (Fresh P/A)	W6 W/SW[a]7	Processing water (treated dam water) Gloves
Skinning	S/SS13 S/SS14 S/SS15 S/SS16 S/SS17	Skinner Racks Trim table Skinning area Drain	F/SF6 F7	trimmed pieces Fish skin	W/SW8	Gloves
Brining	S/SS18 S/SS19	Fish racks Floor	F/SF8	cured&washed fish (treated dam water)		
Smoke- house	S/SS20 S/SS21	smoker cabinet smoker chiller	F/SF9	Smoked fish		
Slicing & Packaging	S/SS22 S/SS23 S/SS24 S/SS25 S/SS26	autoslicer hand slicing machine Reform table (A) Reform table (B) Floor	F/SF10 F/SF11	Smoked salmon from autoslicer Smoked salmon from hand slicing	W/SW9 W/SW10	Gloves from A Gloves from B
Storage Room	S27 S28	Bins (fish waste) Floor & door				
Environ- ment	S/SS29 SS30	Drain from waste tank outside the factory Floor at sawdust			W11 W12 W13 W14 W15 W16	Sea water Sea sediment Dam water Dam sediment Influent Effluent
TOTAL	Swabs 29 + 20[a]		Fish 11 + 7[a]		Water 14+4[a], Sediment 2	

[a] samples collected by the factory staff and delivered to the university laboratory afterwards. An 'S' was added to the samples lab number for the same sample site.

Fish : A fish weighing approx. 3-4 kg was sampled from the harvesting and cleaning process. Pieces of fish, skin or smoked products along the processing line were sampled into stomacher bags.

Water and ice : Water samples were collected using a sterile 1or 2-L Schott bottle (depending on type of water). The chlorine treated seawater and fresh water used in the factory was directly collected from different outlets into sterile 2-L Schott bottles with added 2 ml of 10% sodium thiosulphate solution (Appendix A). Approx. 500 ml of ice was collected into a sterile polyethylene bottle with 0.4 ml of 10% sodium thiosulphate solution added (Appendix A). For the environmental water samples, the sample was collected in the same manner as in 2.2.2.3. A bottle holder (modified golf-ball retriever) with an extension of 3 m was used to collect the dam water sample.

Surface Sediment : At the sea and dam sites, approximately 100 g of sediment, consisting of several subsamples, was collected with a sterile modified syringe. Samples were then placed in a sterile polyethylene bottle.

All 56 samples collected on 13-14 February 1997 were immediately brought to the factory laboratory. All swabs and sediment samples and some fish samples were processed there. The water and some fish samples were refrigerated (4°C) before being transferred to the University's laboratory within 18 hours. The additional 31 samples of the same batch of fish collected by a factory staff were kept on ice and delivered to the university laboratory on the same day of processing (18, 20, and 24 February 1997).

3.2.2.2 Microbiological analysis

The USDA/FSIS method (Dennis and Lee, 1989) currently used in the food industry was employed for isolation of *Listeria* spp. in this study. There were some differences in the amount and preparation of samples but after the samples were in the primary enrichment broth (UVMI), the methods presented in Fig. 2.4 (section 2.2.2.3) were followed.

Swab : Fifty ml of UVMI was added into each bottle of the swabbed samples. 100 ml of UVMI was added for the drain swabs.

Fish, gut, skin and smoked products : Twenty five gram composite samples (flesh, smoked products, and skin) were transferred into a stomacher bag. Twenty five grams of gut and gut contents from a salmon were carefully removed and collected into a stomacher bag. 225 ml of UVMIwas added and stomached (Colworth) for 2 min. Isolation and identification of *Listeria* species including *L. monocytogenes* as described in Fig. 2.4 (section 2.2.2.3) were followed thereafter.

Water and ice : One or two litres of samples, depending on the type of water, was filtered through a prefilter and membrane filter 0.45 μm-pore-size, 90 mm diameter. Ice (500 ml) was filtered through a prefilter and membrane filter 0.45 μm-pore-size, 45 mm diameter. Both the prefilter and 0.45 μm membrane filter were placed in 100 ml UVM1 and the methods described in Fig. 2.4 (section 2.2.2.3) followed

Surface Sediment : Sediment samples in polyethylene bottles were mixed by stirring with a sterile handheld spoon, and 25 grams of sample added to 225 ml of UVM1 and the method given in Fig. 2.4 (section 2.2.2.3) followed.

In the case of presumptive *Listeria* positive isolates, (from the results of biochemical test: see section 2.2.2.3, Table 2.3), the cultures were confirmed using api *Listeria* (bio Mérieux Vitek) test kits.

3.2.3 SUBTYPING METHOD: REP-PCR (REPETITIVE SEQUENCE ELEMENT POLYMERASE CHAIN REACTION)

3.2.3.1 Isolates

Five known *L. monocytogenes* strains, i.e. 2 strains of ET-53 and 3 strains of ET-68, isolated from NWB (see section 2.3.3, Table 2.7) which had been subtyped by the MEE method were also subtyped by the BOX-PCR and REP-PCR methods in order to compare the sensitivity of the methods. A *L. monocytogenes* pathogenic strain, Scott A, and *L. monocytogenes* L5, an isolate from cold-smoked salmon (Table 3.5), were also compared to these 5 NWB strains.

Twenty strains of *L. monocytogenes* (L5 to 19, Table 3.6) isolated from a fish factory environment and its finished products during May-August 1996 were also used in this study. *L. monocytogenes* strains W12, S29 and SS29 (see Tables 3.3 and 3.4) were isolated from the environment outside the factory. Sources of, and relevant information on bacterial isolates, are listed in Table 3.6.

The isolates were streaked on Listeria selective agar. A single colony was suspended in BHI and 0.1 ml was spread on TSA-YE and incubated at 37°C for 24 hr. The methods as outlined in sections 3.2.3.2-4 were followed.

3.2.3.2 Preparation of DNA

Genomic DNA from *L. monocytogenes* isolates was extracted by a modified method of Marmur and Doty (1962) as follows. Cells were scraped from TSA-YE plates and

combined in a 15 ml sterile conical tube. Two ml of saline-EDTA, and 0.2 ml of lysozyme solution were added, mixed well, and then incubated at 37°C overnight. 0.1 ml of 10% Sodium dodecyl sulphate (SDS), and 50 μl of proteinase K solution were added, mixed well, and incubated at 60°C for 30 min. 0.3 ml of 10% SDS was added and incubated at 60°C for 15 min. 1.4 ml of 70.2% sodium perchlorate was added, and shaken using a wrist action for 4 min. Cell lysates were extracted once with 25:24:1 phenol: chloroform:isoamyl alcohol and twice with 24:1 chloroform: isoamyl alcohol solution. Genomic DNA was precipitated by adding 2 volume of ice-cold 95% ethanol. The DNA was dissolved in sterile MilliQ water. The DNA quantitation was performed by spectrofluorimetry at excitation and emission wavelengths of 365 nm using a mini-fluorometer Model TKO-100 and a DNA-specific dye, Hoechst 33258 according to manufacturer's instructions (Appendix A, section A.2.11).

3.2.3.3 rep-primers and rep-PCR amplification conditions

The REP-PCR primers (18-mer) are composed of REP 1RI (5'-III-ICGICGICA TCIGGC-3') and REP 2-I (5'-ICGICTTATCIGGCCTAC-3'). The ERIC-PCR primers (22-mer) are composed of ERIC 1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC 2 (5'-AAGTAAGTGACTGGGGTGAGCG-3'). The BOX-PCR primer, BOX AIR (5'-CTACGGCAAGGCGACGCTGACG-3') is the same for both sides.

The polymerase chain reaction mixture was prepared as described by Versalovic *et al.* (1991) as follows: each 25 μl PCR reaction contained 50 pmol each of 2 primers, 50 ng of template genomic DNA, 1.25 mM of each of 4 dNTPs, 25 mM $MgCl_2$, 10x Reaction Buffer, and 1 U *Taq* DNA polymerase. The amplifications were performed in a DNA thermocycler.

Amplification condition for REP-PCR : 1 cycle at 95°C for 3 min, 30 cycles at 90°C for 30 s, at 40°C for 1 min, at 72°C for 1 min, 1 cycle at 72°C for 8 min and 4°C for 1 min.

Amplification condition for ERIC- and BOX PCR : 1 cycle at 95°C for 5 min, 30 cycles at 90°C for 30 s, at 50°C for 30 s, at 52°C for 1 min, at 72°C for 1 min, 1 cycle at 72°C for 8 min and 4°C for 1 min.

3.2.3.4 Analysis of rep-PCR products

5-μl of gel loading buffer (Appendix A) was added to the amplified PCR products then a 12-μl portion of the suspension was separated on 1.5% agarose gel (10x15 cm) in

TAE buffer (Appendix A). The electrophoresis was run in a continuous buffer system at 70 mA, room temperature (ca 20°C) for 3 h. DNA molecular size markers pUC19 (26-501 bp) and SPP-1 (360-8,510 bp) were used as size standards. DNA fingerprints were visualized by staining the gel with ethidium bromide solution for 10 min on a slow shaker then washing off with tap water. Gels were photographed on a UV transilluminator with Polaroid type 55 film. DNA fingerprints generated from different strains were compared visually. Clonal identity is reflected in isolates having the same DNA fingerprint patterns.

3.3 RESULTS

In collaboration with a fish factory, a visit to the factory was carried out in February 1997. One batch of fish was followed through the consecutive stages used in production of cold-smoked salmon. The processing line was examined twice, i.e. on the harvesting day and at the stage that the selected lot of fish was processed. The 87 samples were tested for *Listeria* spp. (Table 3.3). Collectively, no *L. monocytogenes* was recovered from 78 samples of factory products and processing sample sites but 3 strains of *L. monocytogenes* were isolated from the 9 environmental samples outside the factory (Table 3.4). *L. innocua* and *L. seeligeri* were recovered from the same sample, from a swab from a waste collection pipe at the cleaning site inside the factory (Table 3.4). *L. innocua* was the commonest species found in the aquatic habitat, samples W11-W15 and S29, while *L. welshimeri* was isolated from dam water, sample W13, only (Table 3.4). No *L. ivanovii* or *L. murrayi* were isolated during this survey.

Table 3.4 Occurrence of *Listeria* spp. including *L. monocytogenes* from samples at the fish factory.

Site	Number of samples	Number of samples (%) positive for					
		L. mono-cytogenes	*L. innocua*	*L. seeligeri*	*L. welshi-meri*	*L. ivanovii*	*L. murrayi*
Harvesting	8	0	0	0	0	0	0
Cleaning	8	0	1[a](12.5)	1[a](12.5)	0	0	0
Filleting	16	0	0	0	0	0	0
Skinning	14	0	0	0	0	0	0
Brining	6	0	0	0	0	0	0
Smokehouse	6	0	0	0	0	0	0
Slicing&Packaging	18	0	0	0	0	0	0
Storage room	2	0	0	0	0	0	0
Environment	9	3[b](33)	6[c](66)	0	1[d](11)	0	0
Total	87	3 (3.5)	7 (8)	1 (1)	1 (1)	0	0

[a-d] sample number(s) which were positive for the indicated *Listeria* species,
[a] S6, [b] S29, W12, and SS29, [c] S29, W11-W15, and [d] W13; see the abbreviation in Table 3.3

Fig. 3.1 and Table 3.5 show the results of the preliminary tests for sensitivity of REP-, and BOX-PCR methods. The two strains of ET-53, 53a and 53b, can be clearly separated to different strains designated REPI and II, and BOXI and II respectively (Fig. 3.1 and Table 3.5). The 3 strains of ET-68 contain some identical characteristic bands particularly with BOX-PCR but more than 2 distinct bands can be observed (Fig. 3.1). Hence, the 68a, 68b, and 68c are subtyped into 3 different strains by rep-PCR method. When the methods were applied to *L. monocytogenes* Scott A, and L5, the results show that all of the 5 isolates from NWB and L5 are different from the pathogenic strain, Scott A, and none of the 7 strains tested here was identical (Fig. 3.1 and Table 3.5).
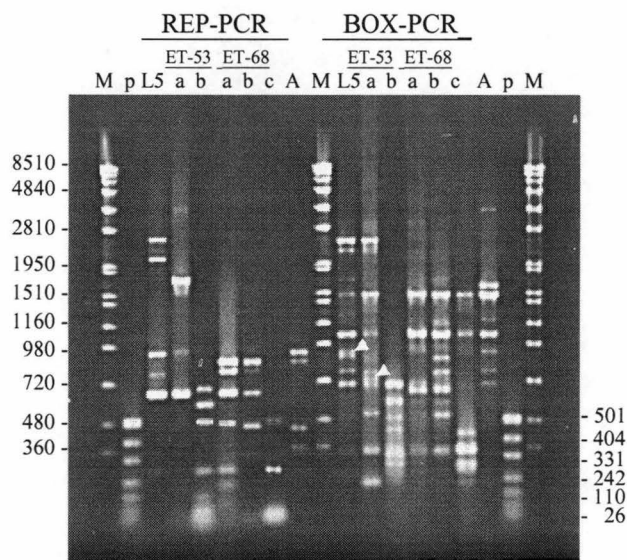
Figure 3.1 rep-PCR fingerprinting patterns from genomic DNA of 7 *L. monocytogenes* strains (Table 3.5); L5, ET-53a, ET-53b, ET-68a, ET-68b, ET-68c, and Scott A (lane A) respectively. The REP-PCR,and BOX-PCR are indicated above the lanes. DNA molecular weight standards (in base pairs), lanes labelled M and/or p, are indicated on the left, in the middle and right. Lanes labelled L5 to A correspond to *L. mono-cytogenes* strains as outline in Table 3.5.

Table 3.5 Numbers, sources, date of collection and subtypes of *L. monocytogenes* isolates used in the study.

PCR number[a]	Source of *L. monocytogenes* isolate	Date of Collection	rep-PCR Rep	rep-PCR Box
L5	Cold-smoked salmon in FB	16/5/96	1	1
53a	Effluent from site 12, round 14 (W12/14)	18/11/94	I	I
53b	Effluent from site 12, round 18 (W12/18)	13/1/95	II	II
68a	Fresh water from site 8, round 21 (W8/21)	24/2/95	III	III
68b	Effluent from site 10, round 21 (W10/21)	24/2/95	IV	IV
68c	Mussels from site 6 , round 21 (S6/21)	24/2/95	V	V
A	*L. monocytogenes* Scott A	-	VI	VI

[a] 53a-b and 68a to 68c were the *L. monocytogenes* isolates taken from the North West Bay study in Chapter 2.

Fig. 3.2a-c show rep-PCR fingerprint profiles obtained for 23 *L. monocytogenes* isolates from the factory (Table 3.6). The distinct REP-, BOX-, and ERIC-PCR products ranged from approximately 30 bp to over 3.6 kb (Fig. 3.2). The three different set of primers gave concordant results by discriminating the 23 *L. monocytogenes* isolates into 4 subtypes (Table 3.6). An obvious relationship among the 20 *L. monocytogenes* isolates (L5 to 19) could be summarised on the basis of those rep-PCR fingerprint patterns to belong to the same subtype, i.e. BOX1, REP1, and ERIC1 (Fig. 3.2 and Table 3.6). Whereas the 3 environmental *L. monocytogenes* isolates, W12, S29 and SS29, gave different fingerprint profiles and were designated different rep-PCR subtypes (Table 3.6).

3.4 DISCUSSION

The method, including the media used for isolation and identification of *Listeria* in environmental samples and salmon in this study, was shown to be sufficiently sensitive in Chapter 2 and else where (Warburton *et al.*, 1991; Hayes *et al.*, 1992). In addition, at least 10 typical colonies on OXF were selected and screened for haemolysis on HBA in order to increase the probability of finding *L. monocytogenes* amongst other competitors especially other species of *Listeria* (Petran and Swanson, 1993).

Although the factory had a *Listeria* contamination problem in the past 7 months, only few positive results were obtained from this extensive investigation. In detail, only 1 sample from the 78 samples (1.3%) collected within the processing factory was found to contain *L. innocua* and *L. seeligeri* (Table 3.4), whereas 7 samples from 9 environmental samples (78%) outside the factory contained *Listeria*; *L. monocytogenes* 33%, *L. innocua*

Figure 3.2 rep-PCR fingerprinting patterns from genomic DNA of 23 *L. monocytogenes* strains isolated from the fish factory (Table 3.6). The BOX-PCR, REP-PCR, and ERIC-PCR patterns are shown in panels A, B, and C respectively. DNA molecular weight standards (in base pairs), lanes labelled M and/or p, are indicated on the left, in the middle and right. Lanes labelled L5 to 19 correspond to *L. monocytogenes* isolates as outline in Table 3.6. Lane 20; W12, lane 21; S29, and lane 22; SS29.

Table 3.6 Numbers, sources, date of collection and subtypes of *L. monocytogenes* isolates used in the study.

PCR Number	Source of *L. monocytogenes* isolate	Date of Collection	rep-PCR REP	BOX	ERIC
L5	FB (cold-smoked salmon)	16/5/96	1	1	1
1	FB (smoked salmon)	17/5/96	1	1	1
2	FB (smoked salmon)	18/5/96	1	1	1
3	FB (salmon Gravalax)	18/5/96	1	1	1
4	salmon, Gravalax	22/5/96	1	1	1
5	swab of table slicing area in UVM	23/5/96	1	1	1
6	swab of skinner blade in UVM	24/5/96	1	1	1
7	FB (smoked salmon fillet)	26/5/96	1	1	1
8	UVM (cold-smoked salmon)	25/5/96	1	1	1
9	FB (cold-smoked salmon)	26/5/96	1	1	1
10	FB (cold-smoked salmon)	29/5/96	1	1	1
11	FB (smoked salmon sliced)	31/5/96	1	1	1
12	swab of curtain chiller in LEB	29/5/96	1	1	1
13	swab of autoslicer	29/5/96	1	1	1
14	swab of smoked salmon reform table	29/5/96	1	1	1
15	swab of autoslicer	30/5/96	1	1	1
16	swab of skin trimmed table	30/5/96	1	1	1
17	FB (sliced smoked salmon)	1/6/96	1	1	1
18	FB (sliced smoked salmon)	3/6/96	1	1	1
19	1 kg sliced smoked salmon mfd. 1/6/96	1/8/96	1	1	1
W12	sea sediment	14/2/97	2	2	2
S29	swab of drain from waste tank outside the Factory	14/2/97	3	3	3
SS29	swab (frozen LEB) of drain from waste tank outside the factory	26/2/97	4	4	4

FB : Fraser Broth, UVM : University of Vermont, LEB : Listeria Enrichment Broth

(67%), and *L. welshimeri* (11%). These results suggested that good hygienic practice and management within the factory have been effectively used to control and prevent the pathogen from spreading into the processing line and products. In addition, the routine sanitation and cleanup procedures in the factory have adequately eliminated *L. monocytogenes* from the processing line and equipment. This, in turn, suggests that it is possible to control *L. monocytogenes* in food products by GMP. However, because of the ubiquity of *L. monocytogenes*, the pathogen can recur and spread throughout the factory and products. Therefore, the development and validation of HACCP plans from harvesting or production to consumption is very important in all processing plants (ICMSF, 1988).

The finding of *L. monocytogenes* in sea sediment (W12, Table 3.4) but not in sea water agreed with the result in Chapter 2 that the sediment served as a better reservoir in aquatic environments for *Listeria* spp. Although *L. monocytogenes* must move into the water column at some stage, the organism dies-off rapidly in seawater (Faud *et al.*, 1989; see 2.3.2.3 Chapter 2). Hence, this may diminish the chance for *L. monocytogenes* to survive in seawater and subsequently to be accessible to contaminate fish or shellfish. The presence of *L. monocytogenes* in marine water, fish or shellfish may indicate a recent contamination. In this study no *Listeria* spp. were found in the effluent discharged into the seawater. The results of this study also demonstrated that the occurrence of *L. monocytogenes* in sea sediment was not correlated with the occurrence in fish living in the ambient water. This result is in agreement with the findings of Jemmi and Keusch (1994).

The rep-PCR method is an effective tool to discriminate between those strains that are not distinguished by biochemical or serological methods (Louws *et al.*, 1994). The method was reported to display a higher degree of discrimination for the *Shewanella* species than DNA sequencing in 16S RNA (S. McCammon, pers. comm.). Jersek *et al.* (1996) have shown that REP- and ERIC-PCR can be used for identification of *Listeria* spp., discrimination of *L. monocytogenes* within and between serotypes and provides a comparable discriminative potential as RAPD combining 3-4 primers.

In this study, the profiles generated from independent DNA preparations extracted from single-colony cultures or from different colony at different times were very reproducible (data not shown). Negative control assays in which no DNA template was added yielded no detectable amplified product.

Using the BOX-PCR protocol (Fig. 3.1), *L. monocytogenes* L5 and 53a appeared to share some common banding patterns, but the presence or absence of some unique bands were noted as accentuated by the arrowheads in Fig. 3.1. Major differences, however, were noted between these strains when the REP-PCR protocol was used. The limited test comparing MEE method and REP-, and BOX-PCR in this study also suggests the PCR method to be more powerful than the MEE method in differentiation of *L. monocytogenes* strains (Fig. 3.1, and Table 3.5). It would still be useful to continue typing the 20 *L. monocytogenes* strains in this study using the MEE technique (see Chapter 2) so that, at least, the environmental strains may be compared. However, because the testing laboratory is located several thousand kilometers from the University of Tasmania, and because they did not have the staff to undertake the testing for the candidate, it was not possible to continue performing the MEE test. Since rep-PCR was demonstrated to be the most powerful method, it was considered to be sufficient to satisfy the aim of *L. monocytogenes* discrimination in this study.

Fig. 3.2 shows that no notable differences were observed between the 20 *L. monocytogenes* strains, i.e. 13 strains were from fish products, and 7 strains were from the factory environmental swabs, collected from 16/5/96 to 1/8/96. To be precise, *L. monocytogenes* strain 19 which was isolated on 1/8/96 from the vacuum-packed sliced cold-smoked salmon was detected to be contaminated soon after manufacturing (1/6/96). The products were kept frozen (-20°C) for 2 months and re-examined to determine the survival of the organism. Finding the same rep-PCR type indicates that the same *L. monocytogenes* clone, collectively called rep 1, resided in the factory over 19 days in the period of 16/5/96 to 1/6/96 and the same clone survived the stress environment. The results (Table 3.6) suggested that there might be a single source of *L. monocytogenes* that was not eliminated during the 19 days of rigorous cleaning process (every 2 hr tables and equipment cleaned, and every 24 hr walls and drains cleaned). Rørvik *et al* (1995) also reported a *L. monocytogenes* clone, ET-6, colonized a smoked salmon plant during an eight month investigation period.

Since only 3 strains of *L. monocytogenes* were found from the environment in this recent survey all of which are different from those 1996 isolates, the source of *L. monocytogenes* contaminated during 16/5/96 to 1/6/96 could not be definitely identified.

It is noteworthy that not all amplicons generated by each primer are specific amplicons. The environmental isolates W12 and S29, when determined from BOX and REP primer sets, were closely related strains as there was only a minor difference (Fig. 3.3). However, using ERIC primers demonstrated more differences hence the 2 *L. monocytogenes* strains are designated as rep-PCR 2 and rep-PCR 3 respectively.

In conclusion, this study indicates that REP-, BOX-, and ERIC-like sequences are prevalent in strains of *L. monocytogenes* and can be exploited to generate genomic fingerprints. The rep-PCR analysis promises a highly discriminating, quick and easy to interpret method for subtyping of *L. monocytogenes*. Each primer set offered unique information for detecting limited polymorphisms within a clonal group or apparent similarities between strains. By using three different primer sets, more specific conclusions concerning diversity or similarity among strains were achieved.

4 PREDICTIVE MICROBIOLOGY AND KINETIC MODEL FOR *LISTERIA MONOCYTOGENES*

4.1 INTRODUCTION

In recent years, the seafood industry has become increasingly concerned with the presence of *L. monocytogenes* in chilled cold-smoked salmon, a "ready-to-eat" food. Since the traditional cold-smoking process contains no listericidal step and no CCP1 to control or eliminate any *L. monocytogenes* that may contaminate the product (Truelstrup Hansen, 1995), there is currently no effective means to guarantee that cold-smoked salmon remains free from *L. monocytogenes*. In addition, typical cold-smoked salmon contains 3-6% salt (water activity ca 0.983-0.964), has a pH of about 6, and is stored and distributed in vacuum packs at 5°C (Dalgaard, 1997). These conditions are suitable for the growth of *L. monocytogenes*, so that if any contamination occurs, the organism may proliferate and reach dangerous levels at the time of consumption (Huss *et al.*, 1995; Dalgaard, 1997). Considering that the minimum infective dose for human listeriosis is still unknown, although some estimates have been suggested (Farber *et al.*, 1996; Buchanan *et al.*, 1997) (see section 1.6.2), it is important to minimise both the incidence and level of *L. monocytogenes* in food to improve the safety of the product. This suggests a need to reevaluate and improve the traditional way of processing, that is, to incorporate one or more hurdles which can inactivate *L. monocytogenes* in the process, prevent its growth or eliminate it (Huss *et al.*, 1995; Truelstrup Hansen, 1995).

The major factors controlling the fate of microbial populations in many foods are the extrinsic factors such as temperature at which the foods are stored, and intrinsic factors (or food environment) such as water activity and pH (Ray, 1996). *L. monocytogenes* is reported to be able to grow at 1°C , with some strains growing at 0.5°C (Junttila *et al.*, 1988), and it can survive at -20°C for up to 2 years (Lehnert, 1960). The minimal water activity for growth of *L. monocytogenes* is reported to be 0.91-0.93 for five different strains at 15°C (Farber *et al.*, 1992) and it can survive for up to a year in 16% NaCl (a_w of 0.883) (Seeliger, 1961). The minimal pH for growth was found to be 4.3 at 30°C and 5.0 at 4°C (Farber *et al.*, 1989b). The full preservation potential of an individual constraint is restricted because of considerations related to the aesthetic, organoleptic and nutritional properties of cold-smoked salmon. However, several constraints may be combined to provide a desired level of stability. This concept was termed "hurdle technology" by Leistner (1985, 1994).

Organic acid has been reported to provide more inactivation effect and growth inhibition of *L. monocytogenes* than inorganic acids at a specified pH (Sorrells *et al.*, 1989; Young and Foegeding, 1993; Buchanan and Golden, 1994). Lactic acid is regarded as a GRAS (Generally Regarded As Safe) additive for which the United States Food and Drug Administration (US-FDA) has no limitation on the concentration used in food products. Lactic acid is the most widely used organic acid in meat products because of its mild acid taste (flavour enhancement), its preserving properties, its liquid form and its natural occurrence in many foodstuffs (Houtsma, 1996). In fresh salmon muscle tissue, lactic acid is present naturally at a level of ~0.2 to 0.6% depending on the amount of anaerobic conversion of fish muscle glycogen to lactic acid (Cutting, 1953). Hence, lactic acid is recognized as a potential hurdle to be combined with the other environmental factors to inactivate *L. monocytogenes*. Application of a suitable level of lactic acid to the processed fish may serve as a preliminary decontamination and a further preservative throughout the shelf-life of the product.

To manipulate a product formulation in the past, it was necessary to perform storage trials and microbial challenge tests to ensure the safety of the product. The outcomes, however, cannot be extrapolated to any other situations or products and any change to the formulation or conditions would require that new challenge tests be performed. Predictive microbiology was introduced as a cost-effective alternative to achieve this purpose (Dalgaard, 1997). The method involves the accumulation of knowledge on microbial physiology and growth responses to a combination of environmental factors (McMeekin *et al.*, 1993). The results can be incorporated into at least two different types of mathematical models; 1) a "kinetic model" which is useful for predicting the shelf-life of foods (Ratkowsky *et al.*, 1982), and 2) a "probability model" (so-called growth/ no growth interface model) which is useful for predicting the conditions when micro-organisms, especially pathogens, might grow or might not grow (Ratkowsky and Ross, 1995). The model predictions need to be rigorously tested for applicability and validity in foods within the range of values of data from which the model was developed (Ross, 1993).

The focus of this chapter is to examine the behaviour of *L. monocytogenes* Scott A, a pathogenic strain, and L5, a wild type strain isolated from cold-smoked salmon, under different conditions of temperature, water activity, pH, and lactic acid, solely or in combination, in defined systems. The models describing growth rate responses of *L. monocytogenes* to those factors are presented in this chapter. The probability models for growth or no growth of *L. monocytogenes* as a response to those factors are presented in subsequent chapter. The performance of both types of models are evaluated in Chapter 6.

4.1.1 PREDICTIVE MICROBIOLOGY

"Predictive microbiology", or "quantitative microbial ecology", was introduced as a reasonably simple, inexpensive and rapid method for controlling microbiological food safety and also for designing new product formulations (McMeekin *et al.*, 1993). The concept has emerged as one of the most active fields of research in food microbiology (see reviews by McMeekin *et al.*, 1993; McClure *et al.*, 1994; Ross and McMeekin, 1994; McMeekin *et al.*, 1997; Roberts, 1997; Whiting and Buchanan, 1997). Predictive microbiology involves accumulating knowledge of the reproducible nature of micro-organism responses to environmental factors such as temperature, water activity and pH which may then be summarized as mathematical equations or models, e.g. kinetic or probability models (McMeekin *et al.*, 1993).

A three-tier system of classification of models was introduced by Whiting and Buchanan (1997), in which models are described as being primary, secondary and tertiary. Primary models are those which describe the response of the micro-organism to a single set of conditions over time and include growth and inactivation/survival models. Secondary models describe the response of one or more parameters of a primary model to changes in one or more of the environmental factors, while tertiary models involve the application of secondary models to generate systems for providing predictions e.g., user-friendly software and expert systems. A general approach for the development of predictive models is summarised and presented in Table 4.1.

Table 4.1 Summary of the general methodology for development of kinetic or probability models.

Stage of model preparation	Kinetic model	Probability model
Data generation	Growth curves are generated in model systems; covers total range of environmental factors (temp., pH, NaCl, etc.)	Growth or no growth are observed in model systems; covers total range of environmental factors
Primary modelling	Growth curves are fitted by sigmoidal growth models	The times (days) at which the growth occurred are recorded, no model generated
Secondary modelling	The effect of controlling factor(s) on kinetic parameters is modelled (Table 4.2)	The effect of controlling factors on probabilistic parameters is modelled (Table 4.2)
Model validation	Predicted values of kinetic parameters are compared to values obtained in product and challenge tests	The growth/no growth interface conditions are compared to observations on products
Tertiary modelling	Validated models are included in application software	Validated models are included in application software

(Adapted from Dalgaard, 1997)

4.1.1.1 Primary models

'Kinetic models' enable the user to calculate the shelf-life of foods or to predict the time span in which significant microbial growth, e.g. of spoilage bacteria, might occur (McMeekin *et al.*, 1993). The traditional method of determining generation time from a primary model for the bacterial growth curve, where one log-ten cycle is equal to 3.32 doublings, is too subjective as 'by eye' curve fitting is used (Fig. 4.1). By using non-linear regression techniques to mathematically quantify the parameters of the curve, all researchers obtain the same generation times given the same set of data i.e., the process becomes objective. A number of mathematical functions have been proposed of which a modified Gompertz function (Gibson *et al.*, 1987) has gained most prominence and is employed in this study because of i) its slightly greater consistency in estimation (Ross, 1993), ii) its wide use in the literature, and iii) growth parameters can be obtained by simple manual calculation from expressions based on the fitted parameters of the equation. The interpretation of the parameters was redefined by McMeekin *et al.* (1993). The form of this function for viable count data may be written as:

$$\text{Log } N_t = A + D \exp\{-\exp[-B(t - M)]\} \tag{4.1}$$
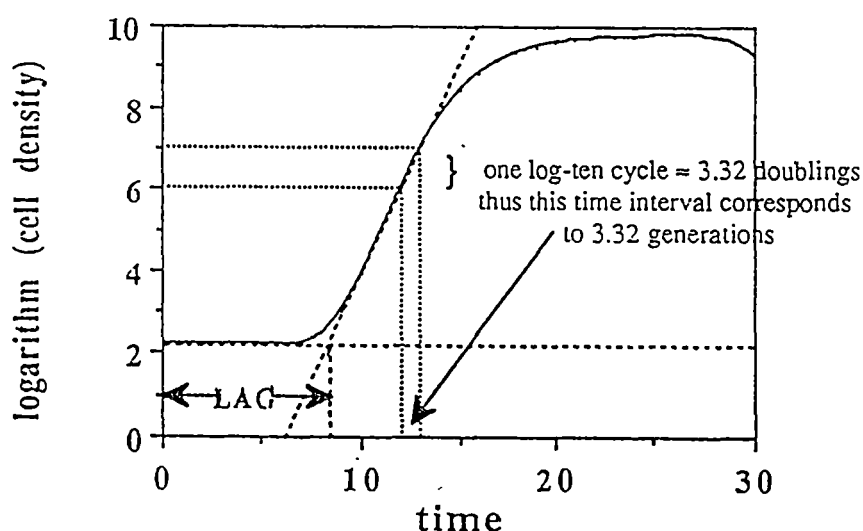
Figure 4.1 A graphical method for the estimation of generation and lag time from a bacterial population growth curve. The slope of the tangent to the steepest part of the curve estimates exponential growth rate. The generation time can be calculated from this tangent as the time for a 0.301 unit increase in log (cell density), i.e. a doubling of the population. The intercept of this tangent with the initial inoculum level (i.e. log $N_{(0)}$) is taken as the end of the lag phase. (After Ross, 1993)

where $Log = Log_{10}$, t = time

N_t = population density at time (t), e.g. CFU/g, or CFU/ml

A = value of lower asymptote or initial level of bacteria (log CFU/g)

D = difference in value of the lower and upper asymptote or number of log cycles of growth

M = time at which rate of the exponential growth rate is maximal (h)

and B is related to the slope of the curve at M such that BD/e is the slope of the steepest tangent, with e = exp(1).

From these parameters, various kinetic properties such as generation time and lag time (Fig. 4.1), a period of adjustment by cells to a new environment, can be calculated. However, several reports indicated shortcomings of the modified Gompertz function. These include the systematic lack of fit of the function (Whiting and Cygnarowicz-Provost, 1992), the overestimation by Eqn. 4.1 of the steepest tangent to the growth curve which leads to falsely fast generation time estimates, and biased estimates of lag phase duration (Whiting and Cygnarowicz-Provost, 1992; Baranyi *et al.*, 1993; Ross, 1993; Dalgaard *et al.*, 1994). Therefore, a factor of 1.131 is recommended to be included to compensate for the overestimation of the fastest rate inherent in the Gompertz function (Whiting and Cygnarowicz-Provost, 1992; Baranyi *et al.*, 1993; Ross, 1993; Dalgaard *et al.*, 1994). Thus, for log (CFU) data:

$$Generation\ time\ =\ \frac{elog2 \times 1.131}{BD}$$

$$=\ \frac{0.925}{BD} \qquad (4.2)$$

$$Lag\ time\ =\ M - \frac{1.13}{B}\{1 - \exp[1 - \exp(BM)]\} \qquad (4.3)$$

The advantages of optical density mesurements (turbidimetric methods) are speed, simplicity and non-invasiveness (McMeekin *et al.*, 1993). Therefore, the method is used for growth rate modelling in this chapter. However, there are some limitations in their use (McMeekin *et al.*, 1993; Ross, 1993). The relationship between concentration and absorbance/ turbidity is only linear over a limited range, corresponding approximately to a tenfold increase in cell numbers. The lower sensitivity limit of detection by turbidity measuring devices is usually such that they are unable to detect bacterial poopulations at densities less than $\sim 10^7$ CFU/ml. Thus, under conditions permitting consistent growth to $\sim 10^9$ CFU/ml in stationary phase, the onset of the stationary phase is not easily measurable, and experiments to determine lag times must be specifically prepared as they

can only be measured on dense populations. Falsely low estimates of cell densities in dense cultures result from a deviation from the response predicted by Beer's Law (i.e. that absorbance is proportional to concentration). In order to obtain accurate estimates of cell density, samples must have, or must be diluted so that they have absorbance <0.3 (Koch, 1981), or that the meatured absorbance be 'corrected' by reference to some correction function relating the observed to the true absorbance (Ross, 1993).

For optical density ($\Delta\%T$) observations, the following function was proposed (Ross, 1993) by analogy with Eqn. 4.1:

$$\Delta\%T_{(t)} = A + D \exp\{-\exp[-B(t - M)]\} \qquad (4.4)$$

where $\Delta\%T_{(t)}$ = the change in %T after time t

A = lower limit of detection of the spectrophotometer or % transmittance of the initial microbial load

B = maximum rate of change of % transmittance

M = time at which rate of change of % transmittance is maximal

D = difference between the lower and upper limits of sensitivity of the spectrophotometer

Thus, the minimum generation time of the %T growth curve can be calculated from the fitted parameters of Eqn. 4.4, with a correction factor of 1.08 (see details in Ross, 1993):

$$Generation\ Time = \frac{1.08 \times 20.5 \times e}{BD}$$

$$= \frac{60.2}{BD} \qquad (4.5)$$

The generation time obtained from the above expression can be converted to the reciprocal, referred to as growth rate (k).

In order to obtain a good fit to the data and reliable parameter estimates with this function, the quality and quantity of the data is extremely important. That is, the points should be spread evenly throughout the growth curve and at least 10 to 15 measurements need to be taken (McMeekin et al., 1993). In addition, reliable estimates of generation time by nonlinear regression are indicated when values for B and D are obtained within 10 iterations (McMeekin et al., 1993).

Considering that viable count (VC) methods remain the standard method of enumeration in food microbiology, the relationship between growth rate estimates from VC and turbidimetric (%T) methods has been investigated. Ross (1993) indicated that the estimates of generation time obtained from VC data are smaller than those from the %T method, and the relationship is constant. Possible explanations for the difference between GT_{vc} and $GT_{\%T}$ could be that 1) non-viable cells may also contribute to the increase in turbidity of the %T measurement, thereby displaying slower generation times than actually occur, and 2) limitation of the spectrophotometer, which can read reliably only within the range $\sim10^7$ CFU/ml to $\sim5\times10^8$ CFU/ml (Ross, 1993) where the culture is already close to the maximum population density and growth rates may already be declining. Thus, based on analysing numerous growth curves of several micro-organism using both methods, the average ratio of maximum specific growth rates (μ_{max}) obtained from VC data and %T was 1.57±0.33 (SD) (Dalgaard *et al.*, 1994). Consequently, a simple calibration factor of 1.5 may be incorporated as:

$$Generation\ Time_{(VC)} = Generation\ Time_{(\%T)} / 1.5 \qquad (4.6)$$

4.1.1.2 Secondary models

The response variable obtained from the primary kinetic model is expressed in time-based units (i.e. a rate, or the time taken for a particular response). To generate a secondary model, there are currently several forms of mathematical model proposed by different research groups of which four main model types are recognised and summarised in Table 4.2. Temperature is regarded as the primary factor regulating the growth of micro-organisms (Curry *et al.*, 1978) with other environmental factors acting independently and additively. Most of the proposed kinetic models have their origins in relationships between temperature and growth rate, with additional factors such as water activity, pH and antimicrobial additives being included subsequently into some of the models (Ross and McMeekin, 1991). For example, consider the square-root type models which are employed extensively in this chapter. Firstly, the effect of temperature was modelled (Ratkowsky *et al.*, 1982), followed by the incorporation of a water activity term (McMeekin *et al.*, 1987). More recently, pH and organic acid terms were included in the model (Presser *et al.*, 1997a). The model can be written as:

$$\sqrt{rate} = b * (T-T_{min}) * (1-exp(c\,(T-T_{max}))) * \sqrt{(a_w-a_{wmin})} *$$

$$\left(\sqrt{1-\frac{10^{pHmin}}{10^{pH}}} * \sqrt{1-\frac{[LAC]}{[U_{min}] * (1+10^{pH-pKa})}} * \sqrt{1-\frac{[LAC]}{[D_{min}] * (1+10^{pKa-pH})}} \right) + e \qquad (4.7)$$

Table 4.2 Mathematical models proposed for use as secondary models in predictive microbiology.

Type of model	Users	References
Kinetic model:		
1. Square root	University of Tasmania, MIRINZ[a], Remonsys	Ratkowsky *et al.* (1983) Gill (1986)
2. Schoolfield	Unilever, UK	Schoolfield *et al.* (1981)
3. Davey's modified Arrhenius	CSIRO, University of Adelaide	Davey (1989), Daughtry *et al.* (1997)
4. Polynomial or response surface model	USDA MAFF[b]	Buchanan *et al.* (1989a) Gibson and Roberts (1989)
Probabilistic model:		
1. Polynomial	University of California MAFF	Genigeorgis *et al.* (1971) Gibson and Roberts (1989)
2. Logistic	University of Tasmania USDA	Ratkowsky and Ross (1995), Presser *et al.* (in press) Whiting and Oriente (1997)
3. Non-linear logistic	University of Tasmania	Presser *et al.* (1997b)

[a] Meat Industry Research Institute of New Zealand (Inc.), [b] the United Kingdom Ministry for Agriculture, Fisheries and Food (Adapted from Ross and McMeekin, 1991)

where b and c are constants of proportionality, T, a_w, and pH are the measured temperature (°C), water activity, and pH of the medium respectively, T_{max} is the notional maximum temperature for growth (°C), T_{min}, a_{wmin}, pH_{min} are the notional minimum temperature, water activity, and pH respectively for growth, and U_{min}, and D_{min} are the notional minimum concentration of undissociated, and dissociated lactic acid respectively which prevent growth, [LAC] is the concentration of lactic acid, and e is the error term.

Early 'probability models' (e.g. Genigeorgis *et al.* (1971), Gibson and Roberts (1989)) predict the likelihood of a specific event such as growth or death or toxin production of the micro-organism of concern in a limited period of time (Ratkowsky and Ross, 1995). The probabilistic approach is important when a pathogenic strain of low infective dose is involved, as the rate of growth of the pathogen is then of lesser importance than the fact that it is present and potentially able to multiply to infectious dose or toxic levels. To define the probability of growth as a function of one or more controlling environmental factors, Ratkowsky and Ross (1995) proposed a logistic regression method and recently there has been a development to a generalised non-linear regression method (Presser *et al.*, 1997b). The latter method enables the parameters T_{min}, a_w, and pH_{min} to be fitted from the data rather than being assumed to be the same as the parameters from kinetic

modelling studies. The form of the expression of the growth limiting factors is suggested by the kinetic model, while the response at a given combination of factors is either presence or absence (i.e. growth/no growth) or probabilistic (employing the fraction of positive responses in n trials). The form of the probability model is a logarithmic transformation of a kinetic model. Eqn. 4.8 presents a probability model when lactic acid is an additional controlling factor (Presser, 1995) which can be written as:

$$\text{logit}(P) = b_0 + b_1 \ln(T-T_{min}) + b_2 \ln(a_w-a_{wmin}) + b_3 \ln(1-10^{pHmin-pH})$$
$$+ b_4 \ln\left(1-\left(\frac{[LAC]}{U_{min}(1+10^{pH-pKa})}\right)\right) + b_5 \ln\left(1-\left(\frac{[LAC]}{D_{min}(1+10^{pKa-pH})}\right)\right) \quad (4.8)$$

where: $\text{logit}(P) = \ln(P/(1-P))$, P is the probability of growth (which has values from 0 to 1), b_0, b_1, b_2, b_3, b_4, and b_5 are coefficients to be estimated, and the other terms are as previously defined in Eqn. 4.7.

Eqn. 4.8 is the basic form of the probability model. An extended form of the model, by incorporating quadratic term(s), e.g. $\ln(T-T_{min})^2$, and/or cross-product(s), e.g. $\ln(T-T_{min}) * \ln(a_w-a_{wmin})$, may be considered to improve the goodness-of-fit of the model.

This type of model enables the incorporation of kinetic data that were developed for other modelling purposes to generate a growth/no growth interface model (Ratkowsky and Ross, 1995). From the fitted model, the interface or boundary between growth and no growth, at some chosen level of probability (e.g. $P=0.5$ which is 50% probability of growth or no growth), can be determined.

The kinetic and early probability models may be considered as the two extremes of a modelling approach where the distinction between the two models is an artificial one. In the probability study, growth is observed earlier when the micro-organism is in a less constrained condition because the organism is able to grow more quickly. Consequently, a high probability of growth is predicted, when a high growth rate (short generation time) is observed at the same condition in the kinetic study. The inclusion of information about the variability of rates of growth in the probability models is also recognised by Baker *et al.* (1990). However, in a more recent development of probability models (Presser *et al.*, 1997b; in press), the observation period was extended to a sufficient time to ensure that either growth was observed or was not possible. Similarly, probability measurements in the present study (Chapter 5) do not depend on the time for a response to be detectable or on the rate of growth of the organism, i.e. they are absolute estimates of the potential for growth.

4.1.1.3 Model validation

Following development of a model, either kinetic or probability, usually with data from micro-organisms grown in laboratory media, scepticism may remain whether the model can reliably predict real situations in foods. Therefore, before a model is accepted for inclusion in a database (tertiary model) it must be evaluated or 'validated'. The validation can be performed in the laboratory to determine the behaviour of the inoculated micro-organism or the natural biota in actual products. The ultimate test of a model is validation of predictions under normal handling with fluctuating environmental conditions, such as during processing, distribution, and storage of product (McMeekin and Ross, 1996). A different approach to validation is taken for data extracted from the literature, allowing a wider validation exercise to be carried out (McClure *et al.*, 1994).

Indices of goodness-of-fit of a model, the "bias" and "accuracy" factors, were introduced by Ross (1996). The bias factor provides an indication of the average deviation of the model from the data, taking account of the signs of the differences, and is described by Eqn. 4.9. A bias factor of 1.0 indicates lack of systematic error and factors of greater and less than 1.0 indicate over- and under-prediction respectively. The accuracy factor provides a measure of absolute difference between the observed and predicted values and is described by Eqn. 4.10. The larger the accuracy factor, the less precise is the average estimate.

$$\text{BIAS factor} = 10^{(\Sigma \log(GT_{observed}/GT_{predicted}))/n} \tag{4.9}$$

$$\text{ACCURACY factor} = 10^{(\Sigma |\log(GT_{observed}/GT_{predicted})|)/n} \tag{4.10}$$

where $GT_{observed}$ is the observed generation time (h), $GT_{predicted}$ is the predicted generation time (h), and n is the number of observations used in the calculation.

4.1.1.4 Tertiary models

Once a fully validated model has been developed, it can be included in user-friendly application software, allowing the information summarised from large amounts of data to become easily accessible and the models to be conveniently applied by many different users (Dalgaard, 1997). Several application software programmes have been developed (see reviews by McMeekin and Ross, 1996; Dalgaard, 1997) e.g., 'Pathogen Modelling Program' developed by the Microbial Food Safety Research Unit of the USDA, USA (Buchanan, 1993) and 'Food Micromodel' developed by MAFF (McClure *et al.*, 1994),

which contain models for several pathogenic micro-organisms and allow growth and thermal death to be predicted for constant environmental conditions input by the user.

Integration of a temperature function (e.g. secondary model) with the time/temperature history by devices such as chemical and physical indicators, electronic temperature integrators or loggers enables the model to analyse the effect of those environmental conditions on the behaviour over time of the organism in question (McMeekin and Ross, 1996). 'Pseudomonas Predictor' developed at the University of Tasmania (Neumeyer *et al.*, 1997a,b) is an example of application software that has the facility to read and interpret temperature profiles collected by temperature loggers in terms of the potential for growth of psychrotrophic Pseudomonads.

4.1.1.5 Application of predictive modelling

Quantitative information regarding microbial behaviour, obtained using the predictive microbiology approach, may be used in various applications (see review by McMeekin and Ross, 1996) including:

• Development of rational quantitative criteria for HACCP (Hazard Analysis Critical Control Point) or other quality assurance procedures,

• Determination of product shelf-life, i.e. the time taken for spoilage micro-organism or pathogens to reach unacceptable levels,

• Formulation and reformulation of products e.g. for determination of optimal combinations of controlling factors that inhibit growth of pathogens or spoilage organisms or eliminate them,

• Technology transfer can be achieved by model simulations where the user may conveniently answer "what-if" questions and which can be presented as an educational tool for food handlers, food scientists or microbiologists.

4.1.1.6 Existing predictive models

Predictive models for the growth of *L. monocytogenes* have been published by several researchers. Those include cubic and quadratic response surface models (Hudson, 1994; Buchanan and Golden, 1995; Buchanan *et al.*, 1997), and square-root type models (Ross, 1993). A probability type model for the survival and growth of *L. mono-cytogenes* using a polynomial model was also generated (Cole *et al.*, 1990). In this model, however, the time to visible turbidity was presented rather than the probability of

growth. These models and their published observations provide useful information to evaluate the models generated in this chapter.

4.1.2 LACTIC ACID

Lactic acid is a short chain organic acid ($CH_3CHOHCOOH$, MW = 90.08) which is produced naturally by controlled fermentation by homofermentative lactic acid bacteria using refined sucrose or other carbohydrate sources or synthetically by hydrolysis of lactonitrile (Shelef, 1994). Lactic acid exists in 2 forms; the D(-) form, and L(+) form. The natural L(+) lactic acid is one of the most widely employed preservative in foods, especially meat products, as discussed in section 4.1. Growth of both Gram-positive and Gram-negative bacteria were reported to be inhibited by lactic acid (Brown and Booth, 1991; Ray, 1996). However, yeasts and moulds were found to be less sensitive than bacteria (Lueck, 1980; Houstma, 1996).

As a weak organic acid, in aqueous solutions, lactic acid is partially dissociated to ionised forms. The equilibrium of the dissociation of a weak acid is dependent on pH as described by the Henderson-Hasselbalch equation:

$$pH = pK_a + \log \frac{[A^-]}{[HA]}$$
(4.11)

The dissociation constant of lactic acid is 1.38×10^{-4} at 22°C ($pK_a = 3.86$) (Ray, 1996).

4.1.2.1 Mechanism of action

Bacteriostasis or bacteriocide can, in principle, be the results of inactivation of, or interference with, one or more of the functional subcellular target groups such as cell components, metabolic enzymes, protein synthesis system, genetic material etc. (Gould, 1989). Normal function of a microbial cell depends on a certain stability of the internal chemical environment. Microbes have developed systems to maintain fluctuations in their cytoplasmic pH, the so-called pH homeostatic process (Booth, 1985). The systems are active mechanisms ranging from use of cell energy provided by ATP and the proton motive force (PMF) to control the permeability of the cell membrane to protons (Montville, 1997). ATP and the PMF are fundamental to cellular energetics and are interconvertible by a membrane-bound BF_0F_1-ATPase that can use ATP to generate a proton gradient and *vice versa* (Montville, 1997). A study of the effect of sodium lactate on acid adaptation of *Listeria* indicated that the proton translocating ATPase played a major role in regulating its intracellular pH (Houstma, 1996).

Lactic acid, as a lipophilic organic acid, has the ability to penetrate the bacterial cell membrane in its undissociated form (Freese *et al.*, 1973; Gould, 1989). On entering the cell, where the pH is higher, the acid dissociates in the cytoplasm, releases protons, and reduces intracellular pH (pH_i). The homeostatic mechanisms of cells overcome this by extruding protons through the proton pump. However, dissociation of the acid within the cell will continue until it reaches equilibrium of dissociated and undissociated acid which is determined by pKa of the acid and the pH_i (Gould, 1989). These processes can cause depletion in energy and eventually a decrease in pH_i (Ray, 1996).

Low cytoplasmic pH can adversely affect the ionic bonds of the macromolecules in cells and disrupt their three-dimensional structures and some functions (Ray, 1996). These changes can also interfere with nutrient transport and energy generation and consequently reduce the growth yield, extend the lag phase, and decrease the growth rate (Freese *et al.*, 1973; Corlett and Brown, 1980; de Wit and Rombouts, 1990). In addition, low pH can reversibly and irreversibly damage cellular macromolecules such as membrane-bound enzymes that subsequently can inflict sublethal-injury as well as lethal injury to cells (Ray, 1996).

A study on the change of intracellular pH of *L. monocytogenes* has demonstrated that the inhibitory effect of various types of weak acids were different even at the same level of pH_i (Young and Foegeding, 1993). Thus, the growth inhibition by acids is not caused by a decrease in pH_i, *per se*, but also involves specific acids effects which may influence metabolic or other physiological activity (Ita and Hutkins, 1991; Young and Foegeding, 1993). On the basis of equimolar total acid, the relative inhibition effect was generally acetic>lactic>citric (Young and Foegeding, 1993).

The above mechanism of weak acid action is supported by several observations in that, lowering the external pH, the proportion of undissociated acid increased, reducing the internal pH_i and enhancing the antimicrobial effectiveness of the acids (Gould, 1989; Ita and Hutkins, 1991; Young and Foegeding, 1993). The effectiveness of the weak acid preservatives against *L. monocytogenes* is also predictably influenced by pH (Petran and Zottola, 1989). Therefore, it has been generally considered that the antimicrobial activity is directly related to the concentration of undissociated acid (Baird-Parker, 1980). However, dissociated acid was also reported to be involved in the antimicrobial activity although less effectively (Eklund, 1983). Very high concentration of lactate anion was suggested to influence metabolic pathways of *Listeria*, particularly when lactate is an intermediate or end-product of metabolism (Houstma, 1996).

The ability of organic acids to induce cell acidification is reported to be greater than inorganic acids which rely only on lowering the external pH (Gould, 1989). The

mechanisms of organic acid inhibition determine their higher antimicrobial effect than inorganic acids. Strong inorganic acids may only exert their influence by the denaturing effect of low pH on enzymes present on the cell surface and on lowering of the cytoplasmic pH due to increased proton permeability when the pH gradient is very large (Corlett and Brown, 1980).

4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

Details of consumables, reagents and media, and equipment used are presented in Appendix A.

4.2.2 GENERAL METHODS

4.2.2.1 Culture preparation

Two strains of *L. monocytogenes*, Scott A and L5, a wild type isolated from cold-smoked salmon, were used over a series of experiments. Stock cultures were maintained at -80°C (Appendix A, A.2.5). To prepare for an experiment, one carrier bead was removed from the -80°C-stored culture, rubbed over the surface of an OXF plate and incubated at 30°C overnight. A single colony of that culture was then transferred to 50 ml TSB-YE in a 250 ml side-arm flask. This was incubated for 18 hr at 30°C with shaking (50±2 rpm). Fifty μl of that culture was transferred to fresh TSB-YE and the incubation repeated. The broth culture was held at 10°C for 1 hr before commencement of the experiment in order to minimise changes in cell density during the inoculation procedure.

4.2.2.2 Inoculation procedures

The amount of inoculum has been reported to have no effect on growth rate (Buchanan and Phillips, 1990), but it may affect the lag phase (Gay *et al.*, 1996). Although lag phase was not studied in these experiments, to maintain reproducibility within the experiment the volume of inoculum was kept constant. The inoculation was prepared by pipetting 0.35-0.40 ml of prepared culture (section 4.2.2.1) into an L-tube (see Appendix A, A.1.6) containing 15 ml TSB-YE. This reduces the %T of media to between 80 and 90%T at 540 nm. The 100%T was set with a sterile TSB-YE blank and periodically checked against the same blank as the experiment progressed. Each inoculated tube was placed in the shaking incubator for ca. 15 sec to mix thoroughly, and then 0.3 ml of the

broth was aseptically dispensed to a well-plate, and the initial pH (pH$_I$) determined using a surface probe pH-meter.

4.2.2.3 Assessment of growth

Cultures were incubated in L-tubes containing 15 ml of TSB-YE on a shaking (33±1 rpm) incubator, i.e. temperature gradient incubator operated normally or isothermally, in a 20°C constant-temperature room. Growth was assessed by measuring the turbidity as %T at 540 nm. Measurement times were chosen to correspond to %T changes of 5-10%T between consecutive readings. This was continued until the %T fell below 5%, or until the rate of change of the %T was zero. The change in pH over time including the final pH was also recorded at every ca. 10%T change using a surface probe pH-meter as previously described for measuring pH$_I$ (see section 4.2.2.2).

In each experiment, monitoring was continued for up to 3 weeks to verify that growth did not occur, enabling the growth rate data to be used and included in the growth/no growth data set (section 4.2.4). At the conclusion of each experiment the final %T was recorded. The %T values were transformed to final optical density (OD) (see Appendix A, A.2.12) to enhance the magnitude when plotted on graph. This enabled the 'cell yield' response to be observed.

Note that 'cell yield' refers to the increase in biomass proportional to a known amount of energy substrate provided (Russell and Cook, 1995). The actual 'cell yield' study is normally prepared using a substrate-limited culture to manipulate the maximum growth to be within the linear range of turbidity measuring device (Krist, 1997). In this study, only "apparent" cell yield was recorded as a final change in OD in an enriched nutrient medium. In some cases, the "apparent" cell yield was "corrected" by a correction function (see Appendix A, A.2.3) to compensate for the non-linearity of the OD-concentration relationship (Koch, 1981) and to provide assurance that the observed "apparent" cell yield is reliable.

At the completion of incubations, the temperature of each tube was measured 5 times over a number of hours with an electronic thermometer. The average of the 5 temperatures was taken as the incubation temperature for calculations and further analysis.

4.2.2.4 Calculation of generation times for kinetic modelling

Percent transmittance readings from kinetic modelling studies were converted to change of

%T (Δ%T) at 'time elapsed since inoculation' (Δt). These values were entered into a SAS[2] PROC NLIN routine, written by Dr. G. McPherson, Mathematics Department, University of Tasmania, which fitted a Gompertz function (Eqn. 4.4) to the data. The analysis by this NLIN procedure gave estimates of the Gompertz parameters B, D, M, and A. If convergence was not obtained within 10 iterations, the values of Δ%T or the change in bacterial numbers (log CFU/g) and Δt were plotted with a tangent drawn "by eye" to the steepest section of the growth plot (Fig. 4.1). The tangent was used to obtain a time value for a change of 24.5%T, the change that corresponds to 1 generation (McMeekin *et al.*, 1993 pp: 84-86). Eqn. 4.4 was then fitted and the generation times calculated using Eqn. 4.5.

4.2.2.5 Analysis of growth responses to pH and organic acid

To account for the growth responses influenced by the effect of each of the forms of lactic acid so that responses at different pH and lactic acid concentrations could be compared, factors to "correct" or "standardise" the observed growth rates (k), based on the pH terms used in the kinetic model (Eqn. 4.7), were developed (Ross, pers. comm.). Eqns. 4.12 and 4.13 were used to standardise k for the growth responses of *L. monocytogenes* Scott A as a function of H^+, and UD at constant temperature respectively. Note that, the lactic acid experiments in this study were prepared at a_w of ~0.96. The addition of lactic acid, up to 450 mM, caused only a small variation of a_w (0.962-0.967). Hence, to simplify the equation, the a_w variation due to lactic acid concentration was omitted.

Although dissociated lactic acid is reported to have little effect on growth rate, this form was taken into account for the correction of growth rate data of *L. monocytogenes* L5 as 450 mM lactic acid data was included (Eqns. 4.14 and 4.15). In addition, an attempt was made to identify the effect of H^+ only, i.e. when HCl was the acidulant. A correction factor to compensate for the difference in a_w was also developed (Eqn. 4.16).

For growth rate data (with lactic acid) of *L. monocytogenes* Scott A:

$$k_{H^+} = \frac{k}{1 - \dfrac{[LAC]}{U_{min} * (1 + 10^{pH-3.86})}} \qquad (4.12)$$

where k is the observed growth rate, k_{H^+} is the growth rate standardised for the undissociated lactic acid present (to identify the response due to H^+ only), [LAC] and U_{min} are as previously defined in Eqn. 4.7.

$$k_{UD} = \frac{k}{1 - 10^{pHmin - pH}} \tag{4.13}$$

where k_{UD} is the growth rate standardised for the effect of H^+, i.e. due to the effect of UD only. k and pH_{min} are as previously defined.

For growth rate data (with lactic acid) of *L. monocytogenes* L5:

$$k_{H^+} = \frac{k}{\left(1 - \frac{[LAC]}{Umin * (1+10^{pH-3.86})}\right) * \left(1 - \frac{[LAC]}{Dmin * (1+10^{3.86-pH})}\right)} \tag{4.14}$$

where k_{H^+} is the growth rate standardised for the undissociated and dissociated lactic acid, revealing the effect of H^+. k, [LAC], U_{min}, and D_{min} are as previously defined.

$$k_{UD} = \frac{k}{\left(1 - 10^{pHmin - pH}\right) * \left(1 - \frac{[LAC]}{Dmin * (1+10^{3.86-pH})}\right)} \tag{4.15}$$

where k_{UD} is the growth rate standardised for the effect of H^+ and dissociated lactic acid, so that the growth rate change due to the effect of UD only is highlighted. k, pH_{min}, [LAC], and D_{min} are as previously defined.

For growth rate data (pH without lactic acid) of *L. monocytogenes* Scott A and L5:

$$k_{0.965} = k_{0.995} * \frac{(0.965 - a_{wmin})}{(0.995 - a_{wmin})} \tag{4.16}$$

where $k_{0.965}$ is the growth rate standardised for the a_w of 0.965. $k_{0.995}$ is the observed growth rate at the a_w of 0.995. a_{wmin} is previously defined in Eqn. 4.7.

4.2.3 KINETIC MODELLING

The effect of 1) temperature, 2) combinations of water activity, pH, and concentrations of lactic acid, and 3) pH and different concentrations of lactic acid on growth rate of *L. monocytogenes* were studied. The range of the combinations tested is given in Table 4.3. Note that two forms of acidulant, i.e. HCl and lactic acid, were used to enable the differentiation of the inhibitory effects due to pH and lactic acid. Filter sterilised 5 M HCl or 4 N NaOH solutions were used for pH adjustment of the media to avoid large changes in volume and concentration of the media.

Table 4.3 Outline of the experimental design covering the conditions tested in kinetic models. Note that the range of those controlling factors are only approximate- full details are presented in Appendix G (Tables G.1-G.4).

Study of	Temp. (°C)	Water activity	pH	Lactic acid (mM)	Number of conditions which growth rates were measured	
					Scott A	L5
1. Temperature[a]	3-37	0.995	7.3	0	30	30
2. pH + lactic acid + a_w[b]	ca. 20	0.92-0.995	5.4, 5.6, and 6.0	0 50	30 28	30 28
3. pH + lactic acid[c]	ca. 20	0.995	4.0-6.8	0	13	13
		0.96	4.4-7.8	20-200	47	47
		0.96	5.4-6.6	450	-	7
Total data for model generation					**148**	**155**

[a, b, and c] Details are given in sections 4.2.3.1, 4.2.3.2, and 4.2.3.3 respectively.

4.2.3.1 Determination of the effect of temperature on growth rate

The effect of temperature on the growth rate of *L. monocytogenes* Scott A and L5 was investigated in the sub-optimum temperature range, from 3 to 37°C at intervals of approximately 1°C, using a Temperature Gradient Incubator (TGI). 15 ml of TSB-YE was added to each of 60 L-tubes, which were then sterilised. The water activity of the sterile broth was measured. The L-tubes were placed in the TGI and allowed to equilibrate overnight prior to beginning the experiment. The methods described in sections 4.2.2.1-4 were followed.

4.2.3.2 Determination of the effect of water activity, pH, and lactic acid on growth rate

A series of over-strength broths of different a_w were prepared by the inclusion of NaCl to the desired level. Due to the volume changes upon addition of large amounts of NaCl, a volumetric flask was used to ensure that final concentrations of the growth medium constituents in a_w adjusted media were the same as in normal preparations. The over-strength media were autoclaved at 105°C for 30 minutes to prevent turbidity of the broths due to formation of precipitates. In a study of the additional effect of lactic acid, 50 mM lactic acid (0.512 g/100 ml) was included as acidulant to the sterile a_w adjusted media in volumetric flask then made up to volume with sterile distilled water. At each water activity level, pH was adjusted to ca. 5.4, 5.7, and 6.1, and then 15 ml of the broth was dispensed into 2 L-tubes for each *L. monocytogenes* strains. The first set of 60 L-tubes were placed in a 20°C constant-temperature room and allowed to equilibrate to the temperature overnight prior to beginning the experiment. The methods as outlined in sections 4.2.2.1-4 were followed.

4.2.3.3 Determination of the effect of pH and lactic acid on growth rate

For the pH-lactic acid study, the experiment was also designed to mimic the water activity of cold-smoked salmon which is ~0.96. Therefore, 4.5% NaCl was also included in the media used.

Sterile over-strength TSB-YE + 4.5% NaCl was prepared in a volumetric flask and made up to final volume with sterile distilled water and filter sterilised lactic acid (88% w/w) to concentrations of 20 mM (1.024 g/500 ml), 50 mM (2.559 g/500 ml), 100 mM (5.118 g/500 ml), and 200 mM (10.236 g/500 ml). In order to determine the growth response of *L. monocytogenes* L5 at high levels of lactic acid, the addition of 450 mM (11.516 g/250 ml) lactic acid was also tested.

In the study of pH without lactic acid, however, no NaCl was added to the medium which already contained 0.5% NaCl. Thus, the water activity was near optimal at ≈0.995. This was intended to enable the bacteria to achieve growth at the lowest pH possible so that pH_{min} could be estimated directly.

For each concentration of lactic acid, the sterile broth was separated equally by weight into two flasks and pH was adjusted to the lowest and highest pH required for each acid concentration. A pH gradient of 15 pH values was prepared aseptically by combining the two broths in varying proportions in sterile containers. The pH of the mixture was

rechecked and adjusted to the desired pH, and then 15 ml was dispensed into 2 L-tubes for each *L. monocytogenes* strain.

The L-tubes were placed in a 20°C constant temperature room and allowed to equilibrate to the temperature overnight prior to beginning the experiment. This also allowed time for any contamination to become apparent, and those tubes to be replaced. The methods outlined in sections 4.2.2.1-4 were followed.

4.2.3.4 Model generation

The kinetic models for *L. monocytogenes* Scott A, and L5, were based on 148 and 155 growth curves respectively, covering the range of sub-optimum conditions. The variables combinations tested are represented diagrammatically in Appendix G, Fig. G.1. Details are presented in Tables G.1 and G.2 for strain Scott A and Tables G.3 and G.4 for strain L5. Growth curves were fitted by PROC NLIN to Eqn. 4.4 and growth rates calculated using Eqn. 4.5.

The model for the temperature-a_w-pH-lactic acid response used in this study, Eqn. 4.7, is a square-root type model and was derived by Ross (1993). A new pH-term, based on the assumption that the growth rate is linearly proportional to hydrogen ion concentration and to undissociated lactic acid concentration, was recently introduced (Presser *et al.*, 1997a). These kinetic models were fitted using a SAS[2] PROC NLIN, a generalised non-linear regression procedure written by Dr. D. Ratkowsky, School of Agricultural Science, University of Tasmania. Goodness-of-fit of the model to the observed data was assessed by root mean square error (RMSE) (Box and Draper, 1987).

4.3 RESULTS

A total of 148 and 155 growth curves were generated for *L. monocytogenes* Scott A and L5 respectively. The fitted models for the combined effects of temperature-water activity-pH-lactic acid on growth rates of *L. monocytogenes* Scott A (Eqn. 4.17a) and L5 (Eqn. 4.18a) are as follows:

$$\sqrt{rate} = 0.148 * (T-1.4) * \sqrt{(aw-0.925)} * \sqrt{1-10^{4.228-pH}} * \sqrt{1-\frac{[LAC]}{3.70*(1+10^{pH-3.86})}} \qquad (4.17a)$$

$$\sqrt{rate} = 0.146 * (T\text{-}0.36) * (1\text{-}exp(0.611 * (T\text{-}40.7))) * \sqrt{(aw\text{-}0.927)}$$

$$* \sqrt{1 - 10^{4.248\text{-}pH}} * \sqrt{1 - \frac{[LAC]}{4.48 * (1+10^{pH\text{-}3.86})}} \qquad (4.18a)$$

where T, a_w, pH, and [LAC] are previously defined in Eqn. 4.7. The parameter estimates and standard errors are given in Table 4.4. It should be noted that the T_{max} term was initially incorporated in the model 4.17a but the fitting was not significantly improved. Furthermore, an irregular T_{max} value of ~38°C was obtained. Therefore, the T_{max} term was omitted in model 4.17a.

Table 4.4 Parameter estimates for *L. monocytogenes* Scott A and L5 fitted to Eqn. 4.7.

Parameter	Scott A	Standard Error	L5	Standard Error
b	0.148	±0.0059	0.146	±0.0073
c	-	-	0.611	±1.21
T_{min} (°C)	1.43	±0.761	0.36	±0.94
T_{max} (°C)	-	-	40.7	±9.12
a_{wmin}	0.925	±0.0014	0.927	±0.0017
pH_{min}	4.228	±0.0058	4.248	±0.0056
U_{min}	3.70	±0.0874	4.48	±0.0578
Root Mean Square Error	0.00514	-	0.00486	-

The root mean square error for √rate, for models 4.17a and 4.18a are relatively small which indicates a good fit to the actual data (Box and Draper, 1987). However, on a closer examination of the growth responses of both strains of *L. monocytogenes* to pH when HCl was used as acidulant (without lactic acid) a sigmoid response is noted (Fig. 4.2). This observations differed from the pH-lactic acid response (see observed data in Figs. 4.3b and 4.4b) predicted by the pH term used in the model (Eqn. 4.16). An attempt to obtain a model with a more accurate description of the response shape was made by sequentially removing the pH-only data (i.e. without lactic acid), i.e. 13 conditions for each strain, from the data set. The following models for *L. monocytogenes* Scott A (Eqn. 4.17b) and L5 (Eqn. 4.18b) were generated:

$$\sqrt{rate} = 0.150 * (T\text{-}0.88) * (1\text{-}exp(0.536 * (T\text{-}41.4))) * \sqrt{(aw\text{-}0.923)}$$

$$* \sqrt{1 - 10^{4.97\text{-}pH}} * \sqrt{1 - \frac{[LAC]}{3.79 * (1+10^{pH\text{-}3.86})}} \qquad (4.17b)$$

$$\sqrt{\text{rate}} = 0.160 * (T-0.60) * (1-\exp(0.129 * (T-51))) * \sqrt{(aw-0.925)}$$

$$* \sqrt{1-10^{4.94-pH}} * \sqrt{1 - \frac{[LAC]}{3.55*(1+10^{pH-3.86})}} * \sqrt{1 - \frac{[LAC]}{1821.9*(1+10^{3.86-pH})}} \qquad (4.18b)$$

where T, a_w, pH, and [LAC] are previously defined in Eqn. 4.7.

The standard errors associated with each of the fitted parameters are small, except for T_{max}. The experimental design, however, was intended to generate a growth rate model for *L. monocytogenes* in which temperatures beyond ~37°C were considered of little practical interest. However, inclusion of the parameter T_{max} to the models provided a better fit. A relatively large standard error for T_{max}, and an inconsistent estimate were obtained due to the lack of data points at high temperature (Table 4.5). The parameter estimates and standard errors are summarised in Table 4.5. The smaller RMSEs obtained from these models (Eqns. 4.17b and 4.18b) show a good fit to the data. The plots of the predictions fitted to the observed data for each of the controlling factors show a satisfactory description of the trends evident in the data (Figs. 4.3(a-c) for Scott A, and Figs. 4.4(a-c) for L5). Several growth characteristics of *L. monocytogenes* response to the controlling factors tested here, in addition to the model generation, deserve further mention.
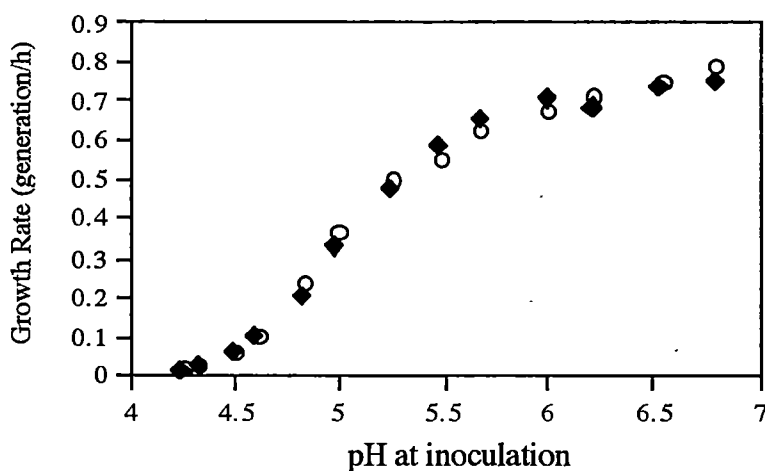
Figure 4.2 The sigmoid response of *L. monocytogenes* Scott A (♦) and L5 (O) growth rate to increasing pH (without lactic acid) in TSB-YE at ~20°C and a_w of 0.995.

Figure 4.3 (facing page).   The observed growth rate of *L. monocytogenes* Scott A compared to the growth rate predicted from the model (Eqn. 4.17b) in:

a) the sub-optimum temperature range from 3 to 37°C,

b) the range of a_w of 0.929-0.995 (NaCl as humectant) in the absence and presence of 50 mM lactic acid, and

c) the range of pH of 4.9-7.8 in the presence of 5.0% NaCl and different levels of lactic acid.

The predicted lines were fitted directly to the observed data without a standardisation for slight difference in pH in b) or temperature in c).

a) *Temperature response*

b) *Aw-pH-Lactic acid response*

c) *pH-Lactic acid response*

The observed growth rate of $L.$ *monocytogenes* L5 compared to the growth rate predicted from the model (Eqn. 4.18b) in:

a) the sub-optimum temperature range from 3 to 37°C,

b) the range of a_w of 0.929-0.995 (NaCl as humectant) in the absence and presence of 50 mM lactic acid, and

c) the range of pH of 4.9-7.8 in the presence of 5.5% NaCl and different levels of lactic acid.

The predicted lines were fitted directly to the observed data without a standardisation for slight difference in pH in b) or temperature in c).

a) *Temperature response*
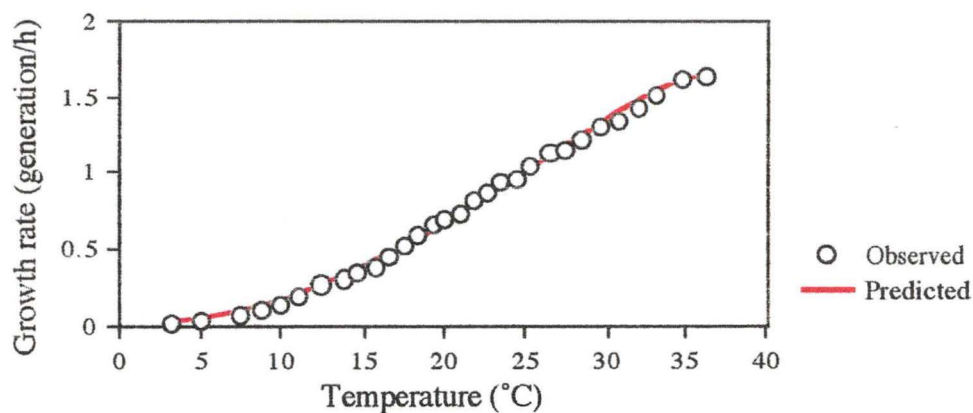
b) *Aw-pH-Lactic acid response*
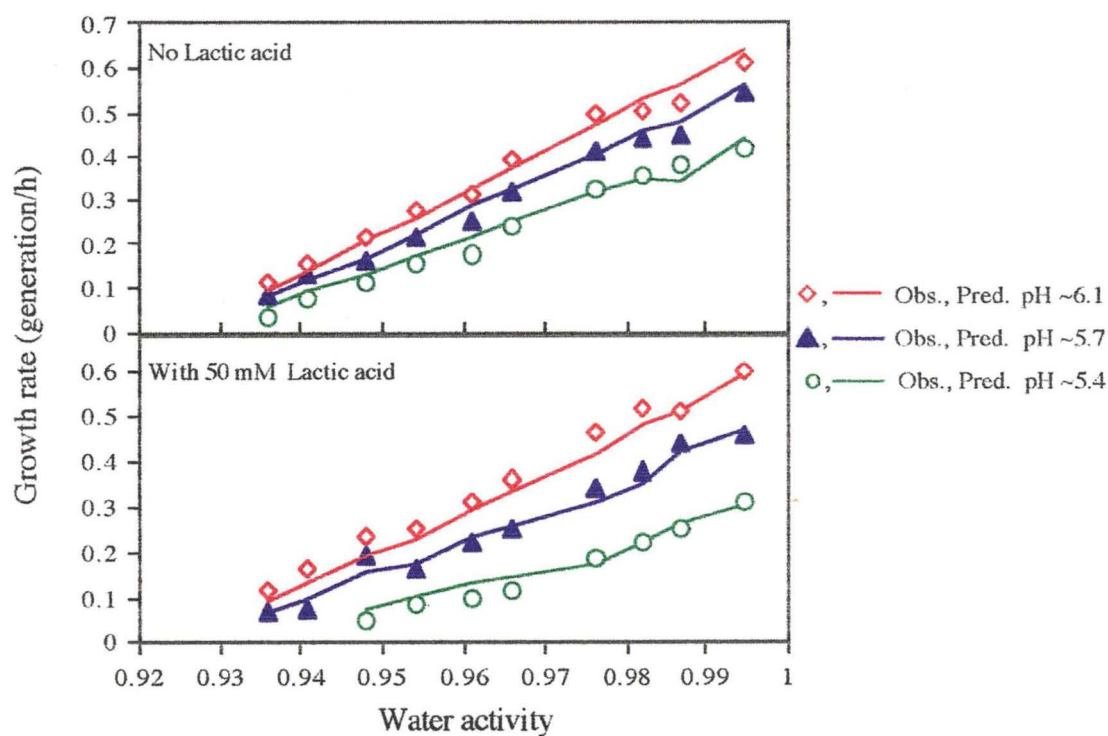
c) *pH-Lactic acid response*
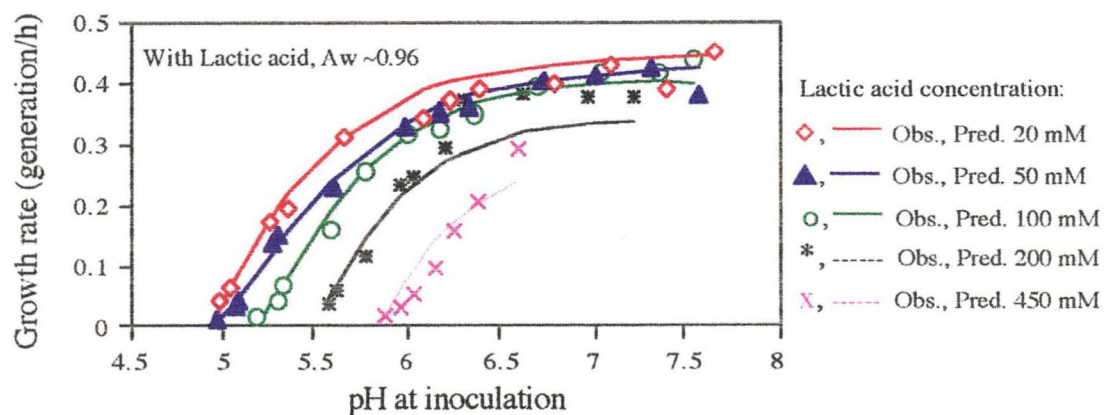
Table 4.5 Parameter estimates for *L. monocytogenes* Scott A and L5 fitted to Eqn. 4.7.

Parameter	Scott A	Standard Error	L5	Standard Error
b	0.150	±0.0038	0.160	±0.0073
c	0.536	±0.6581	0.129	±0.0605
T_{min} (°C)	0.88	±0.4576	0.60	±0.4615
T_{max} (°C)	41.4	±7.09	51.0	±6.13
a_{wmin}	0.923	±0.00084	0.925	±0.00082
pH_{min}	4.97	±0.0131	4.94	±0.00996
U_{min}	3.79	±0.0758	4.55	±0.0608
D_{min}	-	-	1821.9	±301.9
Root Mean Square Error	0.00125	-	0.00074	-

4.3.1 TEMPERATURE RESPONSE

Both *L. monocytogenes* Scott A and L5 grew at temperatures above ~3°C, which was the lowest temperature used in the experiments, to ≥37°C, the highest temperature tested. Similar values of notional minimum temperature (T_{min}) for growth of *L. monocytogenes* Scott A and L5 were estimated from the models which are 0.88°C and 0.60°C respectively (Table 4.5). The growth rates decreased steadily with decrease in temperatures which were successfully described by the fitted models (Figs. 4.3a and 4.4a). In most cultures tested, throughout the temperature range including at the extreme temperatures, the final optical density (cell yield) appeared to be the same (Fig. 4.5). That is, a final transmittance of 4%T (≈1.4 OD) or less was observed in all cultures.
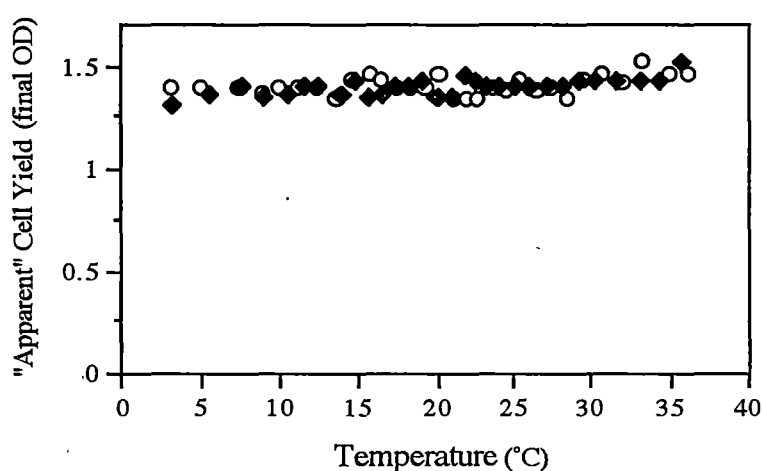
Figure 4.5 Effect of incubation temperature on "apparent" cell yield of *L. monocytogenes* Scott A (♦) and L5 (O) grown in TSB-YE, pH~7.3 and a_w of 0.995.

4.3.2 WATER ACTIVITY-pH-LACTIC ACID RESPONSE

The steady decrease in growth rate toward a lower limiting a_w was observed in both *L. monocytogenes* Scott A and L5 (Figs. 4.3b and 4.4b respectively). Similar estimates of the notional minimum water activity for growth (a_{wmin}), i.e. 0.923, and 0.925 for Scott A and L5 respectively, were obtained from the models (Table 4.5).

For each set of a_w-pH tests the pH_I was adjusted to 5.40, 5.75, and 6.10 as closely as possible. However, an inevitable variation of pH_I of the order of 0.14-0.18 pH unit was found, which slightly affected the growth rate. The non-smooth curves fitted shown in Figs. 4.3b and 4.4b resulted from a slight differences in other environmental conditions, especially the pH mentioned above. Nonetheless, model predictions, which take into account these variations, agree closely with the observed values.

L. monocytogenes grew over the range of water activity from 0.929 to 0.997 in the broths adjusted to three different pH_I without lactic acid. In broth cultures containing 50 mM lactic acid and at $pH_I \approx 5.4$, *L. monocytogenes* growth was prevented at $a_w \sim 0.94$ (Figs. 4.3b and 4.4b). The effect of decreasing pH on the growth rate of *L. monocytogenes* was also demonstrated. The addition of 50 mM lactic acid, nevertheless, contributed little additional effect on growth rate of *L. monocytogenes* at pH ≈ 5.7 and ≈ 6.1 as evident in Figs. 4.6a,b. However, at pH ≈ 5.4 differences in growth rate in broth cultures with and without lactic acid were observed (Fig. 4.6c).

The %reduction in growth rate as a result of the decrease in pH and a_w shown in Figs. 4.3b and 4.4b, was calculated for each a_w value and is presented in Fig. 4.7. When the pH was decreased from ≈ 6.1 to ≈ 5.7, a consistent proportional reduction in growth rate was observed in the broth cultures without lactic acid (Fig. 4.7a). In the presence of lactic acid, a larger proportional change was found at a_w close to a_{wmin}. When the pH was reduced from ≈ 5.7 to ≈ 5.4, the proportional reduction in growth rate increased progressively with the lowering of a_w (Fig. 4.7b). This effect was more pronounced in the broth with lactic acid. Interestingly, these changes were found to be similar to the observed cell yield changes at pH ≈ 5.7 and ≈ 5.4 (Figs. 4.8b,c) as described below.

In most cases at $pH_I \approx 6.1$, with and without lactic acid, the "apparent" yield was found to be the same for all levels of a_w tested (Fig. 4.8a). The change in "apparent" yield as a function of a_w-pH-lactic acid (Figs. 4.8b,c) displays similar trends when compared to the corrected yields (Figs. 4.9a,b). At $pH_I \approx 5.7$ without lactic acid, only the cultures at the extreme a_w (0.93) exhibited a reduced yield, with the addition of 50 mM lactic acid reduced yields were observed at higher a_w (0.95). The greatest effect on growth rate and cell yield was observed at pH ≈ 5.4, particularly when the broths contained lactic acid.

a) Medium pH ≈6.1
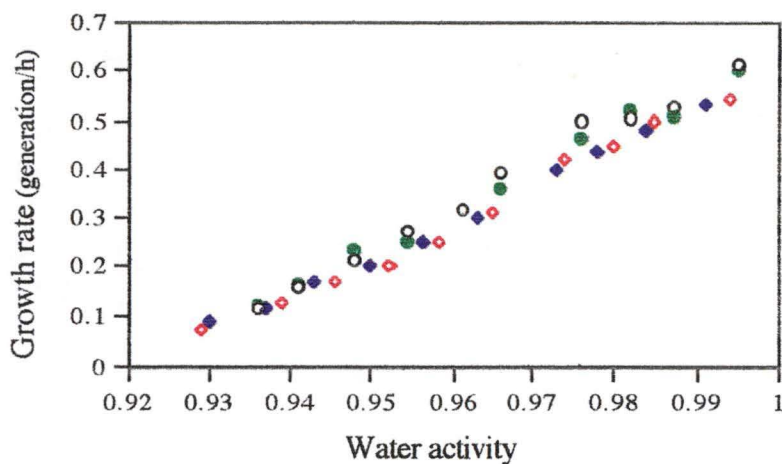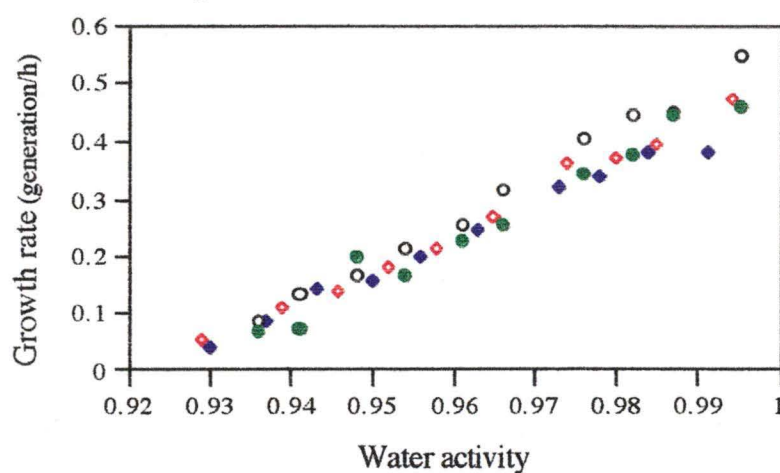
b) Medium pH ≈5.7

c) Medium pH ≈5.4
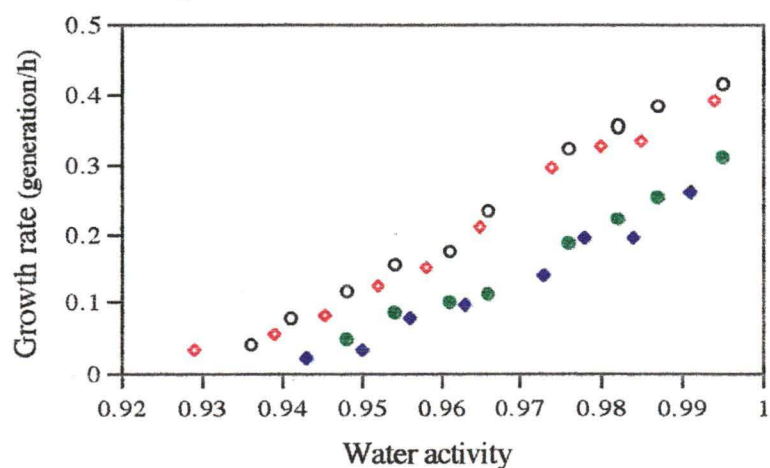
<u>Figure 4.6</u> Growth rate of *L. monocytogenes* as a function of water activity (NaCl as humectant), lactic acid, and pH; a) pH ≈6.1, b) pH ≈5.7, and c) pH ≈5.4. Strain Scott A; growth in the absence (◇), and presence (◆) of 50 mM lactic acid. Strain L5; growth in the absence (○) and presence (●) of 50 mM lactic acid.
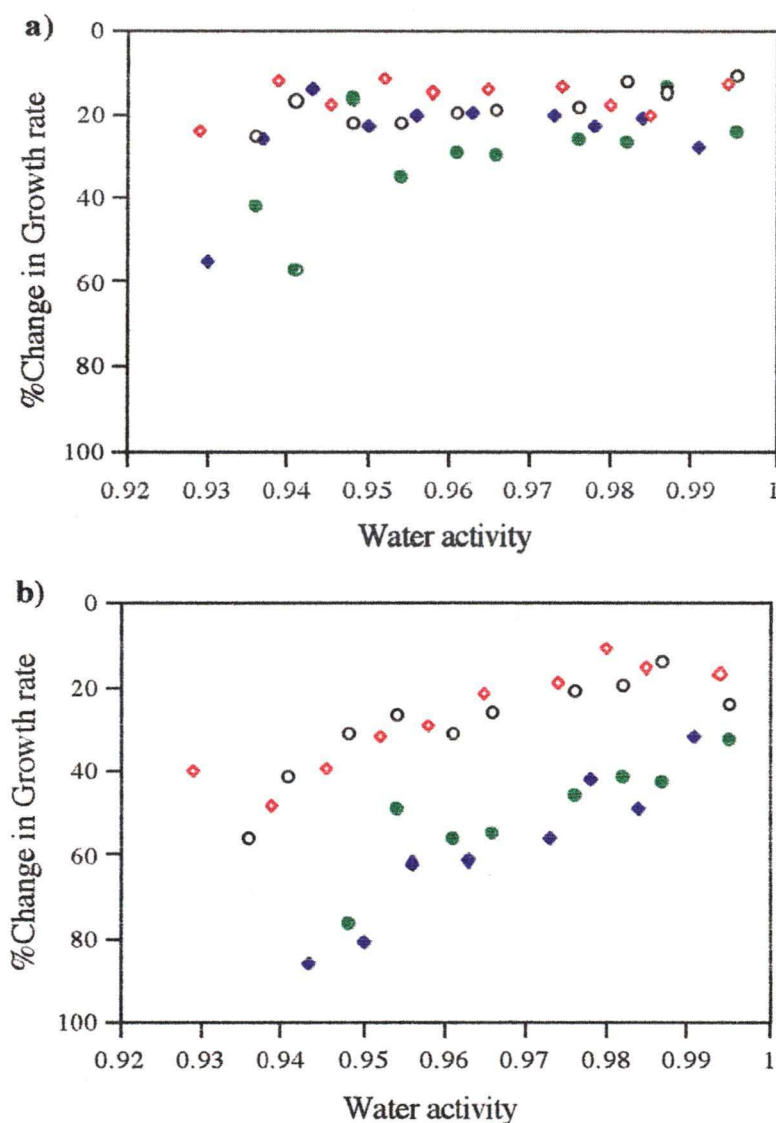
Figure 4.7 %change in growth rate of *L. monocytogenes* as a function of lowering pH and water activity (NaCl as humectant), and addition of 50 mM lactic acid; a) pH change from ≈6.1 to ≈5.7, and b) pH change from ≈5.7 to ≈5.4. Strain Scott A; growth in the absence (◇), and presence (◆) of 50 mM lactic acid. Strain L5; growth in the absence (○) and presence (●) of 50 mM lactic acid.

a) Medium pH ~6.1

b) Medium pH ≈5.7

c) Medium pH ≈5.4
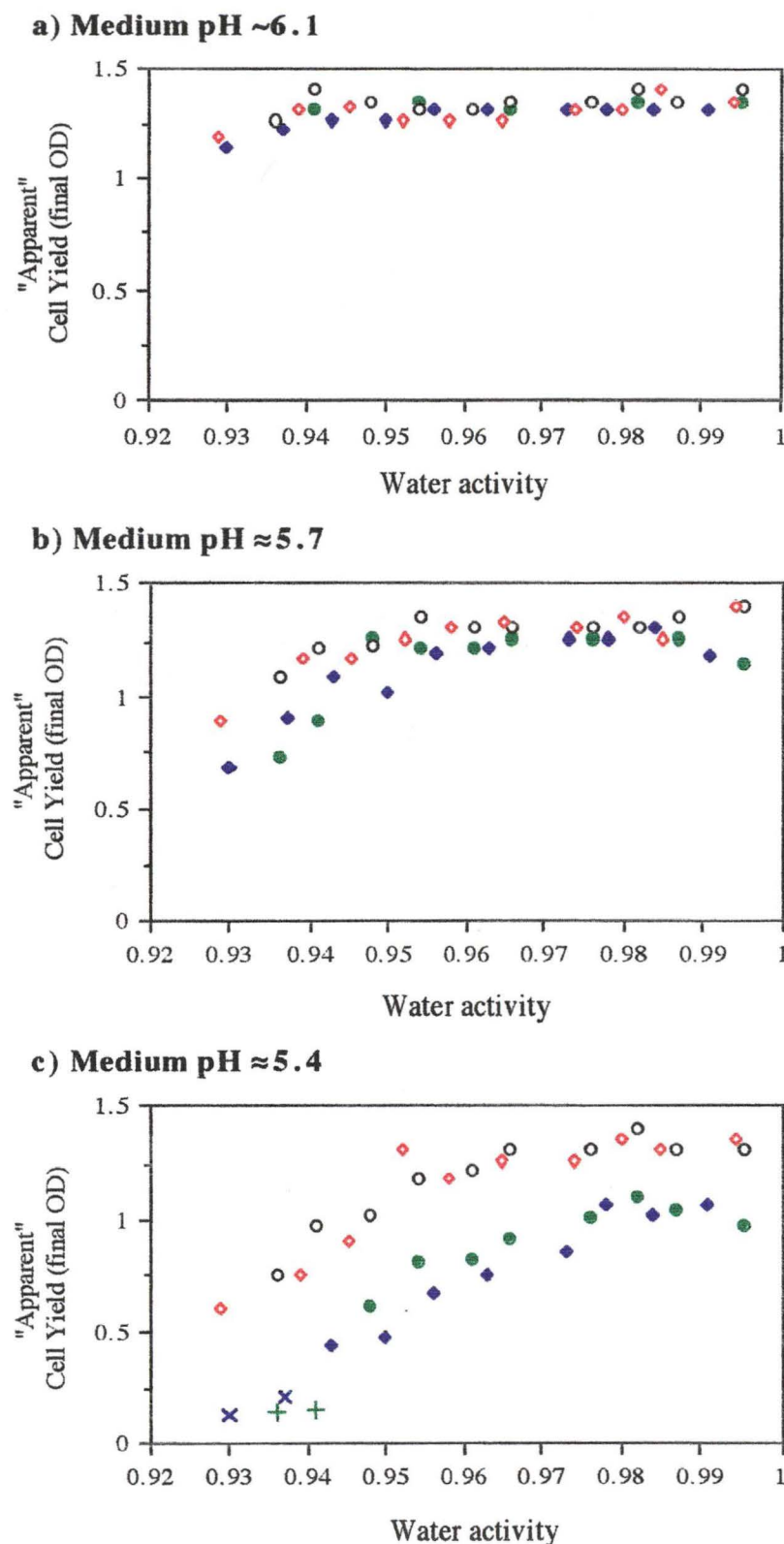
Figure 4.8 "Apparent" cell yield of *L. monocytogenes* as a function of water activity (NaCl as humectant), lactic acid, and pH; a) pH ≈6.1, b) pH ≈5.7, and c) pH ≈5.4. Strain Scott A; growth in the absence of lactic acid (◇), and growth (◆) and no growth (×) in the presence of 50 mM lactic acid. Strain L5; growth in the absence of lactic acid (O), and growth (●) and no growth (+) in the presence of 50 mM lactic acid.

a)

b)
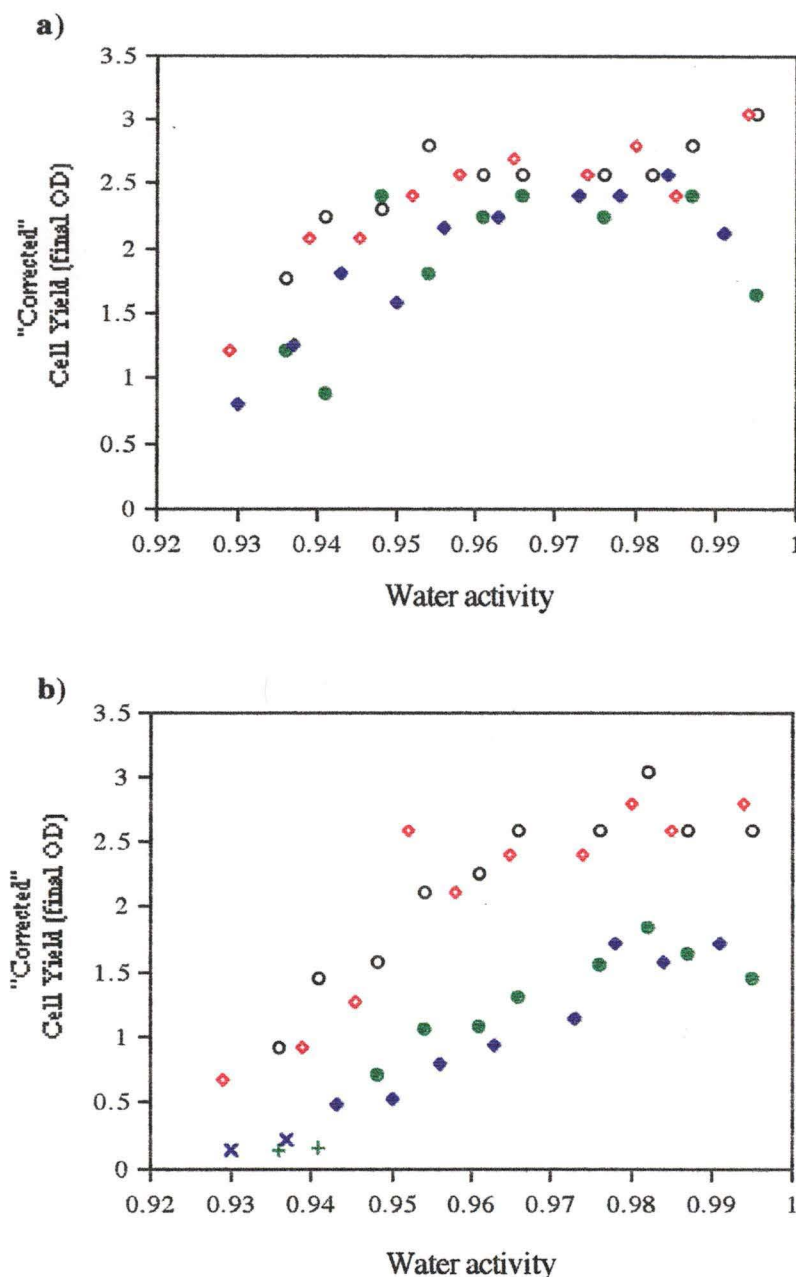
Figure 4.9 The observed cell yield of *L. monocytogenes* was "corrected" for the non-linearity of the OD-concentration relationship (see Appendix A, A.2.3), and plotted against water activity (NaCl as humectant), demonstrating the influence of lactic acid, and pH; a) pH ≈5.7, and b) pH ≈5.4. Strain Scott A; growth in the absence of lactic acid (◇), and growth (◆) and no growth (×) in the presence of 50 mM lactic acid. Strain L5; growth in the absence of lactic acid (O), and growth (●) and no growth (+) in the presence of 50 mM lactic acid. These figures are comparable with Figs. 4.8b,c.

4.3.3 PH RESPONSE

An example of pH change as a function of *L. monocytogenes* growth in TSB-YE is shown in Fig. 4.10. A decrease of ~0.7 to 1 pH unit in the less stressful pH (~6-7.7) cultures was normally observed at the end of incubation (final pH or pH$_f$). This response was typical of all those cultures in which the pH was monitored as growth proceeded, regardless of type or amount of acidulant. An exception, however, was found with the more constrained cultures at pH close to pH$_{min}$ where very slow growth was observed and change in pH was less than in the less constrained cultures. The pH at the midpoint of exponential growth, designated pH$_{mid}$, was estimated from the growth curve (Fig. 4.10).

A plot of pH$_I$ against pH$_{mid}$ for strain Scott A (Fig. 4.11) shows only a slight change in pH at the time the fastest growth of the culture occurred and a linear relationship was observed. A similar response was also found for *L. monocytogenes* L5 (not shown). Thus, pH$_I$ which is the pH which bacteria first encounter, may be used as the modelled variable especially when related to the pH recorded in food monitoring systems. A plot of pH$_I$ versus pH$_f$ (Fig. 4.12) shows the change in pH over the time observed, a constant pH$_f$ was found when pH$_I$ was close to the limit to growth which was not dependent upon the initial pH of the broth but the amount of lactic acid present in the broth.
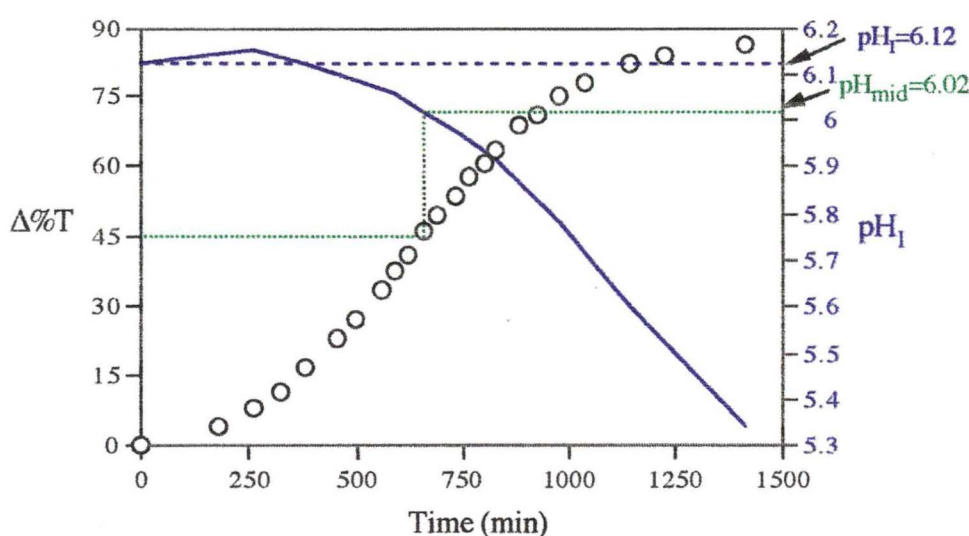
Figure 4.10 Change in medium pH (—) as a function of time and change in %T (O) of *L. monocytogenes* Scott A grown at 20°C in TSB-YE with 50 mM lactic acid. The pH at inoculation (pH$_i$) was 6.12. pH$_{mid}$ is estimated to be the midpoint of the range of pH at which the fastest growth rate was observed for each culture which is at 45 Δ%T for this culture.
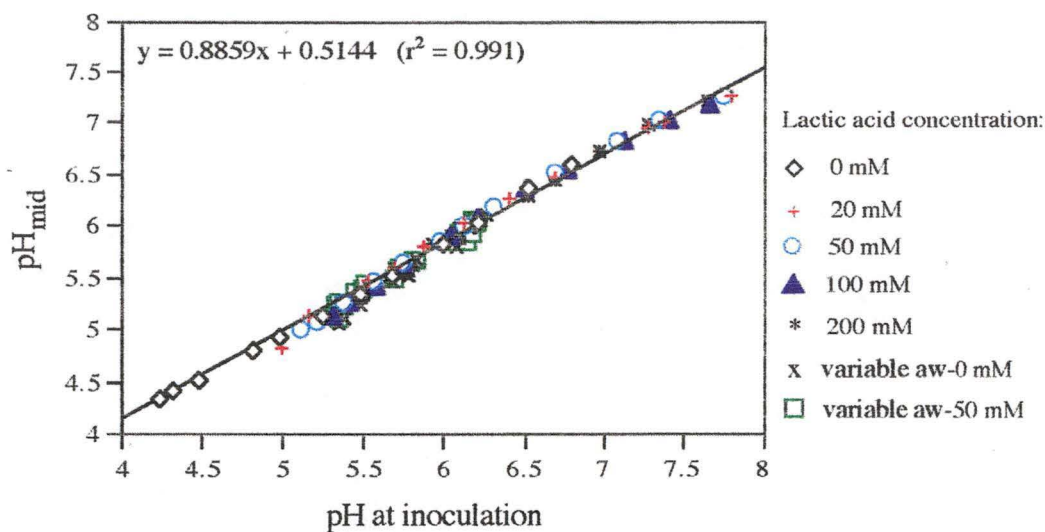
Figure 4.11 Relationship between pH at inoculation and pH_{mid} from *L. monocytogenes* Scott A growth data. pH_{mid} for each growth curve was estimated from the mid point of exponential phase (Fig. 4.10) as described in the text. The line shown was fitted by linear regression; the equation and regression coefficient value are given in the graph.
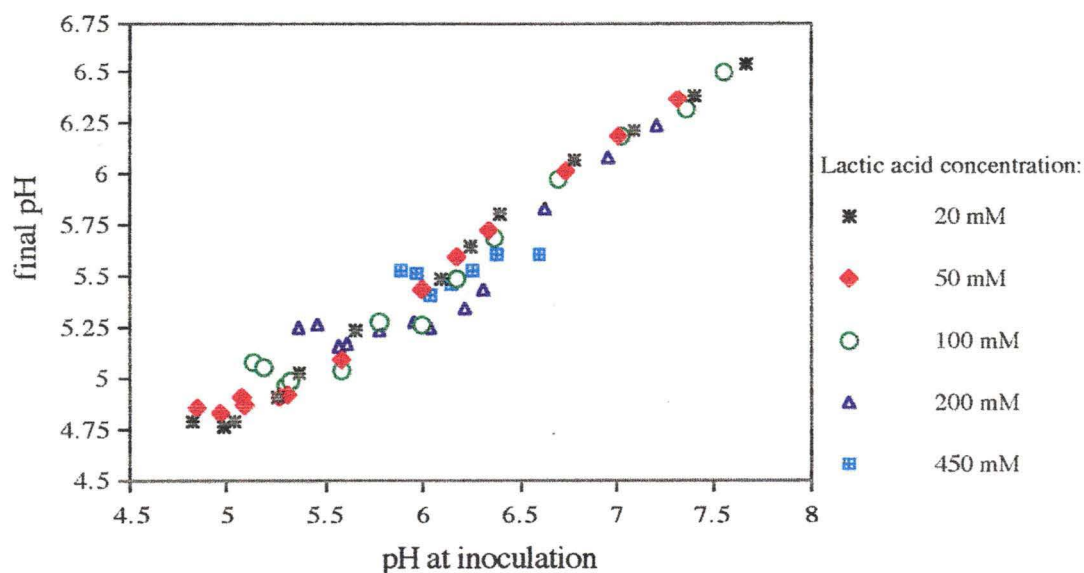
Figure 4.12 Change in medium pH as a result of growth of *L. monocytogenes* L5 at ~20°C in TSB-YE (5% NaCl) with different levels of lactic acid.

The growth rate data for *L. monocytogenes* Scott A and L5 over a range of pH with various levels of lactic acid were plotted directly against the fitted growth rate models and presented in Figs. 4.3c and 4.4c respectively. The effect of pH on the growth of *L. monocytogenes* was more pronounced than that observed for temperature and a_w, discussed in the previous sections. The trends of the effect of pH_I on growth of *L. monocytogenes* are clearly described by the models. A consistent pattern of a smaller effect on growth rate when pH_I was closer to the optimum pH_I, and an increasingly rapid rate of decline in growth rate as pH_I approaches pH_{min}, was observed.

The lowest pH_I at which *L. monocytogenes* was able to initiate growth was observed in media acidified by HCl only, and was 4.23 for Scott A and 4.25 for L5 (Fig. 4.2). These values are very close to the estimated pH_{min} of 4.228 and 4.248 respectively (Eqns. 4.17a and 4.18a). A similar effect was also found in fitting models 4.17b and 4.18b, where the estimates of pH_{min} were close to the lowest pH values in the growth rate data sets for 20 mM lactic acid (Tables 4.5 and 4.6).

The increase in minimum pH_I, and optimum pH_I for the growth of *L. monocytogenes* when lactic acid was the acidulant, was observed to be dependent upon lactic acid concentration. Conversely, when the same pH_I was considered, the growth rate decreased as a consequence of increasing lactic acid concentration (Figs. 4.3c and 4.4c). For example, neither strain of *L. monocytogenes* initiated growth at pH_I 5.46 when the broth contained 200 mM lactic acid. At this level of lactic acid, there was little effect on the growth rate when the pH_I range above 6.2. These pH values are summarised in Table 4.6.

Table 4.6 Summary of the observed pH range with little effect on growth rate and cell yield of *L. monocytogenes* at ~20°C. The minimum (min.) pH for growth and the corresponding [H⁺], and [UD] in relation to lactic acid concentration are also presented. Note that the water activity of the media was ~0.965, except for 0 mM lactic acid experiments in which the a_w was 0.995.

Lactic acid concentration (mM)	pH below which growth rate declines rapidly	pH below which yield fell below 1.3 OD (5%T)	Scott A min. pH	Scott A [H⁺] μM	Scott A [UD] mM	L5 min. pH	L5 [H⁺] μM	L5 [UD] mM
0	5.3	5	4.23	58.9	-	4.25	56.2	-
20	5.5	5.7	4.99	10.2	1.4	4.98	10.5	1.4
50	5.9	6	5.12	7.6	2.6	4.97	10.7	3.6
100	6.0	6.2	5.31	4.9	3.4	5.19	6.5	4.5
200	6.2	6.7	5.59	2.6	3.37	5.57	2.7	3.8
450	>6.6	>6.6	-	-	-	5.88	1.3	4.3

Plots of growth rate of *L. monocytogenes* Scott A as a function of [H⁺], and concentration of undissociated lactic acid are shown in Fig. 4.13. There appears to be a linear relationship between [H⁺], and undissociated lactic acid concentration, and the growth rate when lactic acid was the acidulant. Lines fitted by linear regression show very high regression coefficient values. However, a non-linear relationship was observed when HCl was the acidulant (Fig. 4.13a). Note that the difference in water activity levels between the broths with and without lactic acid, i.e. 0.965 and 0.995 respectively, shown in Fig. 4.13a may also contribute to the large difference in the [H⁺] required for the inhibition effect.

The growth rate inhibition evident in Fig. 4.13 is expected to be due to both [H⁺] and undissociated lactic acid ([UD]). Therefore, the growth rates were corrected to present only the effect due to each of these components (see section 4.2.2.5). Plots of the corrected growth rates for the data with lactic acid and the corrected models prediction versus [H⁺] and [UD] show a simple linear relationship for both strains Scott A and L5 (Figs. 4.14 and 4.15). When lactic acid was the acidulant, the extrapolated values of [H⁺], and [UD] causing complete growth inhibition were found to be 10.8 μM, and 3.8 mM respectively for strain Scott A, and 11.5 μM, and 4.6 mM respectively for strain L5. The data from the 450 mM lactic acid experiments, however, were much more variable and did not conform to those trends (Figs. 4.15a,b). When HCl was the acidulant, the maximum [H⁺] at which *L. monocytogenes* could initiate growth was found to be 58.9 and 56.2 μM, calculated from the minimum growth pH of 4.23 and 4.25 for Scott A and L5 respectively.

The influence of acidity on cell yield of *L. monocytogenes* is presented in Figs. 4.16a,b. pH_I above 5 appeared to be an optimum pH range for *L. monocytogenes*. At pH_I <5 cell yield declined rapidly until no growth was observed (Fig. 4.16a). A linear decline in cell yield as a function of hydrogen ion concentration is revealed in Fig. 4.16b.

The effect of pH on cell yield of *L. monocytogenes* in the presence of lactic acid is shown in Fig. 4.17a. A consistent trend of constant cell yield over the optimum pH range, but declining markedly with the decreasing pH was observed. The similarity of the cell yield and growth rate response as a function of pH and lactic acid concentration were noted (Figs. 4.3c, 4.4c and 4.17a). The approximate pH at which cell yield started to decline below 1.3 OD (5%T) was presented in Table 4.6. Plots of cell yield against each components of lactic acid (Figs. 4.17b-d) suggest the hydrogen ion and in particular, undissociated lactic acid played an important role in the inactivation effect, whereas the concentration of dissociated lactic acid shows no clear relationship (Fig. 4.17d).

a)

b)
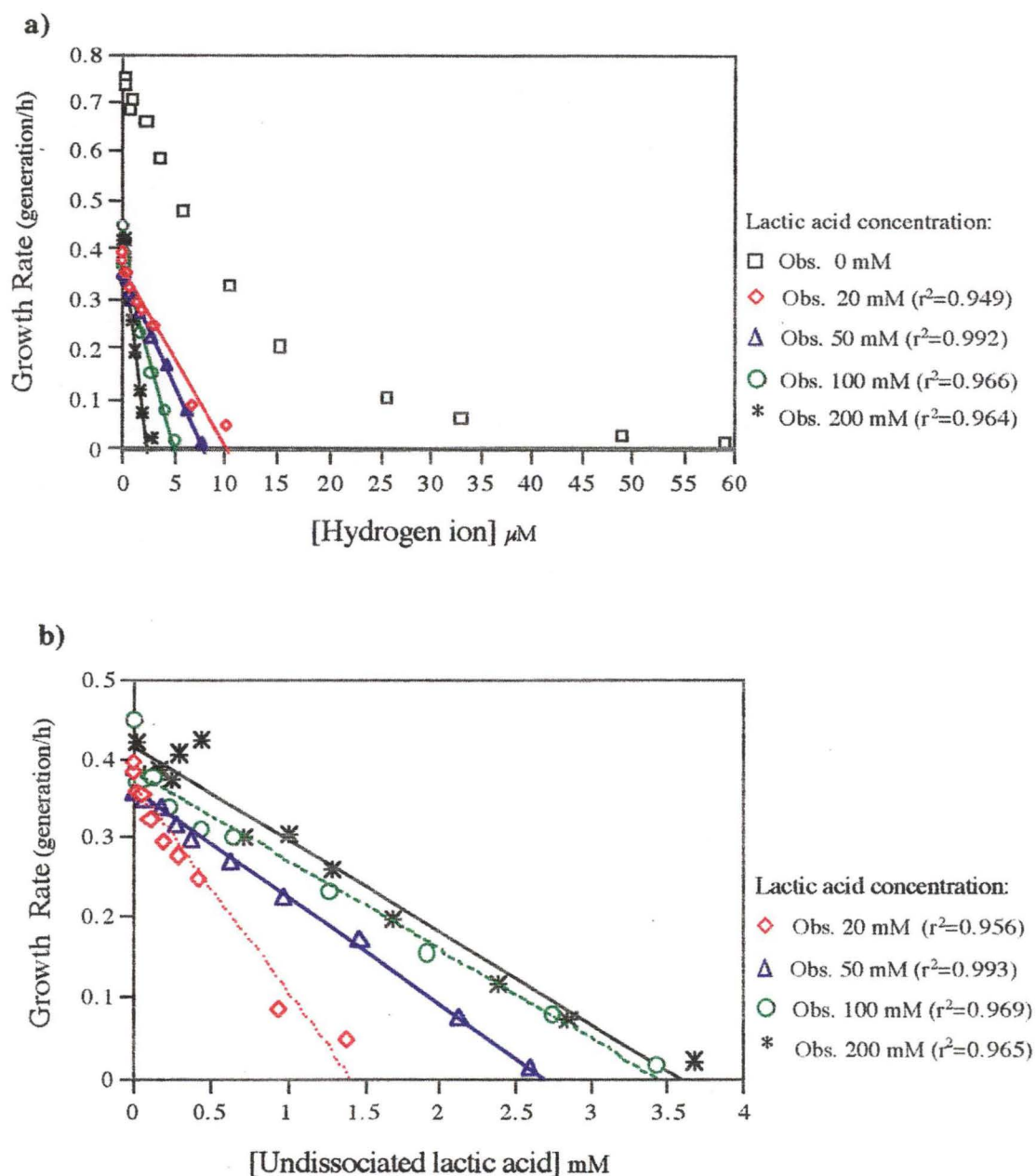
Figure 4.13 Relationship of growth rate of *L. monocytogenes* Scott A to concentration of a) hydrogen ion and b) undissociated lactic acid. The lines fitted were obtained by linear regression (Cricket Graph[3]). The regression coefficient (r^2) for each lines is given in the brackets. Note that the water activities of the broths were different; 0.995 and ~0.96 in the absence and presence of lactic acid respectively.

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[3] CA-Cricket Graph III 1.5.2. One Computer Associates Plaza Islandia, NY 11788-2000 USA.
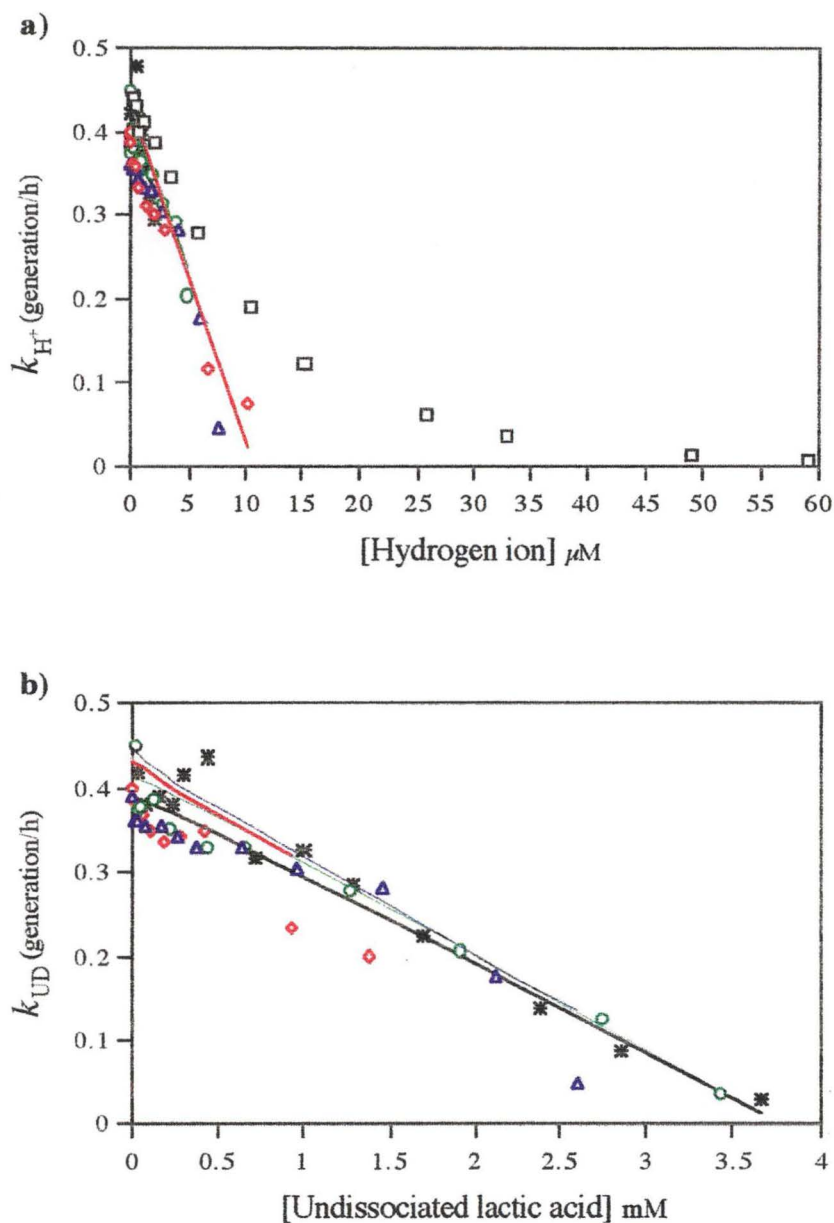
Figure 4.14 Growth rate of *L. monocytogenes* Scott A standardised for lactic acid effect (k_{H^+}) and H$^+$ effect (k_{UD}) using Eqns. 4.12 and 4.13 respectively, and plotted against the concentration of a) hydrogen ion and b) undissociated lactic acid respectively. The fitted lines were plotted from the standardised model predictions (Eqns. 4.12 and 4.13) for total lactic acid (mM); 20 ($\diamond$), 50 ($\triangle$), 100 ($\bigcirc$), and 200 ($*$). The growth rate data in the absence of lactic acid ($\square$) was standardised using Eqn. 4.16 for the water activity of 0.965, which differed from that of the other experiments ($a_w = 0.995$).
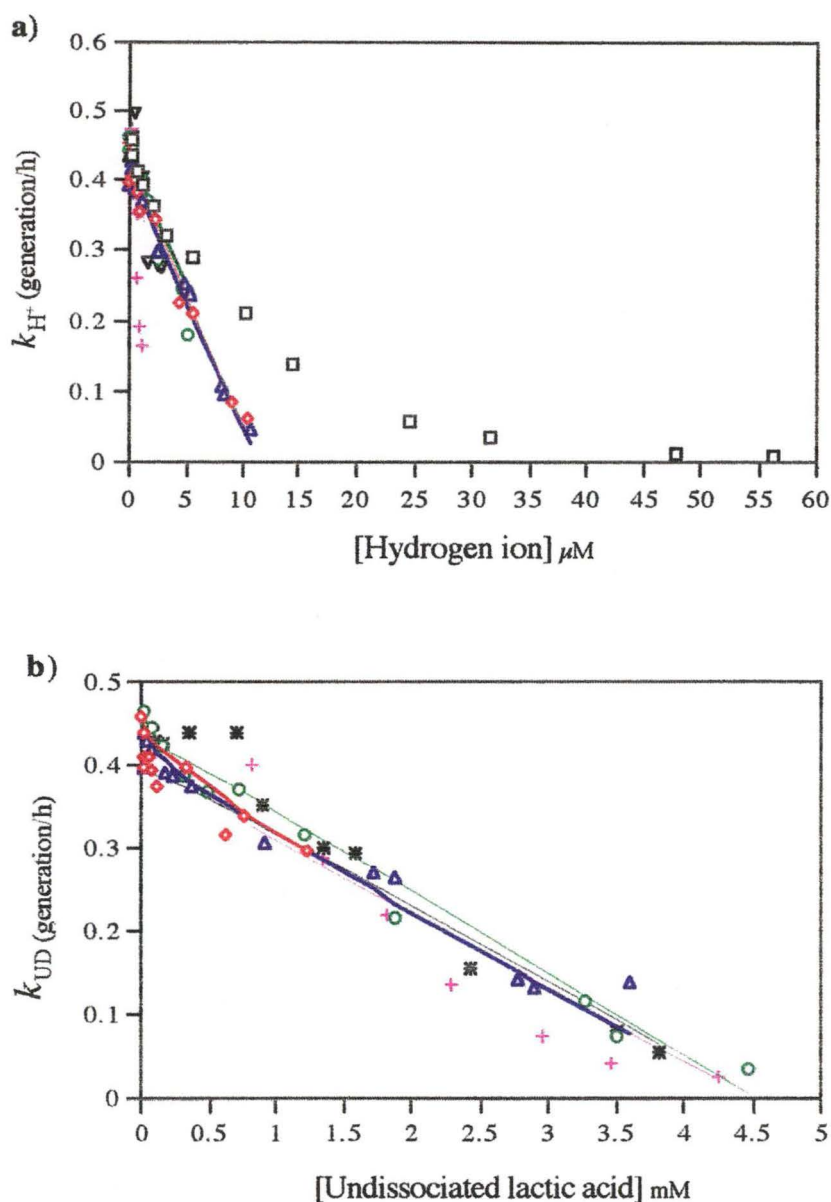
Figure 4.15 Growth rates of *L. monocytogenes* L5 obtained from different pH and levels of lactic acid combinations were standardised for lactic acid effect (k_{H+}) and H$^+$ effect (k_{UD}) using Eqns. 4.14 and 4.15 respectively, and plotted against the concentration of a) hydrogen ion and b) undissociated lactic acid respectively. The fitted lines were plotted from the standardised model predictions (Eqns. 4.14 and 4.15) for total lactic acid (mM); 20 (◊), 50 (Δ), 100 (○), 200 (∗), and 450 (+). The growth rate data in the absence of lactic acid (□) was standardised using Eqn. 4.16 for the water activity of 0.965, which differed from that of the other experiments (a_w = 0.995).
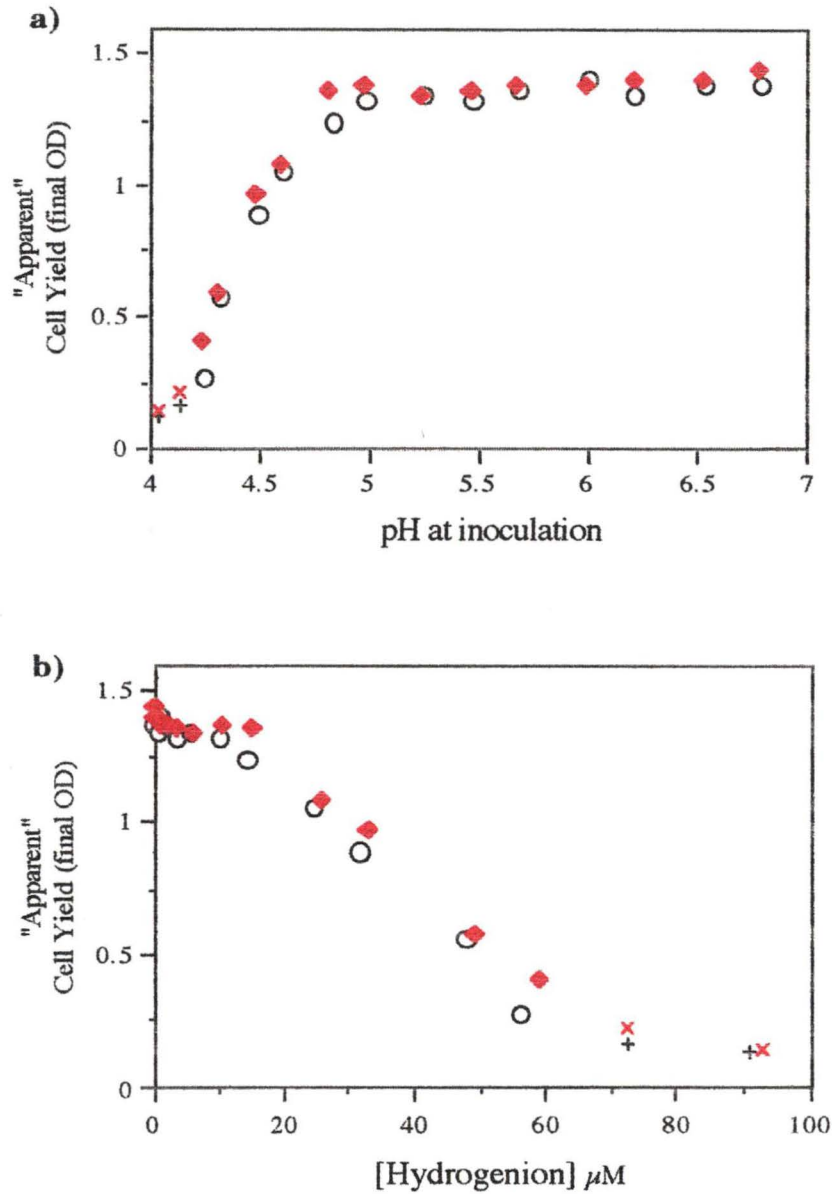
Figure 4.16 Effect of acidity (HCl as acidulant on cell yield of *L. monocytogenes* Scott A (♦) and L5 (O) as a function of a) pH, and b) hydrogen ion concentration. Cross signs (×, +) indicate that no growth of strains Scott A and L5 respectively were observed under these experimental conditions.

Figure 4.17 (facing page). Effect of lactic acid concentration (mM); 20 (□), 50 (○), 100 (◆), 200 (✳), and 450 (◇) on cell yield of *L. monocytogenes* L5 as a function of a) pH, b) hydrogen ion concentration, c) undissociated lactic acid concentration, and d) dissociated lactic acid concentration. Cross signs (×) with different colours refer to the observed of no growth at each concentration of lactic acid. Note that Figs. 4.16(b-d) demonstrate only relationship between cell yield response and single active component of lactic acid. The combined effect from other components must also be taken into account.
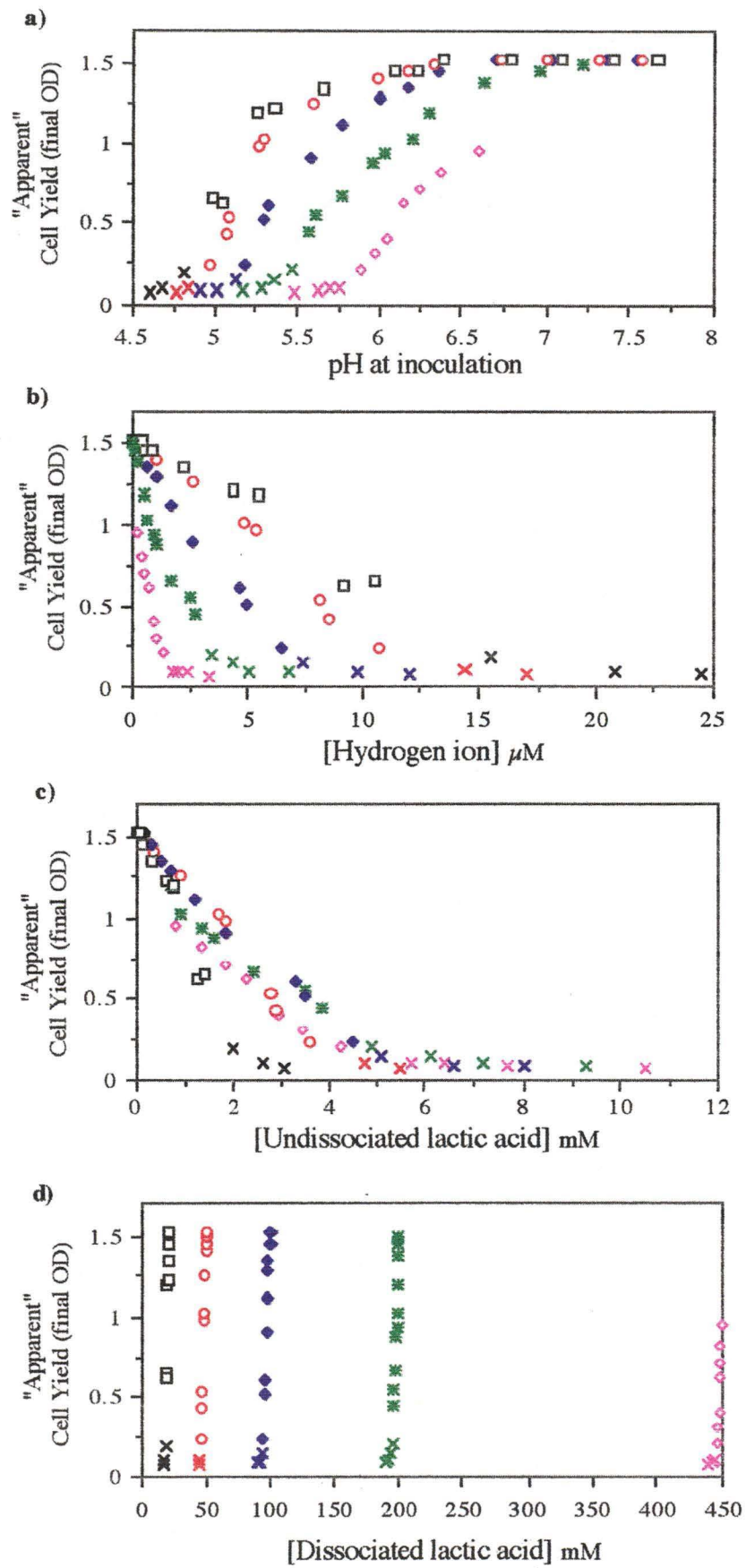
4.4 DISCUSSION

Mathematical models to predict the growth rate of *L. monocytogenes* Scott A and L5 when temperature, water activity, pH, and lactic acid are the controlling factors were developed in this chapter. The models are square-root type models (McMeekin *et al.*, 1993) with the incorporation of pH and organic acid terms recently introduced by Presser *et al.* (1997a). The square-root models describing the effects of temperature and water activity terms are well established in the literatures (Ratkowsky *et al.*, 1982; McMeekin *et al.*, 1987; Ross and McMeekin, 1991; Ross, 1993). The novel pH and organic acid terms were also reported to accurately describe the shape of the growth rate response of *E. coli*, i.e. steeply rising from pH_{min} to an asymptote and exhibiting a plateau at a range of pH near optimum (Presser *et al.*, 1997a). This pH term has been compared, and found to perform better, than those of previous square-root models (Adams *et al.*, 1991; Wijtzes *et al.*, 1993) which were derived simply by substituting pH terms to the prototype form of temperature (Ratkowsky *et al.*, 1982) or water activity (McMeekin *et al.*, 1987) terms in the square-root model.

Other types of model such as polynomial models having pH and organic acid as one of the controlling factors have also been developed for *L. monocytogenes* (McClure *et al.*, 1991; Buchanan and Golden, 1995; Buchanan *et al.*, 1997). Although all models for pH are empirical, polynomial models generally employ a high number of parameters and are too complex to allow determination of the response shape from the terms given by the equation. In addition, the use of high order polynomials tends to generate more errors of the measured values (Baranyi and Roberts, 1995). Lower accuracy of the model prediction when compared to the analogous square-root model prediction was also pointed out by Delignette-Muller *et al.* (1995). Considering all these aspects, square-root models enable an intuitive understanding of the basis of the mathematical function describing the response to each factor. Further, they may be used to clarify the cause of inhibition (Presser *et al.*, 1997a) as demonstrated by the separate influence of each component of lactic acid in this chapter (section 4.2.2.5).

A good description of growth responses by the square-root type models for *L. mono-cytogenes* Scott A and L5 (Eqns. 4.17b and 4.18b respectively) demonstrated by the simultaneous plotting of observed and predicted response indicate the appropriateness of the models (see Figs. 4.3 and 4.4 respectively). The parameter estimates derived from the models for the growth rate response of *L. monocytogenes* to temperature, water activity, and pH were compared to the literature and are separately discussed in the subsequent sections (4.4.2.1-4.4.2.3). The cell yield response of *L. monocytogenes* for all controlling factors tested in this chapter are discussed later (section 4.4.2.4).

The heightened awareness of the practical limitations of the models generated have been discussed extensively (McMeekin *et al.*, 1993; Baranyi *et al.*, 1996). The model predictions should be generated only by interpolation, i.e. within the data range used to generate a model. This 'interpolation region' is described by Baranyi *et al.* (1996) as a 'minimum convex polyhedron' which encloses all the combinations tested. A conservative design which measures the full range of each variable at two or more values of each other variable is required to accomplish this purpose.

The methodology of minimum experimental design proposed by Ross (1993) was employed in this study for the development of growth models for the combined effects of temperature-water activity-pH-lactic acid for *L. monocytogenes* Scott A and L5. The full data sets cover a range of the 4 parameters (see Appendix G, Fig. G1). Considering the interpolation region, the models generated are, thus, subjected to the limitation of smaller variables space. Nonetheless, the plotting of predicted and observed responses demonstrate the appropriateness of models to be used for prediction within the data range used to generate the models.

4.4.1 TEMPERATURE RESPONSE

The growth response of *L. monocytogenes* Scott A and L5 over the sub-optimum temperatures (Figs. 4.3a and 4.4a) were consistent with the published reports (Duh and Schaffner, 1993; Bajard *et al.*, 1996). Under the conditions tested here, the fastest growth of *L. monocytogenes* Scott A and L5 were found to be 33.6 and 36.6 minutes at 35.8°C and 36.2°C respectively. The values are consistent with the report by Ross (1993) of 33.6 and 34.8 minutes at 35.6 °C and 37.2 °C for strains Scott A and Murray B respectively.

In laboratory media broth, *L. monocytogenes* was reported to exhibit growth at temperatures of -2°C and 0°C (Bajard *et al.*, 1996). A range of minimum temperature for growth from -0.4 to -0.1°C and 0.5°C was observed by Walker *et al.* (1990). Other reports of the minimum growth temperature for *L. monocytogenes* range from 0.5°C to 3.0°C (Junttila *et al.*, 1988), 1.84°C (Duh and Schaffner, 1993), >2°C (Gill *et al.*, 1997), and 3.3°C (Wilkins *et al.*, 1972) in various media broth.

The estimates of the notional minimum temperature (T_{min}) were reported to range from -2.55 to -1.75°C (Wijtzes *et al.*, 1993), -2.2 to -2.4°C (Grau and Vanderlinde, 1993), -1.16 to -0.16°C (Duh and Schaffner, 1993), and 1.2°C (Gill *et al.*, 1997).

In this study, the minimum temperature for growth of 3°C and the estimated T_{min} derived for *L. monocytogenes* Scott A and L5 (Tables 4.4 and 4.5) are higher than some of the

literature values cited above. The reason for the relatively high estimates is unknown. This discrepancy may affect the performance of the models when applied to independent data sets for *L. monocytogenes*, especially when the reported temperature is close to $3°C$ which is the observed lowest temperature. The inferior performance of a model when extrapolated beyond the range of data used for model generation is recognised (Ross, 1993; Baranyi *et al.*, 1996). An attempt to obtain additional growth data of *L. monocytogenes* at lower temperatures was made but resulted in a similar minimum temperature for growth of $3°C$ (J. Kettlewell, unpublished). A method of growing the inoculum at low temperature, used by Walker *et al.* (1990), may help lower this minimum growth temperature and consequently produce a lower value of T_{min}.

The other possible reason for the "high" estimate was recently suggested by Bajard *et al.* (1996). Those authors indicated an unexpected behaviour of *L. monocytogenes* that its growth at sub-optimum temperature did not obey the square-root model, i.e. that a straight line is obtained when the square-root of growth rate is plotted against sub-optimum temperature. The authors described a change in slope of the square-root plot caused by a faster growth of *L. monocytogenes* than expected at the temperature $\leq 15°C$. Thus, those authors proposed two straight lines to be fitted to the square-root plot which gave a lower T_{min} of $-5°C$ instead of $4°C$. The suitability of the square-root type model to predict growth responses of *L. monocytogenes* is also questioned by other researcher (T. Ross, pers. comm.). However, the good fit of the square-root models to the observed data evident in Figs. 4.3 and 4.4 indicates that the models is sufficient to describe the kinetic behaviour of *L. monocytogenes* to within the ranges tested of the respective environmental factors.

The models predictions, especially at the highest temperature tested in this study ($\sim 36°C$), were found to be improved when T_{max} term was included. Apart from this, a relatively similar performance of the models, with or without T_{max}, was observed. Therefore, the inconsistent estimates of T_{max} compared with literature reports ($\sim 46°C$) are not anticipated to affect the model performance when applied to foods in the interpolation region, i.e. temperature up to $36°C$.

4.4.2 WATER ACTIVITY RESPONSE

Several researchers (Tapia de Daza *et al.*, 1991; Nolan *et al.*, 1992) reported the value of minimum water activity (NaCl as the humectant) for growth of *L. monocytogenes* to be 0.92 or 0.91-0.93 by Farber *et al.* (1992). The estimates of the notional minimum water activity (a_{wmin}) was 0.912-0.916 (Wijtzes *et al.*, 1993), and 0.92-0.93 (Ross, 1993).

In this study, the minimum water activity for growth of *L. monocytogenes* Scott A and L5 were found to be 0.929 and 0.936 respectively (Figs. 4.3b and 4.4b) which were the lowest a_w used in the experiments. The a_{wmin} of 0.923-0.927 (Tables 4.4 and 4.5) obtained here are generally consistent with the above literature values.

Similar growth responses of *L. monocytogenes* to water activity were reported by Ross (1993). It should be noted that the growth rate data for the effect of water activity were used directly in the model generation and fitted without standardisation for the pH differences in each set of the experiments. Despite a slight variation in pH in each block of experiments, a proportional decrease in growth rate with the lowered pH in relation to lower water activity can be observed (Figs. 4.3b and 4.4b). The inclusion of 50 mM lactic acid to the broth cultures enhanced the growth inhibitory effect at pH ≈ 5.4 (Fig. 4.6c) but the effect could not be discerned at pH ≈ 5.7 and ≈ 6.1 (Figs. 4.6a,b). This is because at pH 5.7 and 6.1, there is very little [UD]. At pH 5.4, the [UD] becomes relatively large in comparison to U_{min} and produces a measurable growth rate reduction (Table 4.7).

Table 4.7 Comparison of the amount of undissociated lactic acid in the broth cultures at different pH with the presence of 50 mM lactic acid.

pH of broth cultures with 50 mM lactic acid	undissociated lactic acid (mM)
5.4	1.4
5.7	0.7
6.1	0.3

The increase in the proportional reduction in growth rate, especially at water activity levels approaching the limit (Fig. 4.7), suggests there is an interaction, in a synergistic manner (Gould and Jones, 1989), between the low pH and osmotic stress. This finding is contrary to the report of Cole *et al.* (1990) who suggested, on the basis of the form of the polynomial model used in that study, the effect between salt concentration and [H⁺] was completely additive and not synergistic or interactive. However, a synergistic effect may be found in the responses they reported, e.g. at [H⁺] of 0.1 μmol/L growth occurred at all levels of 0 to 10%NaCl, but became slower to be detected with the increasing [H⁺], and was completely inhibited at 10%NaCl when [H⁺] ≥ 7.3 μmol/L (Fig. 3 in Cole *et al.*, 1990).

The synergistic effect between water activity and [H⁺] in growth rate reduction reported here was more pronounced when lactic acid was the acidulant (Fig. 4.7). A similar explanation may apply to this observation in that the undissociated lactic acid has a

greater cytoplasmic pH lowering effect which results in a more potent stress on bacterial cells compared to [H⁺] only at the same pH. The lower the pH, the higher the [UD] (Table 4.7) and the stronger the inhibition effect.

A fundamental "theme" of the square-root type models that there is no interaction between each controlling factor, i.e. each term is independent although being multiplied by each other. The proportional change reported above was, thus, anticipated to be consistent throughout the range of water activity, i.e. 12% reduction in growth rate when pH was decreased from ≈6.1 to ≈5.7 and 22% reduction from ≈5.7 to ≈5.4. There are several explanations possible for these observations including; 1) the square-root model may, assuming that the finding is correct, not describe the actual response of the micro-organism to these factors and may require further development, 2) the growth rate obtained from the Gompertz function fitted to turbidimetric data, especially at the low water activities may be subject to the limitation of the turbidity measuring devices (see section 4.1.1.1) and display slower growth rate than the actual maximum specific growth rate (Dalgaard *et al.*, 1994). This is because the culture's growth rate is being measured toward the end of exponential growth. This systematic error could result in the enlargement of the change in growth rate as reported above. Further study, using a more sensitive method such as viable count may help to clarify whether there is an artefact in the turbidimetric method.

4.4.3 PH RESPONSE

The minimum pH for the growth of *L. monocytogenes* was reported to be 4.3 by Farber *et al.* (1989b), or 4.39 by George *et al.* (1988), for HCl as the acidulant. In this study, *L. monocytogenes* Scott A and L5 was found to be able to grow at levels as low as pH 4.23 and 4.25 respectively. This lower pH limit for growth, particularly for *L. mono-cytogenes* Scott A, was determined from an optical density experiment but was corroborated by an identical experiment which compared the optical density measurement with the viable count and bacterial cell viability using a fluorescent anionic membrane potential probe as the indicator (Jepras *et al.*, 1995) assessed by direct microscopic visualisation (J. Kettlewell, unpublished),

The estimates of notional minimum pH (pH_{min}) of 4.228 (Eqn. 4.17a) and 4.248 (Eqn. 4.18a) for *L. monocytogenes* Scott A and L5 respectively, which are only 0.002 pH units lower than the observed minimum growth pH, demonstrate the effect of data range used in model generation. The pH_{min} estimated from Eqns. 4.17b and 4.18b without the low pH data sets gave a higher pH value of 4.97 and 4.94 respectively, and is thus likely to be affected by the range of data used in the fitting process. Similar findings were also

reported by Ross (1993). The pH$_{min}$ derived in this chapter are generally higher than the report of Wijtzes (1996). That author generated various estimates of pH$_{min}$ of 3.84 from pH data between 4.6-7.4, and 4.15 and 4.03 from pH data in the range pH≤6.3, and ≤6.7 respectively, which suggests that the model he used is inadequate to fully describe the pH response.

The pH response of *L. monocytogenes*, in the presence of lactic acid in this study can be described as a plateau of unaffected growth rate over a range of optimum pH and a continuous decline toward the pH$_{min}$ (Figs. 4.3c and 4.4c). This finding is similar to previous reports of *L. monocytogenes* behaviour (Ross, 1993), and also other micro-organisms such as *Vibrio parahaemolyticus* (Miles, 1994) and *E. coli* (Presser *et al.*, 1997a). However, a sigmoid pH response of *L. monocytogenes* was found in this chapter when HCl was the sole acidulant (Fig. 4.18). This is in contrast to Wijtzes (1996) who described the pH response of *Lactobacillus curvatus* as a symmetrical parabolic curve over an entire range of growth pH. That author also used an expanded square-root model for entire temperature range (Ratkowsky *et al.*, 1983) to model pH response of *L. monocytogenes* for the pH range from 4.6 to 7.4 as discussed above. A
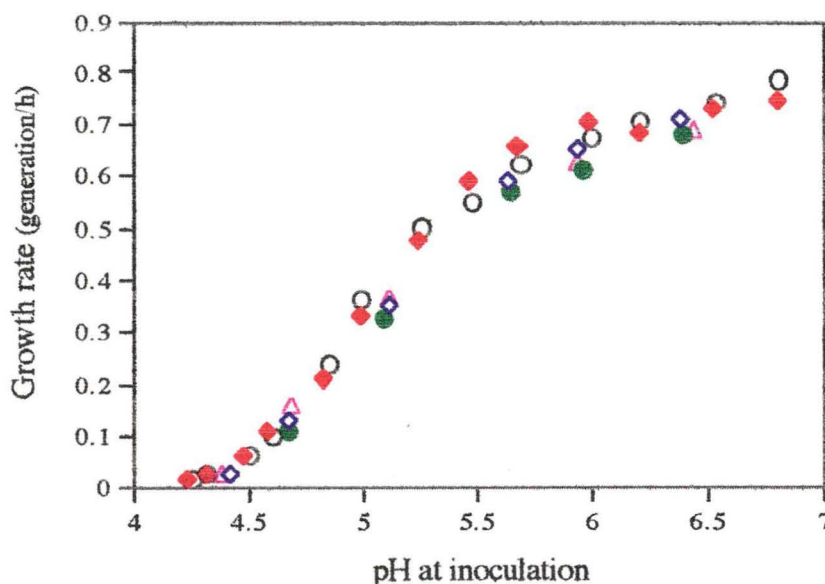
Figure 4.18 A sigmoid growth response to pH (HCl as acidulant) of various strains of *L. monocytogenes*. The data were obtained from two independent experiments i) this study; Scott A (♦) and L5 (O), and ii) Experiment; Scott A (◇), L5 (●) and MC23 (△) (D. Miles, unpublished) grown in TSB-YE at ~20°C, a$_w$ of 0.995.

similar, sigmoid, pH-growth rate response, with HCl as the acidulant, is evident in the data of Petran and Zottola (1989) (not shown). In addition, an independent experiment in this laboratory using *L. monocytogenes* Scott A, and L5 and strain MC23 also exhibited a similar sigmoid response (Fig. 4.18) (D. Miles, unpublished). Thus, this sigmoid response of *L. monocytogenes* to pH in the absence of lactic acid requires further investigation in order to understand the actual underlying response and to be able to develop a kinetic model which accurately describes all pH responses.

An inherent problem of modelling pH is that it changes over the period of bacterial growth as shown in Fig. 4.10. The ability of the organism to maintain pH homeostasis within the limit suitable for growth or survival is well documented (Booth, 1985; Eklund, 1989; Montville, 1997). In the broth cultures at the optimum pH range, a decrease in pH of ~1 pH unit by the end of the growth of *L. monocytogenes* was recorded. In more constrained conditions, i.e. lower pH_I, a smaller change in pH to a somewhat constant levels of pH_f at a range of pH_I (Fig. 4.12) was observed. These pH_f values were anticipated not to be below the minimum pH_I that *L. monocytogenes* can initiate growth for each lactic acid concentration. However, slightly lower values of pH_f were found at all levels of lactic acid. A possible explanation is that the growth of the organism had already ceased at the pH close to its minimum pH prior to the measured pH_f, but that cells were still metabolically active (Brown and Booth, 1991) and reduced the pH to lower than the pH limits for growth appropriate to that concentration of lactic acid (Fig. 4.12).

Organic acids are generally more inhibitory to micro-organisms than inorganic acids due to their lipophilic nature (Gould, 1989). In this study, in the presence of even low concentration of lactic acid, e.g. 20 mM, *L. monocytogenes* was unable to grow to the minimum pH for growth (pH 4.23) in the absence of lactic acid, i.e. the limiting pH for growth of *L. monocytogenes* increased as a function of lactic acid concentration. Similar findings for *L. monocytogenes* have been noted before (Ahamad and Marth, 1989; Sorrells *et al.*, 1989; Conner *et al.*, 1990). Increasing inhibition due to pH as the lactic acid concentration increases was also reported for *E. coli* (Presser, 1995). In the presence of 200 mM lactic acid, the pH value for complete growth inhibition at ~20°C was 5.46 which is consistent with the finding by Ross (1993). In other studies where lactic acid was the acidulant (Ahamad and Marth, 1989; Sorrells *et al.*, 1989), insufficient information regarding the pH or the total concentration of lactic acid employed was given to enable comparison with those reports.

Weak organic acid, in aqueous solution, dissociates corresponding to its pKa (Corlett and Brown, 1980; Gould, 1989). The effectiveness of weak acid is, therefore, assumed to be proportional to the concentrations of each components present which are strongly pH-dependent. Several reports suggest that growth inhibition is not primarily due to

hydrogen ions but to the concentration of undissociated molecule (Baird-Parker, 1980; Ahamad and Marth, 1989). The dissociated molecule, however, was reported to be a far less effective inhibitor, i.e. 10-600 times less inhibitory than the undissociated acid (Eklund, 1983), so that, in this study, it was considered to have a measurable effect only when a very large amount (450 mM) was applied to *L. monocytogenes* L5.

The data reported here have shown that both $[H^+]$ and [UD] have inhibitory effects on the growth rate of *L. monocytogenes* and the effects of both are linear (Fig. 4.13). The amount of each component required to exhibit equal growth rate inhibition varied in according to the concentration of lactic acid and pH. This is as predicted by the model, but is contrary to the finding of Presser *et al.* (1997a) who reported that the inhibition of growth rate of *E. coli* was equal for equal for equal undissociated lactic acid concentration, regardless of pH or lactic acid concentration (0 to 100 mM).

The reduced growth rate in relation to $[H^+]$ or [UD] shown in Figs. 4.13 should not be misinterpreted to be the effect from single component only. The growth rate was affected by several variables (see Eqn. 4.7) of which temperature and a_w are considered to be almost constant in these experiments. The calculated growth rate for each concentration of lactic acid varied according to the pH, and [UD]. This indicates the need to be able to understand the underlying influence of each component of lactic acid.

The advantages of the square-root type model, especially its modular form, enabled a separate calculation for the growth inhibition effect caused by each component of lactic acid, either k_{H+} (growth rate standardised for lactic acid effect) or k_{UD} (growth rate standardised for $[H^+]$ effect) to be clarified. In this way, the combination effect on the growth rate inhibition contributed by each of the components of lactic acid can be perceived more clearly (Figs. 4.14 and 4.15). For example, the minimum pH_I for growth of *L. monocytogenes* Scott A increased to 5.12 when 50 mM lactic acid was added. Thus 7.6 μM of hydrogen ions or 2.6 mM of undissociated lactic acid exhibited an equal inhibitory effect on growth rate of *L. monocytogenes* as k_{H+}:k_{UD} equal to 0.049: 0.046 (1:1) generation/h respectively (Figs. 4.14a,b). Increasing the concentration of lactic acid appeared to increase the ratio of the inhibition effect caused by [UD] at the lower extremes pH_I for growth, e.g. for strain Scott A k_{H+}:k_{UD} in the broth with 100 and 200 mM lactic acid were 0.2:0.036 (1:5.6) and 0.29:0.029 (1:10) respectively. This emphasizes the dominant effect of the undissociated lactic acid in relation to the inhibition due to lactic acid. The models (also being standardised) satisfactorily predicted the apparent linear relationship between these standardised growth rates and the $[H^+]$ and [UD]. Extrapolation of this relationship generates the values for complete growth inhibition which suggested that strain L5 (4.6 mM) was slightly more tolerant of lactic acid than Scott A

(3.8 mM). This may be related to the origin of the strain L5 (cold-smoked salmon) which has been reported to naturally contain up to 100 mM of lactic acid (Dalgaard and Jørgensen, 1998).

The standardised growth rate at 450 mM lactic acid shows a slower growth rate than predicted, especially for [H⁺], where only small amount of [H⁺] exhibited great influence on growth rate (Fig. 4.15). A similar anomaly was reported by Presser *et al.* (1997a) who suggested there may be a synergistic or additional inhibitory effect occurring under conditions of very high lactic acid concentration.

The growth rate inhibition related to [H⁺] in the absence of lactic acid observed in this study indicates a non-linear relationship which is different from that due to lactic acid (Fig. 4.13a). Standardisation for the differences in water activity between each block of experiments in the absence or presence of lactic acid have been made (Figs. 4.14a and 4.15a) but did not help explaining this non-linear response. This finding is inconsistent with the linear response previously reported (Buchanan *et al.*, 1993; Presser *et al.*, 1997a). Again, this suggests a need for further investigation to resolve these differences.

4.4.4 CELL YIELD-GROWTH RATE RESPONSE OF *L. MONOCYTOGENES* TO THE ENVIRONMENTAL FACTORS

A comprehensive study of cell yield in *L. monocytogenes* was not attempted in this chapter but an understanding of the mechanisms underlying these responses may be advantageous, in particular for the growth or no growth response experiments described in Chapter 5. Thus, it is useful to reiterate that 'cell yield' used here is only a general observation of the increase in OD of broth cultures as a function of environmental factors. The limitation of the turbidity measuring device is recognized (McMeekin *et al.*, 1993). However, modification of the observed "apparent" yield using a correction function (Dalgaard *et al.*, 1994) (Fig. 4.9) demonstrated similar trends to the "apparent" yields (Figs. 4.8b,c). Furthermore, the lack of effect on "apparent" cell yield over a range of sup-optimum temperature (Fig. 4.5) is also identical to a specific study of cell yield of *L. monocytogenes* Scott A as shown in Fig. 4.19 (J. Kettlewell, unpublished). The "apparent" yield reported here was also compared to the cell yield study of *E. coli* (Krist, 1997). A similar response, i.e. a constant yield over a range of temperature (Fig. 4.5) and water activity (Fig. 4.8a), and a continuous drop of yield as the effect of pH (Fig. 4.16) were found. Therefore, the information obtained from cell yield responses reported here is comparable and appropriate to consider.
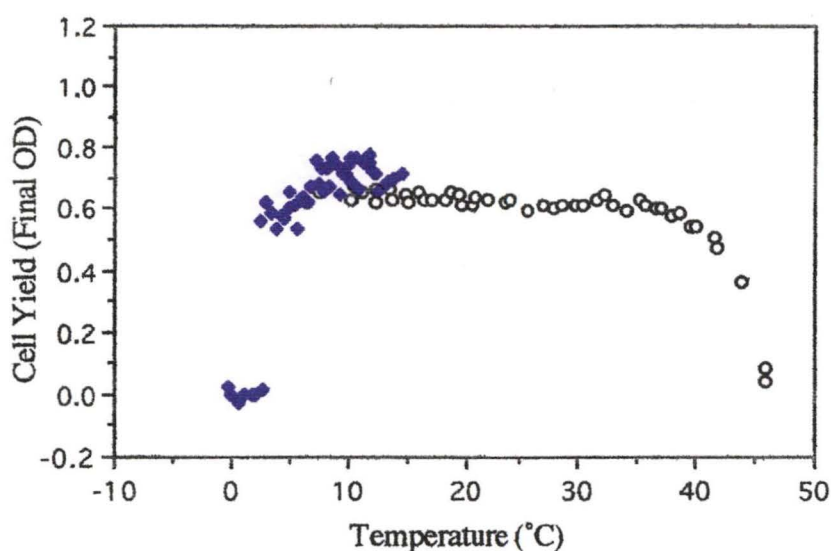
Figure 4.19   Cell yield response of *L. monocytogenes* Scott A as a function of temperature. The results were obtained from two independent set of experiments; (♦) and (O) in 10%TSB (Oxoid) with minimal broth (Difco), a_w of 0.997, and pH 7.2. Reproduced from data of J. Kettlewell (unpublished).

Stressful environments are reported to affect micro-organisms in several ways which force cells to divert energy from growth to the increased requirements of "maintenance" (Gould, 1989). The responses of *L. monocytogenes* to stressful environments has been studied in some detail by several researchers. At low temperature, *L. monocytogenes* responded by rapidly taking up high concentrations of substrates against the concentration gradient using a cold-resistant sugar-transport system (Wilkins *et al.*, 1972).   Under osmotic stress, compatible solutes such as K^+, glycine betaine, carnitine, and glutamate were reported to be accumulated by *L. monocytogenes* (Patchett *et al.*, 1996; Verheul *et al.*, 1995; Smith, 1996). Under acid stress, the cell retained its optimum intracellular pH by extruding the excess protons through the proton pump (Young and Foegeding, 1993). All of these maintenance mechanisms are energy-dependent processes which result in less energy available for growth and which have been interpreted to lead to the extending of lag phase and generation time (Wilkins *et al.*, 1972; Verheul *et al.*, 1995; Ray, 1996). Continual stress can cause depletion of energy and, eventually, cessation of growth and death occurs (Gould, 1989).

The decrease in growth rate of *L. monocytogenes* over a range of growth temperature and water activity at pH ≈6.1 (Figs. 4.3a,b and 4.4a,b) might be explained by the above maintenance mechanisms, i.e. the energy available for growth was diminished. The apparent consistent yield (Figs. 4.5 and 4.8a), however, indicates that the energetic

efficiency of growth (biosynthesis) was unaffected by both factors although it occurred at a slower rate. A study by Wilkins *et al.* (1972) also indicated a slow uptake and incorporation of L-leucine in protein synthesis as a result of low temperature. Similar findings were reported by ter Steeg *et al.* (1995) for *L. innocua* and Krist (1997) for *E. coli*. The changes in growth rate and cell yield, as the responses to temperature or water activity, were suggested by Krist (1997) to be non-coupled mechanisms, i.e. change in one does not of necessity relate to the change of the other. That author also indicated there were critical values at the temperature or water activity close to their respective limits to growth, where a rapid decline in cell yield was observed (Krist, 1997). The critical values concept also applies to *L. monocytogenes* as was confirmed by J. Kettlewell (unpublished) who observed a sharp drop in cell yields at temperatures below 3°C and a more gradual drop above ~37°C (Fig. 4.19).

Unlike the effect of temperature or water activity, increasing acidity not only reduced growth rate (Figs. 4.3c and 4.4c) but also cell yield (Fig. 4.15). The increasing energy demand for maintenance functions within the cell under acid stress, when HCl was the only acidulant, is shown by the linear decline in yield of *L. monocytogenes* with increasing hydrogen ion concentration. The strong yield-reducing effect of pH on *L. innocua* has been reported (ter Steeg *et al.*, 1995). Reduced yield of acid stressed *E. coli* was also found by Krist (1997). In the presence of lactic acid, the yield response of *L. monocytogenes* was found to be related to the combined effect of hydrogen ions and undissociated acid while the dissociated acid did not appear to exert any effect on yield (Fig. 4.16).

A similar trend of rapid decline in yield at the edge of the optimum pH range, when plotted against pH, was found in both absence or presence of lactic acid. A parallel change in yield with the growth rate (Figs. 4.4c and 4.16a) was also observed in the cultures with lactic acid present. However, the sigmoid response of growth rate when HCl was the sole acidulant, as discussed previously, did not parallel with the reduced yield (Figs. 4.2 and 4.15). These observaitions may imply a specific effect of undissociated acid from the hydrogen ion on pH homeostatic disturbance.

In Fig. 4.8 or 4.19, the reduced yield, at the lower pH $\approx$5.7 and $\approx$5.4, especially when approaching the water activity limit may be explained by the combined effect of increasing acidity and osmotic stress on bacterial cells. This was also enhanced by the addition of lactic acid of which the greatest effect was found at pH $\approx$5.4 where the concentration of undissociated acid is highest (Table 4.7). The trends of these changes in cell yield were analogous with the proportional changes in growth rate (Fig. 4.7) which may imply that there exists a very close relationship between these properties, and perhaps the reliability of turbidity-based growth rate measurements under severely growth rate limiting

conditions, i.e. reduced yield is likely to cause the turbidimetric growth rate measurements to be made later in the exponential phase when the growth rate is much slower than the maximum specific growth rate. To reiterate, further study with a specific design to verify these responses, is required.

4.4.5 INTER-STRAIN VARIABILITY

In this study, 2 strains of *L. monocytogenes* were used for the purpose of; investigating the responses to the controlling factors of a pathogenic strain, Scott A, which is known to be able to grow in foods, inparticular cheese, and cause outbreaks. Also this strain is generally been employing by several researchers, thus, for a comparison of the studies., The responses of a strain originated from cold-smoked salmon, L5, although has never been associated with any outbreaks is also of interest to investigate whether there is any or substantial different in the responses in relation to its origin. Also, if possible, a strategy to inactivate this type strain may be found.

The overall responses of both strains to temperature, water activity, and pH (HCl was the acidulant) were generally similar. In the presence of lactic acid of 50 and 100 mM, however, the strain L5 was able to grow to a somewhat lower pH (~0.1 pH unit) than strain Scott A. This resulted in a higher undissociated lactic acid concentration of ~1 mM (see Appendix G, Tables G.1 and G.3), and a higher estimate of parameter U_{min} (~0.8 mM) for strain L5. Apart from this small difference, the similarity between the estimates of the parameters T_{min}, a_{wmin}, and pH_{min} for both strains were obtained which suggests that a single model may be sufficient for this species for a given temperature, humectant, and acidulant.

5 GROWTH LIMITS OF *LISTERIA MONOCYTOGENES*

5.1 INTRODUCTION

The application of predictive microbiology provides insight concerning the responses of micro-organisms to their environment. The kinetic behaviour of *L. monocytogenes* was described in Chapter 4. While the infective dose of the potentially fatal foodborne pathogen *L. monocytogenes* is still unknown, an understanding of how to prevent its growth or, preferably, eliminate it from foods is more of interest. Another approach of predictive microbiology, a probabilistic study, can be applied to gain information about the combination of conditions that prevent growth of *L. monocytogenes*. The probabilistic study gathers qualitative data, i.e. growth or no growth, generating a 'probability model' and defining the boundary between conditions which permit growth and those which do not.

The data employed in an earlier probability model were time-limited kinetic data (Ratkowsky and Ross, 1995). Genuine growth and no growth data, i.e. with a sufficient time allowed for any possible growth to occur, were employed in the study of Presser *et al.* (in press). The probability model was initially generated using a logistic regression method in which the parameter estimates, e.g. T_{min}, a_{wmin}, pH_{min}, and U_{min} were fixed constants with the values obtained from kinetic modelling studies (Presser, 1995; Ratkowsky and Ross, 1995; Presser *et al.*, in press). The development of the method to a generalised non-linear (NLIN) regression was recently proposed (Presser *et al.*, 1997b). This method enables the parameters to be estimated from the observed data.

The concept of using several constraints, including lactic acid, was described in Chapter 4 (section 4.1) and is continued in this chapter. The limits to growth of *L. monocytogenes* Scott A and L5 were examined over a range of pH, sub-optimum temperature and water activity, and lactic acid concentrations. The data sets were combined with the kinetic data in Chapter 4 and probability models, using the NLIN procedure, were generated. The model will enable one to predict the effects of single, or combinations of, controlling factors that can inactivate or prevent growth of *L. monocytogenes*.

5.2 MATERIALS AND METHODS

5.2.1 MATERIALS

Details of consumables, reagents and media, and equipment used are presented in Appendix A.

5.2.2 METHODS

5.2.2.1 Inoculation procedures

L. monocytogenes Scott A or L5 inoculum was prepared as described in 4.2.2.1. The optical density of the culture at 540 nm was measured and adjusted with fresh media to give an absorbance of 0.8. This density was observed in the previous growth study (section 4.2.2.1) to correspond to bacterial cells in the late exponential phase of growth. The inoculum was occasionally kept in an ice bath (4°C) to stabilise the concentration of the inoculum during the inoculation of the multiwell plates.

Under aseptic conditions, 100 μl of inoculum was added into each 50 ml TSB-YE, mixed well and pH immediately measured. An Electronic Digital Pipette was used to facilitate dispensing two ml of each broth into 4 wells of each of 4 24-well plates (4×6 wells). Two wells were prepared for negative, (sterile TSB-YE, pH 7.2) and another 2 for positive (TSB-YE, pH 7.2 containing 100 μl of the inoculum), controls in each well-plate. In this manner, 2 well-plates were used for each of 10 pH levels for each lactic acid concentration and six replicates were incubated at 4°C, 10°C and 20°C using constant temperature rooms, at 6°C and 8°C in waterbaths, and at 30°C in an incubator.

In a comparative study of the effect of water activity, pH, and lactic acid, duplicates were prepared to be incubated at near optimal temperatures for growth yield, i.e. 20°C (constant temperature room), and 30°C (incubator). Duplicate plates, using a standardised ecometric technique (Mossel *et al.*, 1980; 1983), were prepared with the positive control broth for each set of the experiments for each level of lactic acid to serve as an estimate of the initial inoculum density. This standardised ecometric technique was calibrated to viable counts. The results are presented in Appendix F.

5.2.2.2 Assessment of growth

The well plates were examined daily. Each set of experiments was observed for up to 90 days. Growth was judged from the visible increase in turbidity of the broths. The day on which growth was first observed was recorded. The broth then was aseptically mixed

by pipetting up and down, and 0.3 ml was dispensed for pH measurement. Verification of *L. monocytogenes* from each growth broth was performed by streaking onto TSA-YE, for purity checking, and incubated at 30°C for 24-48 hr. Typical colonies were sub-cultured onto OXF for demonstration of typical *Listeria* colonies, and incubated at 37°C for 24-48 hr.

In cases where the visible turbidity did not noticably increase, or only a deposit occurred, a standardised ecometric technique (Appendix F) was performed and compared to the results of the inoculum referred to in section 5.2.2.1. A single spread plate was occasionally employed to further verify the results.

5.2.3 PROBABILITY MODELLING

Three sets of experiments were undertaken. In the first, the effect of combinations of temperature, pH and different concentrations of lactic acid on the growth limits of *L. monocytogenes* Scott A and L5 were studied. To test a hypothesis that temperature and water activity act to inhibit microbial growth by a common mechanism, the effect of combinations of water activity, pH, and different concentrations of lactic acid on growth limits of *L. monocytogenes* were determined in the second experiment. In the third, the effect of different concentrations of lactic acid, from 0 to 500 mM, was also tested at 5°C and 20°C. The range of the combinations tested is shown in Table 5.1.

Table 5.1 Outline of the experimental designs covering the conditions tested in probability models. Note that the range of those controlling factors are only approximate-full details are presented in Appendix G (Tables G.5 and G.6).

Study of	Temp. (°C)	Water activity	pH	Lactic acid (mM)	Number of Conditions tested	
					Scott A	L5
1. Temperature + pH + lactic acid[a]	4 to 30	0.994	3.9-6.5	0, 10, 20, 30, 50	213	221
2. a_W + pH + lactic acid[b]	20 and 30	0.929, 0.940, 0.954, 0.965	4.4-5.8	0, 20, 50	141	130
3. lactic acid + temperature[c]	5 and 20	~0.96	~6.0	0-500	-	15
Total					354 (+148+17)[d]	366 (+155+20)[e]
Total data for model generating					**519**	**541**

[a, b, and c] Details are given in sections 5.2.3.1, 5.2.3.2, and 5.2.3.3 respectively; [d] the 148 growth and 17 no growth data from kinetic study (Chapter 4); [e] the 155 growth and 20 no growth data from kinetic study (Chapter 4).

It should be noted that two forms of acidulant (similar to the studies in Chapter 4), i.e. HCl and lactic acid, were used so that the inhibitory effect due to pH or lactic acid could be distinguished. Filter sterilised 5 M HCl or 4 N NaOH solutions were used for pH adjustment of the media.

5.2.3.1 Determination of effect of temperature, pH and concentration of lactic acid on growth limits

Sterile over-strength TSB-YE was prepared in a 1 L volumetric flask and made up to final volume with sterile distilled water and filter sterilised lactic acid (88% w/w) to concentrations of 10 mM (1.026 g/L), 20 mM (2.053 g/L), 30 mM (3.079 g/L), and 50 mM (5.118 g/L). TSB-YE with no lactic acid was also prepared in the same manner. Each medium was aseptically dispensed to 10 screw-cap bottles and adjusted to 10 different pH levels. Broths were kept for a week at room temperature to help reveal any contamina-tion. The methods described in sections 5.2.2.1-2 were followed.

5.2.3.2 Determination of effect of water activity, pH, and concentration of lactic acid on growth limits

The potential effect of temperature and a_w in combination with pH and different levels of lactic acid was studied. Three levels of lactic acid, i.e. 0, 20 and 50 mM, and four levels of water activity, 0.929, 0.940, 0.954, and 0.965 were selected. 200 ml of each combination was prepared as described in 4.2.3.2. The methods as outlined in sections 5.2.2.1-2 were followed.

5.2.3.3 Determination of effect of lactic acid concentrations-pH and temperature on growth limits

In additional to the lactic acid-pH responses tested in Chapter 4, a preliminary deter-mination of the effect of different levels of lactic acid, at 5°C and 20°C, on *L. mono-cytogenes* L5 at conditions close to that typical of cold-smoked salmon, i.e. pH ~6.0 and water activity of ~0.96 was also studied. Sterile over-strength TSB-YE+4.5% NaCl was prepared in a volumetric flask and made up to final volume with sterile distilled water and filter sterilised lactic acid (88% w/w) to concentrations of 0 to 400 at 50 mM intervals, and 500 mM for the experiment at 5°C, and from 200 to 400 at 50 mM intervals for the experiment at 20°C. Fifty ml of the TSB-YE adjusted to each concentration of lactic acid, at pH ~6.0, was dispensed into separate 250 ml side-arm flasks. Broths were kept overnight at room temperature to help reveal any contamination. 100 μl of inoculum was

added into each 50 ml TSB-YE as described in section 5.2.2.1. The media were incubated at 5°C and 20°C in water baths shaking at ~33±1 rpm. Growth was assessed by measuring the turbidity as %T at 540 nm in addition to the methods described in section 5.2.2.2.

5.2.3.4 Model generation

The data from the kinetic studies in Chapter 4 combined with the data from the probability studies were used to generate "probability of growth" models based on Eqn. 4.8 using SAS[2] PROC NLIN. This generalised non-linear regression procedure is an advanced form of the LOGISTIC procedure employed in earlier model development (Presser *et al.*, in press). In this procedure, the parameters T_{min}, a_{wmin}, pH_{min}, and U_{min} were allowed to be estimated rather than being fixed constants. However, a fixed value for one or more of these parameters may sometimes be necessary to obtain a good fit (D. Ratkowsky, pers. comm.).

For evaluation of the goodness-of-fit of the model, the parameter estimates obtained from the PROC NLIN procedure were used as fixed constants and processed by PROC LOGISTIC. The area 'c' under the receiver operating characteristic (ROC) obtained from the latter method was set as a criteria for goodness-of-fit, e.g. $c > 0.9$ is considered outstanding discrimination (see details in section 2.2.4). In addition, the model performance was assessed from the Hosmer and Lemeshow Goodness-of-Fit value generated from the PROC LOGISTIC. A model with $p>0.05$ is considered satisfactory (Lemeshow and Le Gall, 1994).

Probabilities of growth predicted by the models were compared to the observed growth or no growth data. The growth/no growth interface at $P=0.5$ or 0.1, which is 50% or 10% probability of growth was calculated using the 'Solver' routine of Microsoft Excel[4].

5.3 RESULTS

The growth and no growth data for *L. monocytogenes* Scott A and L5 consisted of 519 and 541 conditions of temperatures, a_w, pH, and lactic acid (Appendix G, Tables G.5 and G.6 respectively). The variables combinations tested cover the entire sub-optimum

[2] SAS (Statistical Analysis System) (1997). SAS/STAT Guide for Personal Computers, Version 6.12 Edition, SAS Institute Inc., SAS Circle, Box 8000, Cary, North Carolina 27512-800, USA.

[4] Microsoft® Excel (1997). User Guide 2, Version 5.0, Microsoft Corporation, One Microsoft Way, Redmond, WA 98052-6399 USA.

ranges for growth of *L. monocytogenes* and are represented diagrammatically in Appendix G, Fig. G.2. Approximately 1,560 and 1,380 observations (numbering between 1 and 4 replicates at each condition) were made for Scott A and L5 respectively.

The parameter estimates of the probability models were initially obtained from the full data sets (519 and 541 conditions for Scott A and L5 respectively). However, it was noted that the estimates of U_{min} obtained by fitting Eqn. 4.8 were always very close to the highest [UD] at which the data were available (16.18 and 15.19 mM for strains Scott A and L5 respectively). Attempts were made to estimate U_{min}, without encountering this problem, by removing from the full data sets the high [UD] data for no growth and which had a very low predicted probability of growth ($\leq$ 0.005). Thus, the remaining 483 and 488 conditions, respectively were employed in the model generation for strains Scott A and L5. The probability models presented here are the extended forms of Eqn. 4.8 for which the coefficients and parameter estimates and their associated standard errors are given in Table 5.2. The fitted growth/no growth interface models for *L. monocytogenes* Scott A and L5 which will be referred to as Eqns. 5.1 and 5.2 respectively are as follows:

$$\text{logit } P = -112.176 + 42.857*\text{Ln}(T+2) - 39.489*\text{Ln}(a_W-0.913) - 146.326*\text{Ln}(1-10^{3.65 \text{ pH}}) + 6.821*$$

$$\text{Ln}(1-\text{LAC}/(5.83*(1+10^{\text{pH}-3.86}))) - 7.517*\text{Ln}(T+2)^2 - 6.027*\text{Ln}(a_W-0.913)^2 - 113.241*$$

$$\text{Ln}(a_W-0.913)*(\text{Ln}(1-10^{3.65-\text{pH}}) - 31.629*\text{Ln}(T+2)*\text{Ln}(1-10^{3.65-\text{pH}}) + 16.695*$$

$$\text{Ln}(1-\text{LAC}/(5.83* (1+10^{\text{pH}-3.86})))*\text{Ln}(1-10^{3.65-\text{pH}}) \tag{5.1}$$

$$\text{logit } P = -49.614 + 50.738*\text{Ln}(T+2) + 1.814*\text{Ln}(a_W-0.927) + 77.326*\text{Ln}(1-10^{3.66-\text{pH}}) + 19.990*$$

$$\text{Ln}(1-\text{LAC}/(5.84*(1+10^{\text{pH}-3.86}))) - 9.166*\text{Ln}(T+2)^2 - 47.960*\text{Ln}(a_W-0.927)*\text{Ln}(1-10^{3.66-\text{pH}})$$

$$-43.459*\text{Ln}(T+2)*\text{Ln}(1-10^{3.66-\text{pH}}) - 3.951*\text{Ln}(1-\text{LAC}/(5.84*(1+10^{\text{pH}-3.86})))*\text{Ln}(T+2) \tag{5.2}$$

where all the terms are as previously defined in section 4.1.1.2, Eqn. 4.8.

In the process of model development, it was found that T_{min} had to be fixed to a constant value to facilitate the model fitting and this also yielded a somewhat better fitting model. The T_{min} values of 0.88 and 0.60 obtained from the kinetic modelling for *L. mono-cytogenes* Scott A (Eqn.4.17b) and L5 (Eqn. 4.18b) respectively were initially used as the constants. However, better models (smaller weighted SS) were obtained when a T_{min} of -2°C (representative of literature values) was used as a constant.

The T_{min}, and the converged values for a_{wmin}, pH_{min}, and U_{min} for strains Scott A and L5 (Table 5.2) obtained from PROC NLIN were fixed as the constants for the models evaluation using PROC LOGISTIC, and identical values of the coefficients were found.

Table 5.2 Parameter estimates for *L. monocytogenes* Scott A and L5 fitted to Eqn. 4.8.

Coefficient & parameter	Estimates: Scott A	Standard Error		Estimates: L5	Standard Error	
		NLIN	LOGISTIC		NLIN	LOGISTIC
b_0 (Intercept)	-112.176	47.55	11.31	-49.614	41.47	5.38
b_1 (T)	42.857	21.19	4.06	50.738	25.56	4.89
b_2 (a_w)	-39.489	24.68	5.90	1.814	0.69	0.21
b_3 (pH)	-146.326	232.21	32.53	77.326	153.70	27.43
b_4 (UD)	6.820	1.78	0.59	19.990	5.53	3.78
b_5 (T^2)	-7.517	3.17	0.75	-9.166	3.80	0.92
b_6 (a_w^2)	-6.027	4.35	0.84	-	-	-
b_7 (T*pH)	-31.629	48.74	5.87	-43.459	74.59	8.44
b_8 (a_w*pH)	-113.24	178.78	12.56	-47.960	79.12	5.17
b_9 (UD*pH)	16.695	31.22	3.16	-	-	-
b_{10} (UD*T)	-	-	-	-3.951	1.38	1.14
T_{min} (°C)	-2.00	2.55	-	-2.00	2.58	-
a_{wmin}	0.913	0.0088	-	0.927	0.00085	-
pH_{min}	3.65	0.6565	-	3.66	0.7162	-
U_{min}	5.83	0.6656	-	5.84	0.6608	-
Goodness-of-fit Statistic	p=0.3329	-	-	p=0.7217	-	-
c	0.980	-	-	0.989	-	-

The standard errors including the Hosmer and Lemeshow Goodness-of-Fit values and the areas 'c' under the receiver operating characteristic (ROC) derived from PROC LOGISTIC fitting are also presented in Table 5.2. (Standard errors for the coefficients obtained from PROC LOGISTIC fitting are much smaller than those from PROC NLIN because the T_{min}, a_{wmin}, pH_{min}, and U_{min} were held fixed, not estimated from the data.)

The performance of both models is gauged by the Hosmer and Lemeshow Goodness-of-Fit values with p>0.05. The high values of 'c' also show the good association of predicted probabilities and observed responses with 97.9%, 98.8% concordant and 1.9%, 1.1% discordant for Eqns. 5.1 and 5.2 respectively.

The model predictions of the growth/no growth interface for Eqn. 5.1 (strain Scott A) when P=0.5 and/or P=0.1 and 0.9 (50% and/or 10% and 90% prediction of growth respectively) are compared to the observed data in Figs. 5.1 and 5.2 for temperature-pH-lactic acid response, Figs. 5.5-5.7 for a_w-pH-lactic acid response, and Fig. 5.11 for lactic acid-pH response. Likewise, the fitted data of Eqn. 5.2 (strain L5) are presented in Figs. 5.3 and 5.4 for temperature-pH-lactic acid response, Figs. 5.8-5.10 for a_w-pH-lactic acid response, and Fig. 5.12 for lactic acid-pH response.

The standardised ecometric method used for growth determination, especially at the conditions close to the growth limits, was a reliable assessment and indicated whether the numbers of cells in the culture had increased or decreased. For cultures at near optimum pH in which growth was observed there was always a decrease in pH of 1-2 pH units. At pH close to its growth limits, *L. monocytogenes* appeared to increase pH to neutralise its environment. It is noteworthy that the growth that occurred at near limiting pH appeared to achieve a lower final turbidity when compared to positive controls or cultures grown at more moderate pH.

5.3.1 TEMPERATURE-PH-LACTIC ACID RESPONSE

The anticipated pH range of the media prepared in the experiments for each concentration of lactic acid covered the growth/no growth interface well except at 4°C where growth occurred only in the higher pH media (Figs. 5.1 and 5.3).

The growth/no growth interfaces at $P=0.5$ fitted by Eqns. 5.1 and 5.2 accurately describe the interface between conditions at which growth is, or is not, observed. Similar trends of the temperature-pH_I effect on the growth limits were observed at all levels of lactic acid tested for both strains. At temperatures from 10 to 30°C similar values of minimum pH_I for growth were observed, with the optimum temperature that supported pH tolerance revealed to be ~20°C. A rise of the limiting pH_I of ~1 pH unit, occurred when the incubation temperature was 4°C. The minimum pH_I for growth at 20°C in the absence of lactic acid were 4.36 and 4.35 (from the probability experiments) for *L. monocytogenes* Scott A and L5 respectively. Note that the next lowest pH values tested at which growth was not observed, were 4.18 and 4.23 respectively. The lowest pH which permitted growth, obtained from the growth rate experiment without lactic acid, also demonstrated that ~20°C was the optimum temperature for growth.

At all temperatures, there was an increase in the minimum pH_I at which growth occurred, related to the lactic acid concentration (Figs. 5.1a and 5.3a). In the presence of 30 mM lactic acid at 10°C and 30°C neither *L. monocytogenes* Scott A nor L5 behaviour was well described by the fitted growth/no growth interface (Figs. 5.1e and 5.3e respectively). An example of the growth/no growth interface at $P= 0.1$ is presented in Fig. 5.1c. The model prediction displayed a shift of the interface toward more stringent conditions when the 10% probability of growth level was selected. A small difference of pH (0.1 pH unit) between the 50% probability of growth to 10% or 90% of growth was noted (Figs. 5.1e).

Plots of temperatures versus concentration of hydrogen ion and undissociated lactic acid are presented in Figs. 5.2a,b and 5.4a,b for Scott A and L5 respectively. The highest

20.7°C were obtained from the growth rate experiment data in Chapter 4 of 58.9 μM and 56.2 μM for Scott A and L5 respectively. The combined effects of [H$^+$] and [UD] on growth rate in broth cultures with lactic acid were demonstrated in Chapter 4. Similar variation was observed in this growth/no growth interface study, in that increasing lactic acid concentration resulted in an increase of the pH$_i$ at the interface corresponding to lower [H$^+$] and increase of [UD]. For example, at 20 mM lactic acid, the values of pH, [H$^+$] and [UD] predicted from model 5.1 at the interface (P=0.5) were 4.54, 28.8 μM and 3.45 mM respectively. At 50 mM lactic acid, the predicted interface (P=0.5) was at pH 4.90 which corresponds to 12.6 μM [H$^+$] and 4.17 mM [UD].

Figure 5.1 (facing page). Growth/no growth interfaces at $P=0.5$ for *L. monocytogenes* Scott A (Eqn. 5.1), showing interaction between pH and temperature in determining minimum conditions for growth at water activity of ~0.993 and in the presence of 0, 10, 20, 30, and 50 mM lactic acid (a). Comparison between the observation of growth (●, ○) and no growth (×, +) from the probabilistic and kinetic experiments respectively and the predicted interface at b) 0 mM, c) 10 mM, d) 20 mM, e) 30 mM, and f) 50 mM lactic acid. Predicted g/ng interfaces at $P=0.1$ are shown as black lines in c) and e), and at $P=0.9$ as a green line in e) to illustrate the abruptness of the transition from high to low probability of growth.

a) Predicted G/NG Interface (*P*=0.5)

b) 0 mM LAC

c) 10 mM LAC

d) 20 mM LAC

e) 30 mM LAC

f) 50 mM LAC

a) Temp. vs [H$^+$]

[H$^+$] μM

Temperature ($^\circ$C)

0 mM

10 mM

20 mM

30 mM

50 mM

b) Temp. vs [UD]

[UD] mM
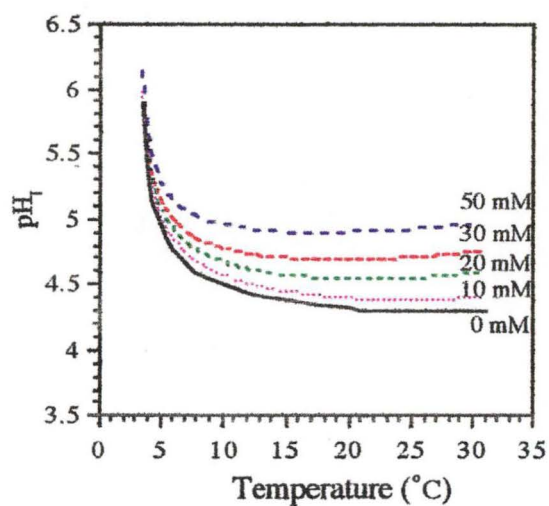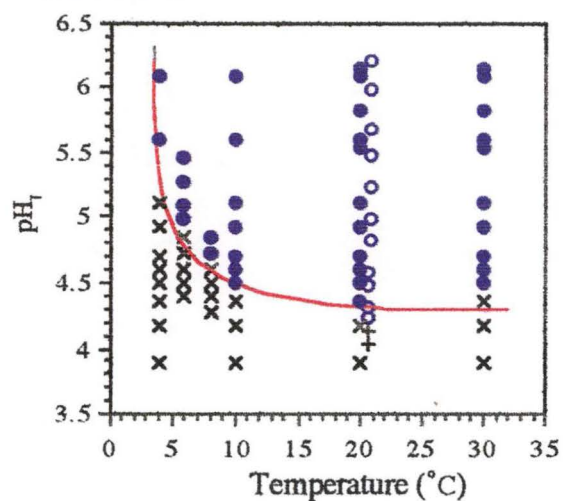
Temperature ($^\circ$C)

50 mM

30 mM

20 mM

10 mM
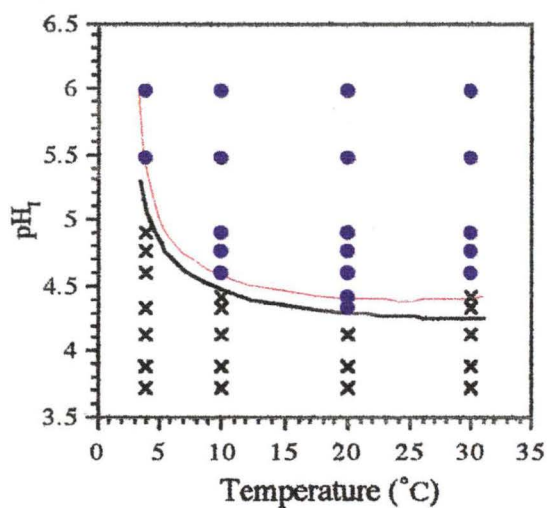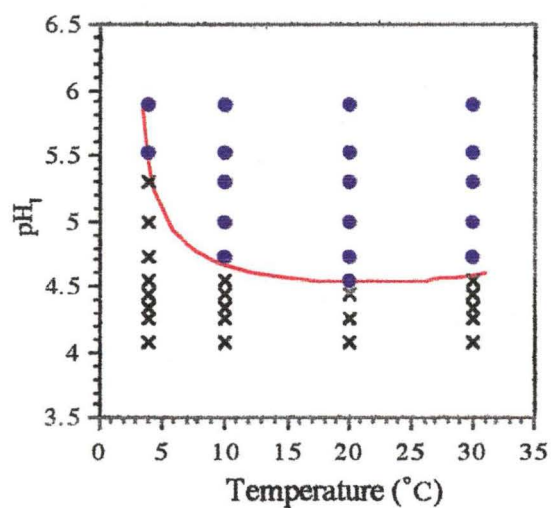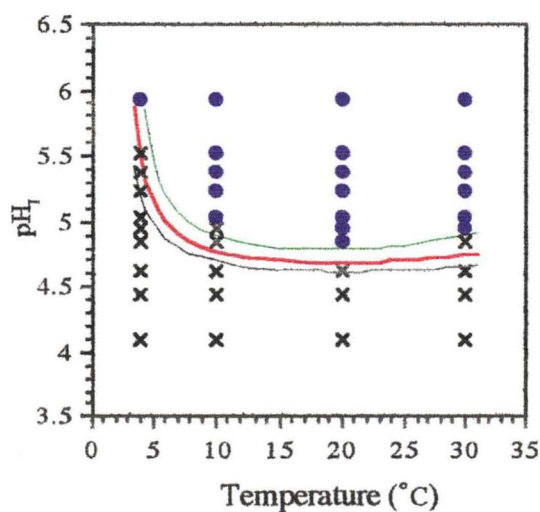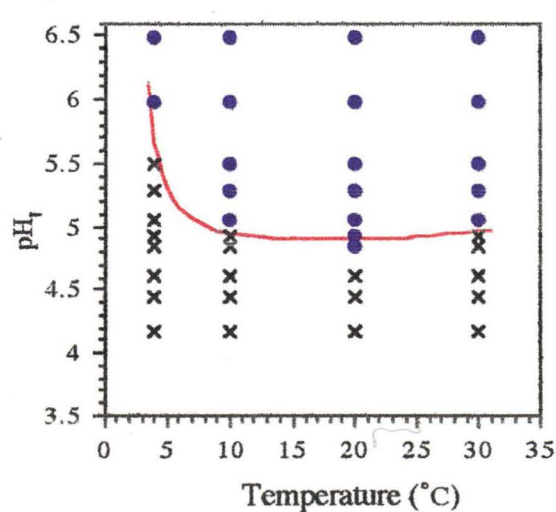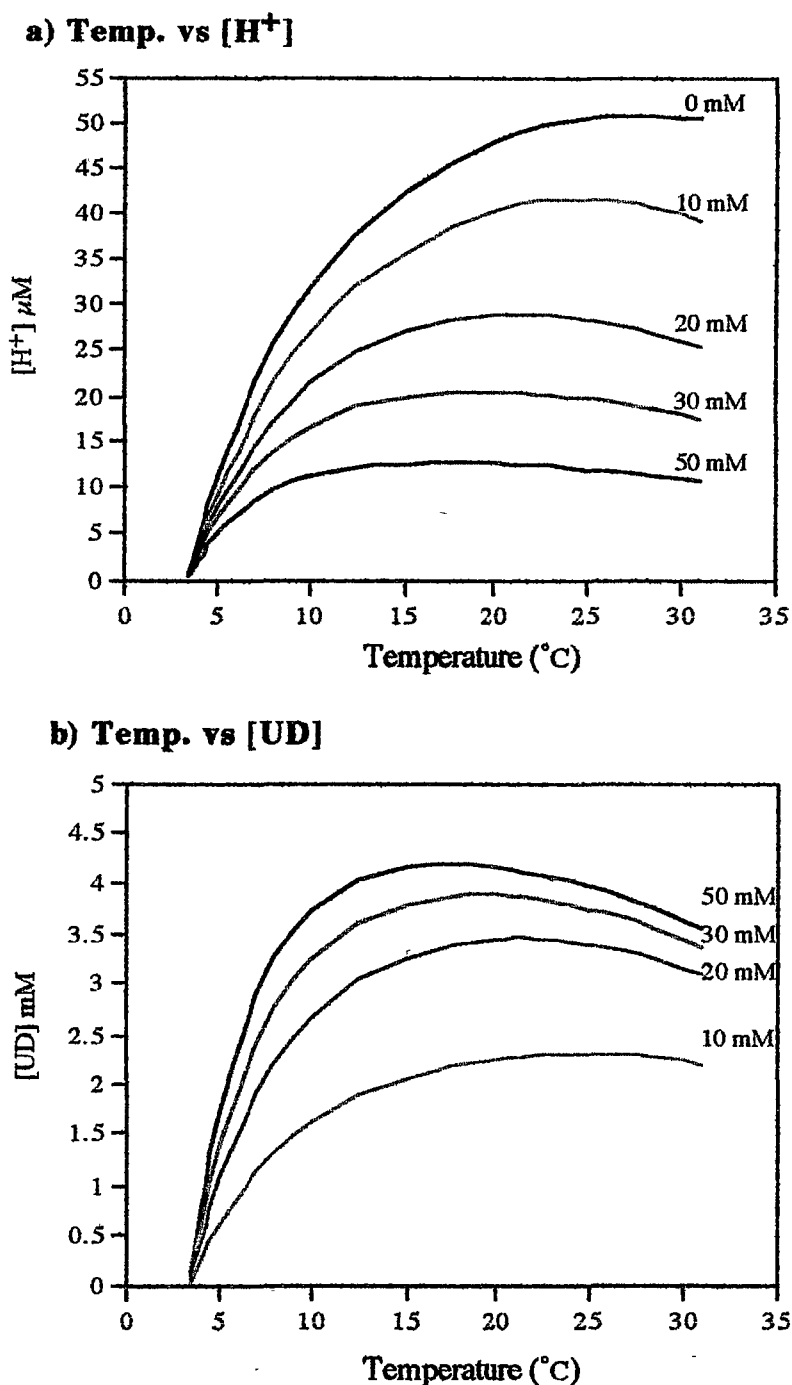
Figure 5.2 Growth/no growth interfaces at P=0.5 predicted by Eqn. 5.1 for *L. monocytogenes* Scott A as a function of temperature and different levels of lactic acid; 0, 10, 20, 30, and 50 mM presented as: a) [H$^+$]; and b) [UD]. The spaces below and above each line represent the conditions predicted for 50% probability of growth and no growth respectively. The observed growth and no growth data compared to each of the growth/ no growth interfaces are not presented here but corresponded to the pH$_i$ displayed in Fig. 5.1. The water activities in these tests were in a narrow range (0.992-0.994).

Figure 5.3 (facing page). Predicted growth/no growth interfaces at $P=0.5$ for *L. mono-cytogenes* L5 (Eqn. 5.2), showing interaction between pH and temperature in determining minimum conditions for growth at water activity of ~0.993 and in the presence of 0, 10, 20, 30, and 50 mM lactic acid (a). Comparison between observed growth (●, ○) and no growth (×, +) data from the probabilistic and kinetic experiments respectively and the predicted interface at b) 0 mM, c) 10 mM, d) 20 mM, e) 30 mM, and f) 50 mM lactic acid.

a) Predicted G/NG Interface (*P*=0.5)

b) 0 mM LAC

c) 10 mM LAC

d) 20 mM LAC

e) 30 mM LAC

f) 50 mM LAC
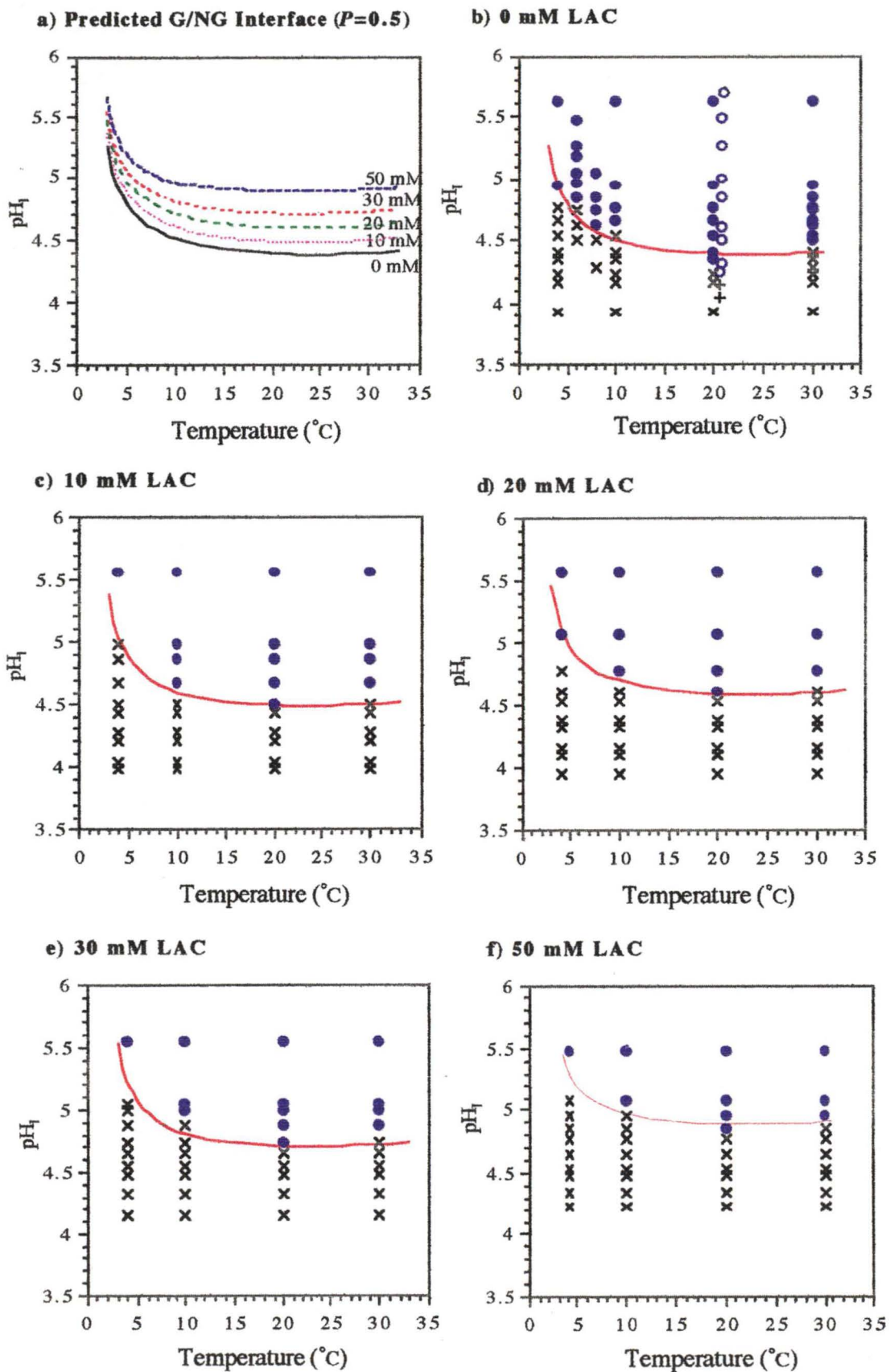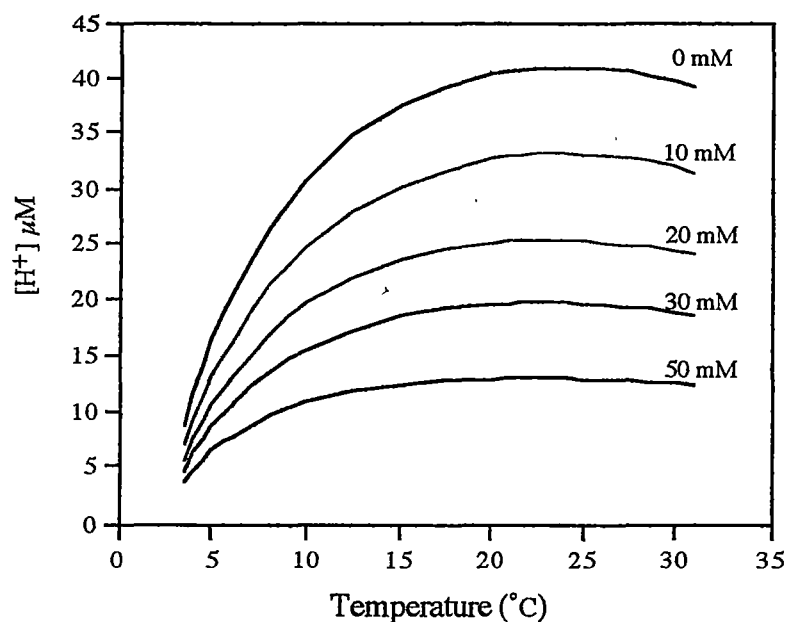
a) Temp. vs [H⁺]

b) Temp. vs [UD]
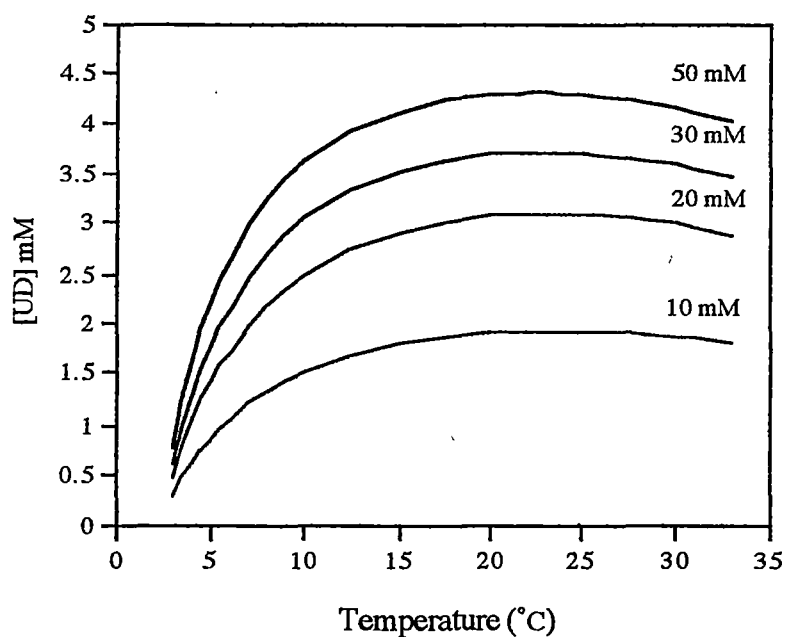
Figure 5.4 Growth/no growth interfaces at $P=0.5$ predicted by Eqn. 5.2 for *L. mono-cytogenes* L5 as a function of temperature and different levels of lactic acid; 0, 10, 20, 30, and 50 mM presented as: a) [H⁺]; and b) [UD]. The spaces below and above each lines represent the conditions predicted for 50% probability of growth and no growth respectively. The observed growth and no growth data compared to each of the growth/no growth interfaces are not presented here but corresponded to the pH_I displayed in Fig. 5.3. The water activities in these tests were in a narrow range (0.992-0.994).

5.3.2 WATER ACTIVITY-PH-LACTIC ACID RESPONSE

The potential of reduced water activity (NaCl as humectant) to increase the minimum pH_I at which *L. monocytogenes* can initiate growth is demonstrated in Figs. 5.5-5.7 for Scott A and 5.8-5.10 for L5. The optimum water activity for growth, in the broths without lactic acid, was found to be 0.995. For both strains, when HCl was the acidulant, the effect of water activity appears to increase gradually with the decrease in water activity. This effect was more pronounced for water activity close to the a_w limit, especially at 30°C.

An increase in pH of growth/no growth interface was found with the addition of lactic acid. This effect, however, appeared to be constant over the range of a_w ≥0.95, i.e. similar values of the minimum pH_I for growth were observed, but increased gradually when a_w was less than 0.95. When the water activity approached the a_w limit, an immediate rise in pH at the growth/no growth interface, especially at 30°C was observed, similar to that observed in the absence of lactic acid.

Anomalous results were found from the data obtained from the kinetic studies, where growth at 20°C, in the presence of lactic acid, occurred at higher pH_I than in growth/no growth experiments (Figs. 5.5c,d and 5.8c,d for Scott A and L5 respectively). These differences of 0.2-0.3 pH units were found especially in the broth cultures with 50 mM lactic acid at a_w< 0.94.

At 20°C *L. monocytogenes* appeared to be more tolerant to pH_I than at 30°C (Figs. 5.5a and 5.6a, and 5.8a and 5.9a). Plots of the growth/no growth interface as a function of [H⁺] and [UD] are presented in Figs. 5.7 and 5.10 for Scott A and L5 respectively. The fitted models, which satisfactorily describe the growth/no growth interface of the observed data, also demonstrate the higher concentrations of [H⁺] and [UD] at which growth occurred at 20°C than at 30°C. The linear decline in the amount of [H⁺] and [UD] required for growth inhibition as water activity is reduced was predicted by the probability models both for Scott A and L5.

Figure 5.5 Growth/no growth interfaces (*P*=0.5) predicted by Eqn. 5.1 for *L. monocytogenes* Scott A, showing interaction between pH and water activity in determining minimum conditions for growth at 20°C and in the presence of 0, 20, and 50 mM lactic acid (a). Comparison between observed growth (●, ○) and no growth (×, +) data from probabilistic and kinetic experiments respectively and the predicted interface at b) 0 mM, c) 20 mM, and d) 50 mM lactic acid.

a) Predicted G/NG Interface (*P*=0.5), 30°C
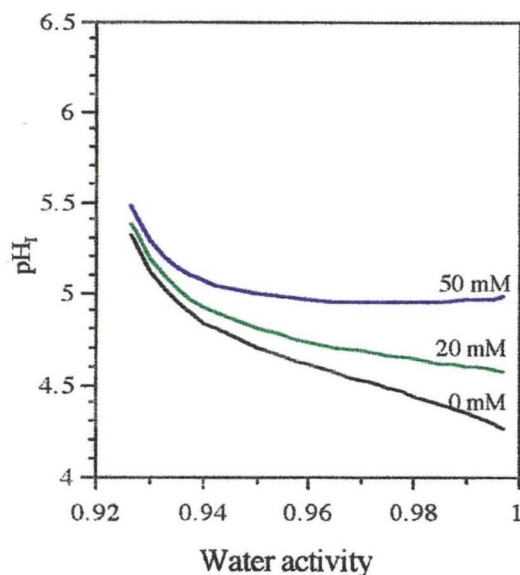
b) 0 mM LAC, 30°C
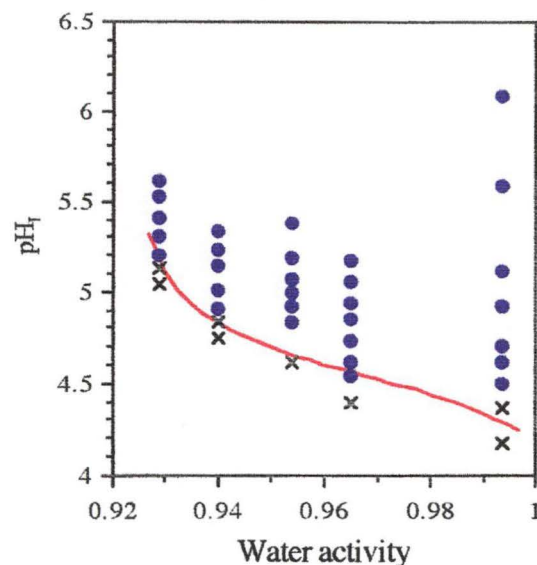
c) 20 mM LAC, 30°C
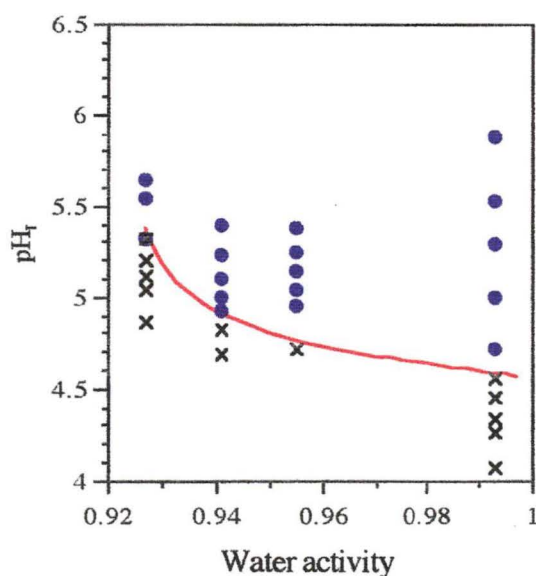
d) 50 mM LAC, 30°C
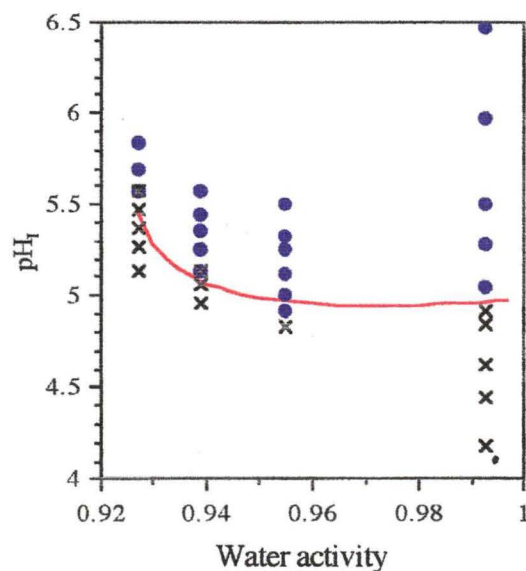
Figure 5.6 Growth/no growth interfaces (*P*=0.5) predicted by Eqn. 5.1 for *L. mono-cytogenes* Scott A, showing interaction between pH and water activity in determining minimum conditions for growth at 30°C and in the presence of 0, 20, and 50 mM lactic acid (a). Comparison between observed growth (●) and no growth (×) data from probabilistic experiments and the predicted interface at b) 0 mM, c) 20 mM, and d) 50 mM lactic acid.

a) a_w vs [H$^+$]

b) a_w vs [UD]
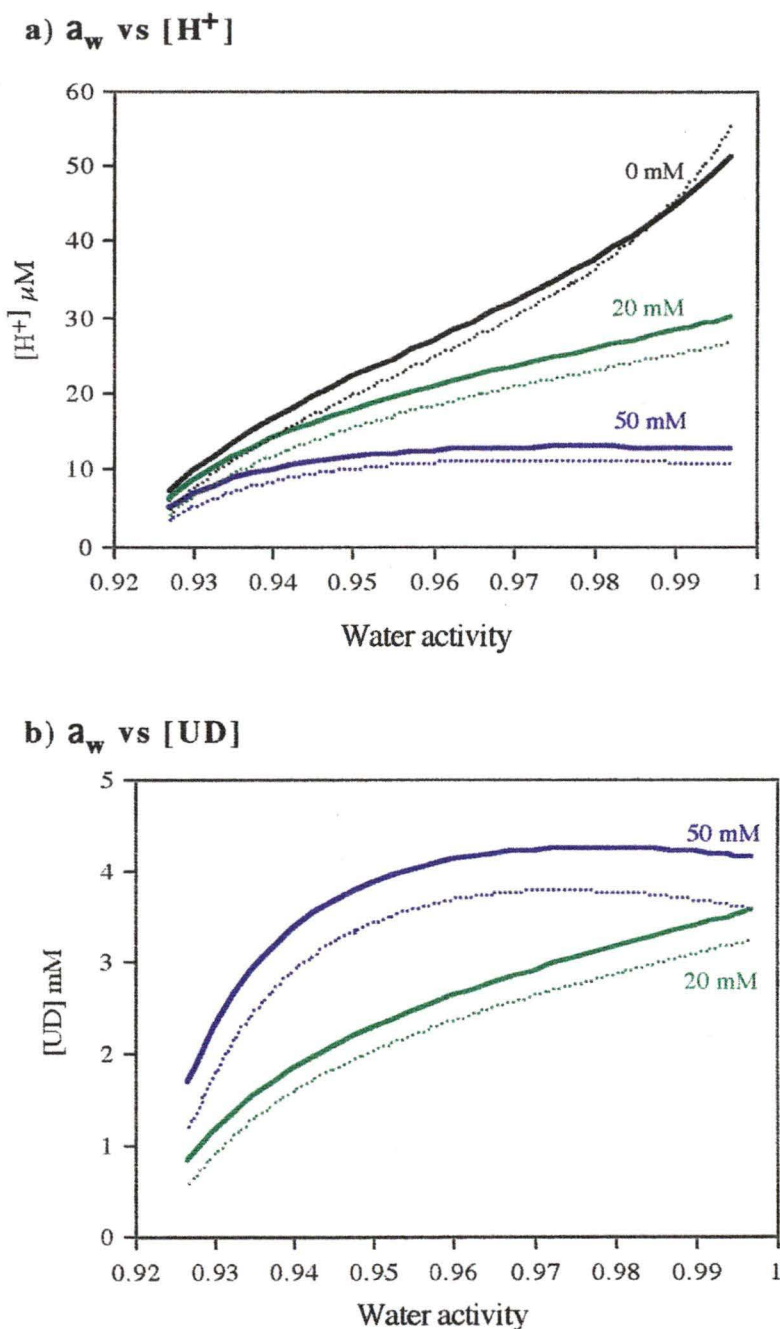
Figure 5.7 Growth/no growth interfaces at P=0.5 predicted by Eqn. 5.1 for *L. mono-cytogenes* Scott A at 20°C (solid lines) and 30°C (dotted lines) as a function of water activity and various levels of lactic acid (0, 20, and 50 mM) presented as: a) [H$^+$]; and b) [UD]. The spaces below and above each lines represent the conditions predicted for 50% probability of growth and no growth respectively. The observed data fitted to each of the growth and no growth responses are not presented here but corresponded to the responses displayed in Figs. 5.5 and 5.6 for 20°C and 30°C respectively.

a) Predicted G/NG Interface (*P*=0.5), 20°C
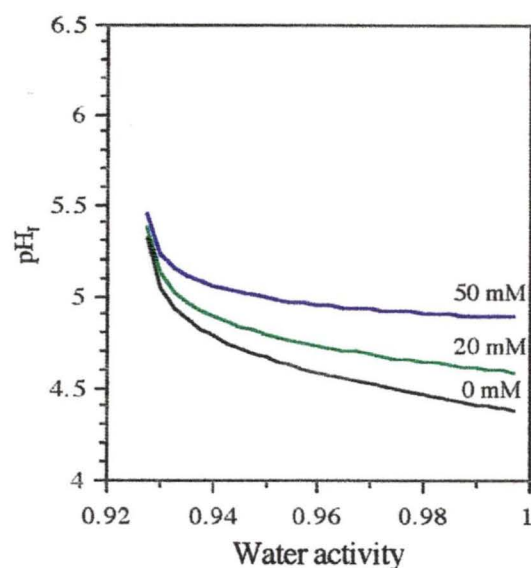
b) 0 mM LAC, 20°C
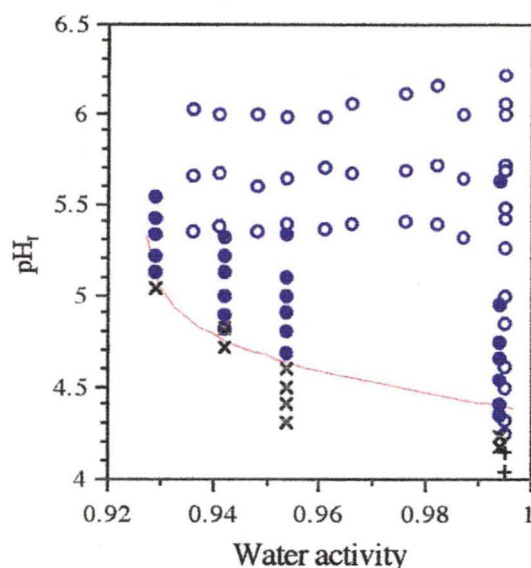
c) 20 mM LAC, 20°C
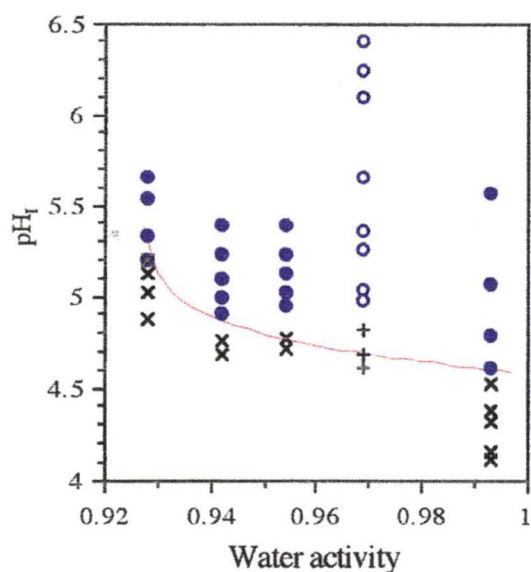
d) 50 mM LAC, 20°C
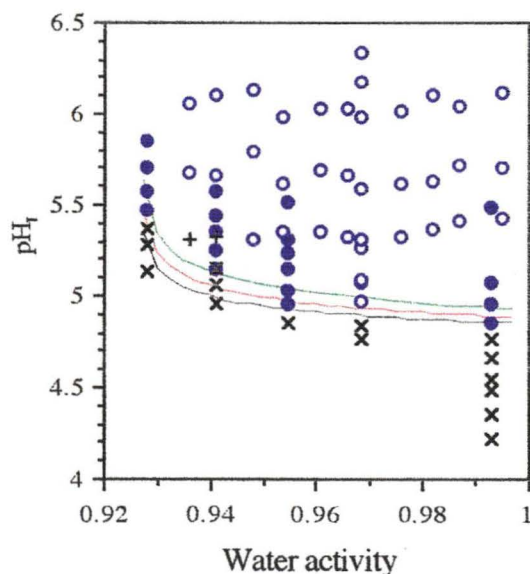
<u>Figure 5.8</u> Growth/no growth interfaces (*P*=0.5) predicted by Eqn. 5.2 for *L. mono-cytogenes* L5, showing interaction between pH and water activity in determining minimum conditions for growth at 20°C in the presence of 0, 20, and 50 mM lactic acid (a). Comparison between observed growth (●, ○) and no growth (×, +) data from probabilistic and kinetic experiments respectively and the predicted interface at b) 0 mM, c) 20 mM, and d) 50 mM lactic acid. Predicted growth/no growth interfaces at *P*=0.1 and 0.9 are shown as black and green lines respectively in d) to illustrate the abruptness of the transition from high to low probability of growth.
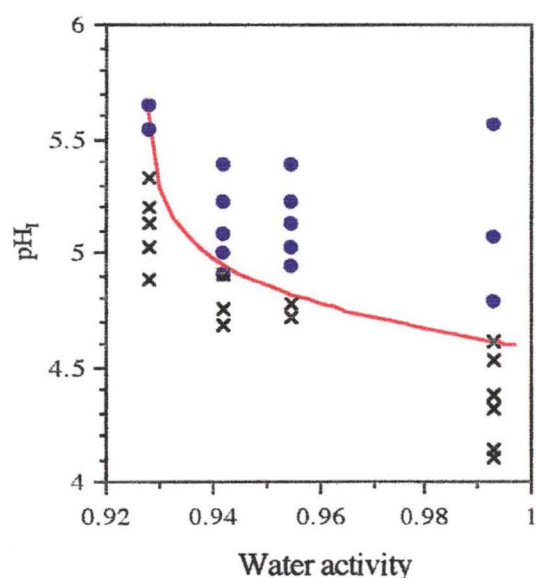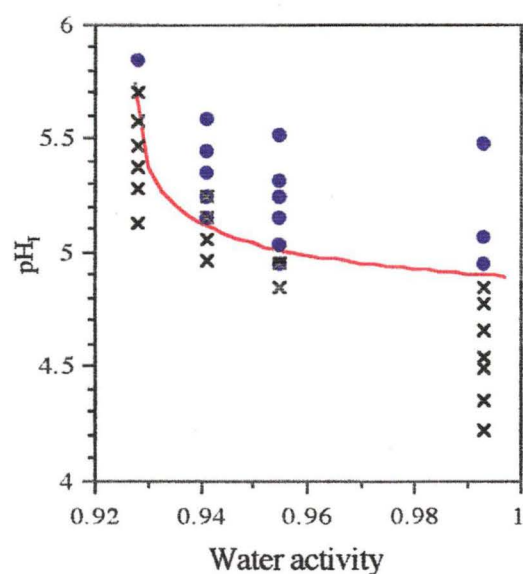
Figure 5.9 Growth/no growth interfaces (*P*=0.5) predicted by Eqn. 5.2 for *L. mono-cytogenes* L5, showing interaction between pH and water activity in determining minimum conditions for growth at 30°C in the presence of 0, 20, and 50 mM lactic acid (a). Comparison between observed growth (●) and no growth (×) data from the probabilistic experiments and the predicted interface at b) 0 mM, c) 20 mM, and d) 50 mM lactic acid.

a) a_w vs [H$^+$]
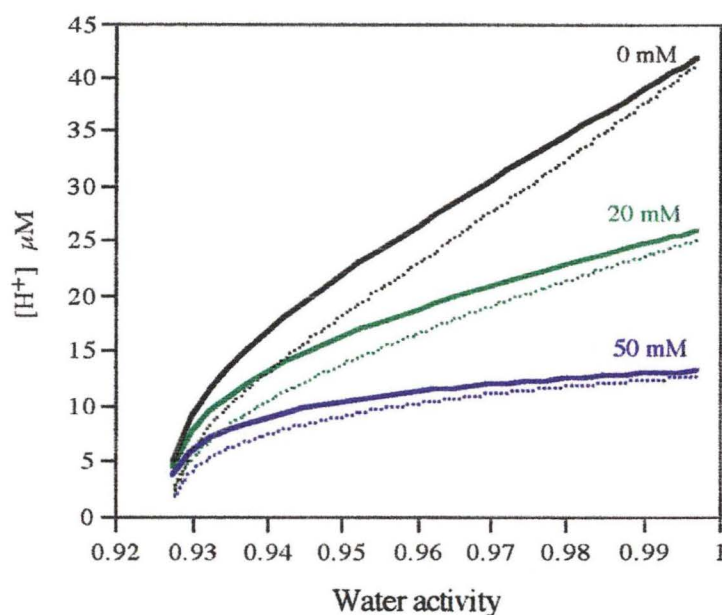
b) a_w vs [UD]
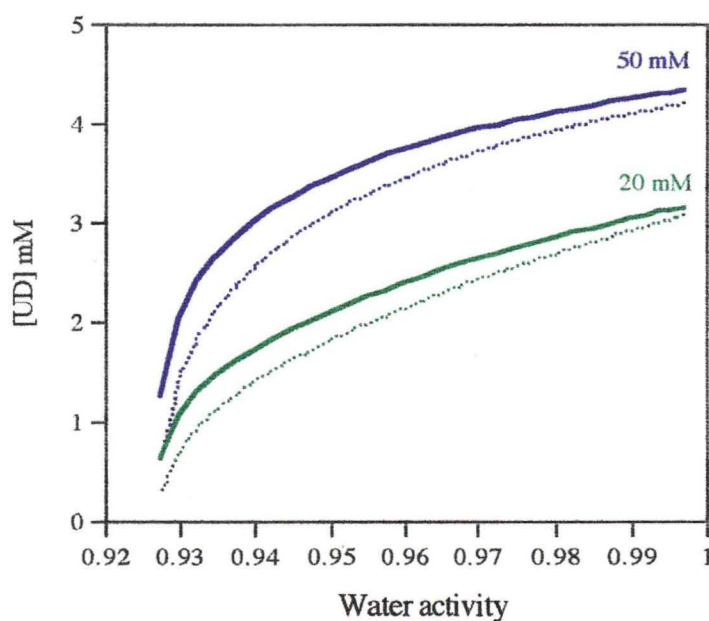
Figure 5.10 Growth/no growth interfaces at P=0.5 predicted by Eqn. 5.2 for *L. mono-cytogenes* L5 at 20°C (solid lines) and 30°C (dotted lines) as a function of water activity and various levels of lactic acid (0, 20, and 50 mM) presented as: a) [H$^+$]; and b) [UD]. The spaces below and above each lines represent the conditions predicted for 50% probability of growth and no growth respectively. The observed data fitted to each of the growth and no growth responses are not presented here but corresponded to the responses displayed in Figs. 5.8 and 5.9 for 20°C and 30°C respectively.

5.3.3 LACTIC ACID-PH RESPONSE

Plots of the change in pH_I at which growth could occur, including the corresponding [H^+] and [UD] as a function of lactic acid concentrations, were prepared for a range of lactic acid concentrations at a fixed temperature (~21°C) and a_w (~0.96) (Figs. 5.11 and 5.12 for Scott A and L5 respectively). Similar trends of an increase in pH limits with the increase in lactic acid concentration were found in both strains. The lowest pH_I at which growth was observed in broth without lactic acid was 4.54 for *L. monocytogenes* Scott A (Fig. 5.11a). The critical pH_I below which growth was not observed at 450 mM lactic acid was 5.88 for *L. monocytogenes* L5 (Fig. 5.12a).

Figs. 5.11a, b and 5.12a,b show the amount of each component, i.e. [H^+] or [UD], presented at the growth/no growth interfaces. It should be noted that the observed growth and no growth, and the predicted growth/no growth interfaces depicted in those Figures are not standardised to reveal the effect of only one component. The apparent responses, therefore, result from the combined effect of both [H^+] and [UD] which are co-dependent and must be taken into account when considering the effect of lactic acid. The lowest amount of [H^+] required for growth inhibition of *L. monocytogenes*, in the absence of lactic acid, was ~30 μM (at ~21°C and a_w of 0.96). The lowest amount of [UD] required for growth inhibition of *L. monocytogenes* was ~4.5 mM.

Figure 5.11 (facing page). Growth/no growth interface of *L. monocytogenes* Scott A at a_w of ~0.96 and 22°C (an average of the temperatures for this set of data) as a function of lactic acid concentration and a) pH at inoculation, b) [H⁺], and c) [UD]. Comparison between the observed growth (●, ○) and no growth (×, +) data from the probabilistic and kinetic experiments respectively. Note that Figs. 5.6 b,c demonstrate only the fitted growth/no growth interface and each active component of lactic acid. The combined effect from other components must also be taken into account. The black, red and green lines are the fitted models (Eqn. 5.1) for P=0.1, 0.5, and 0.9 respectively to illustrate the abruptness of the transition from high to low probability of growth.

a) [Lactic acid] vs pH_I
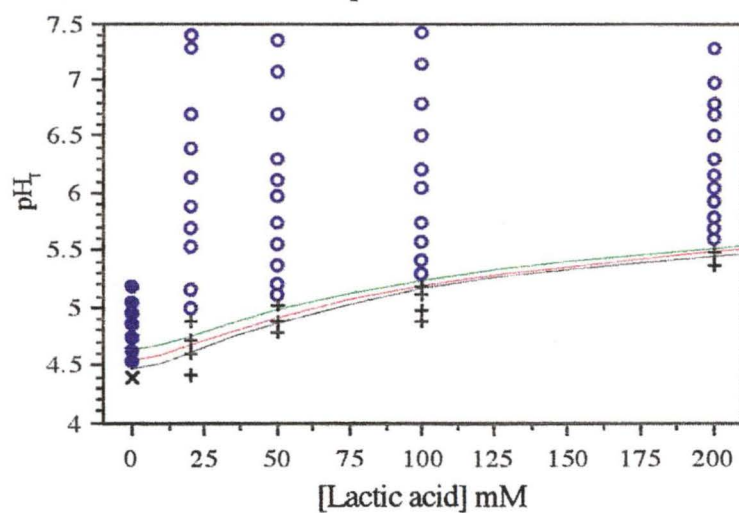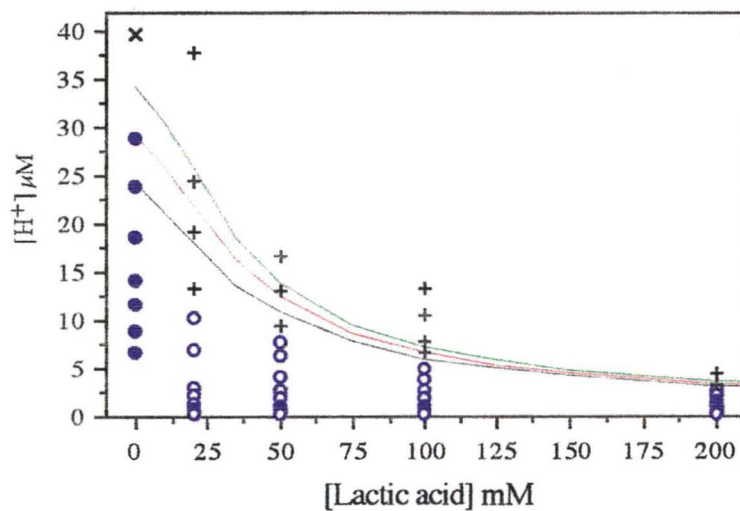
b) [Lactic acid] vs [H$^+$]

c) [Lactic acid] vs [UD]
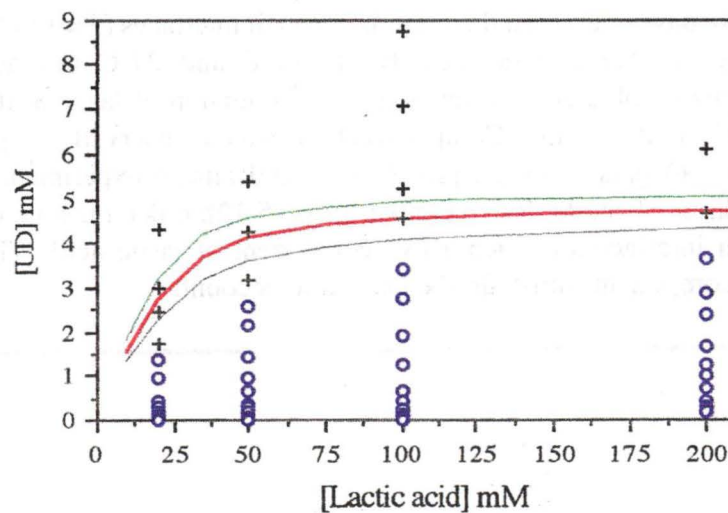
Figure 5.12 (facing page). Predicted growth/no growth interfaces (P=0.5) from Eqn. 5.2 for *L. monocytogenes* L5 at water activity of ~0.96 and 21°C (an average of the temperatures for this set of data) as a function of concentration of lactic acid and a) pH at inoculation, b) [H$^+$], and c) [UD]. Comparison between the observed for growth (●, ○) and no growth (×, +) data from the probabilistic and kinetic experiments respectively showing the goodness of model fit. Note that Figs. 5.12b,c demonstrate only the fitted growth/no growth interface and each active component of lactic acid. The combined effect from other components must also be taken into account.

a) [Lactic acid] vs pH$_I$
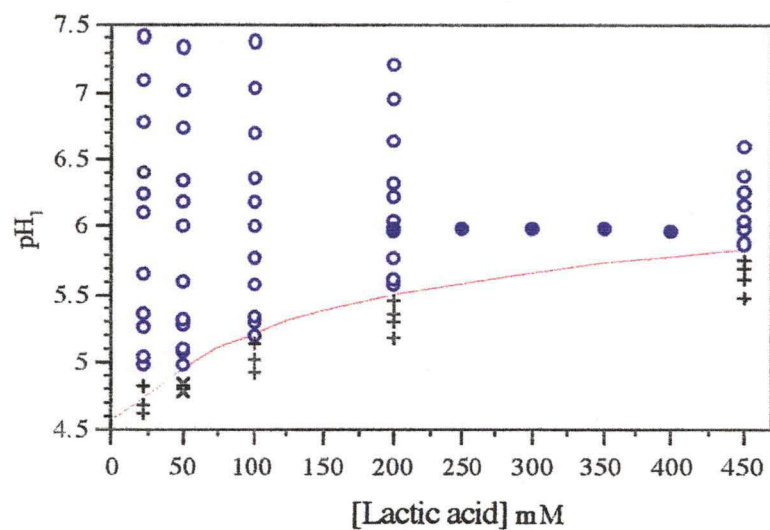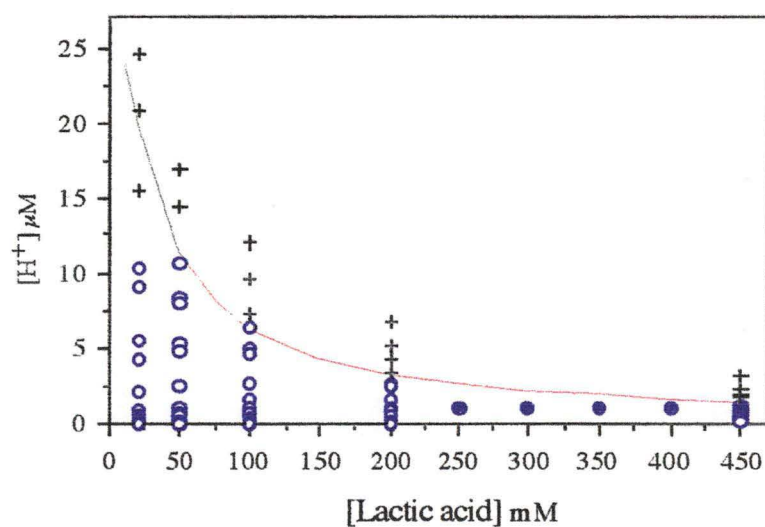
b) [Lactic acid] vs [H$^+$]

c) [Lactic acid] vs [UD]
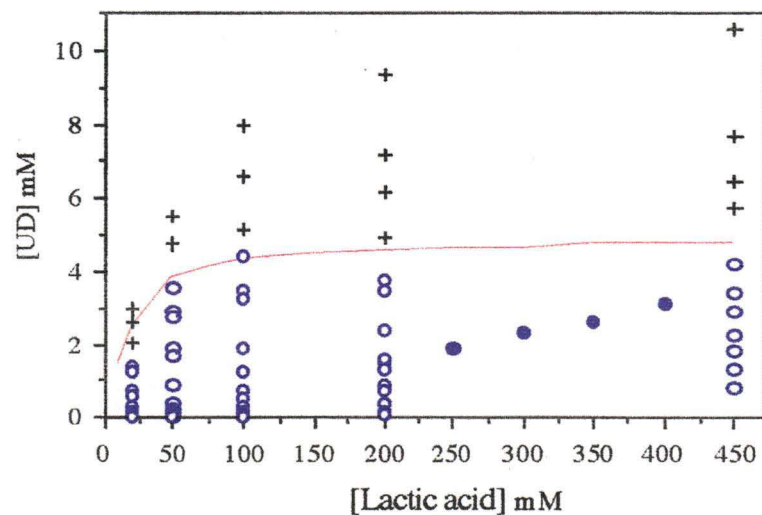
5.4 DISCUSSION

The "probability" or "growth/no growth interface" models developed in this chapter demonstrate a different approach of predictive microbiology where the rate and extent of growth, especially for pathogens, is of less interest than the possibility of growth. For pathogens like *L. monocytogenes* the infective dose of which is still unknown, small numbers in foods may present a hazard, especially to susceptible consumers. In particular, in foods that support growth of *L. monocytogenes*, especially if there is temperature abuse, there is potential for the organism to proliferate. Understanding its growth limits due to stressful environmental conditions would identify conditions for controlling its growth in foods and may serve as a built-in CCP throughout the shelf-life of products.

The probability models presented here are based on a binary response, i.e. growth or no growth, within a limit of time (90 days) sufficient to ensure any possible growth would be detected. The growth, as defined, was assessed by visual determination and verified by a standardised ecometric technique (Appendix F). The methods proved to be reliable and convenient for screening for growth in relatively large numbers of combinations of inhibitory factors. The reading of absorbance, especially in automated systems, was reported to face some sensitivity limitations (McClure *et al.*, 1991). The quadruplicate cultures prepared for nearly all conditions also served as a rigorous assessment to help confirm the likelihood of growth. A high degree of replication is considered favourable for the generation of datasets for probability models, especially at the stressful conditions. Generally, similar occurrence in all replicates were observed except at the conditions close to the interfaces where the growth, no growth (survival) or death are more variable (T. Ross, pers. comm.).

The experimenal design covered more than 500 different environmental conditions for *L. monocytogenes*. The probability models (Eqns 5.1 and 5.2) were generated using the SAS[2] NLIN procedure instead of the LOGISTIC procedure as previously employed (Ratkowsky and Ross, 1995; Presser *et al.*, in press). With this procedure, the parameters were allowed to be estimated from data, instead of being fixed to constant values. The models, however, appeared to perform better when T_{min} was fixed as -2°C. The reason for this is unclear but may be related to the large amount of growth and no growth data at temperatures $\geq 4°C$ and only one growth condition at temperature of 3°C.

The range of temperature and water activities tested was not extended beyond growth limits. However, pH values lower than the anticipated minimum growth pH for each level of lactic acid were included. Some of these extreme pH conditions, however, were found to substantially affect the estimate of the parameter U_{min}, resulting in a value

markedly different from the estimate obtained from the kinetic study (Chapter 4) and also from the observations. If the definition of U_{min}, i.e. the notional minimum concentration of undissociated lactic acid which prevents growth (see section 4.1.1.2) is appropriate, then the U_{min} values from different experiments are expected to be consistent. However, observations from *L. monocytogenes*, for example strain L5, show no growth at UD ≥ 4.6 mM, but using all the extreme pH conditions U_{min} was estimated to be 15.2 mM. This is simply a consequence of the arithmetic fact that one cannot take the logarithmic of zero or of a negative number. The general form of the expression is $Ln(1-[UD]/U_{min})$. As 15.19 mM was the highest [UD] used in the study for *L. monocytogenes* L5, then U_{min} must be greater than this value to prevent this mathematical problem. To overcome this limitation, the data for very high undissociated acid values where no growth could possibly occur were systematically removed (see section 5.3). This resulted in a U_{min} of 5.84 mM (Table 5.2 and Eqn. 5.2), a more consistent estimate, without affecting the performance of the model in any perceptible way. The U_{min} derived from these probability models were only slightly higher than the values obtained from kinetic models in Chapter 4. It should be noted that no other probability models for *L. monocytogenes* exist in literature, thus all of the parameter estimates being compared were derived from kinetic studies (see section 4.4.1). The a_{wmin} estimated from the models were consistent with the kinetic models and published reports (see section 4.4.1.2).

The growth or no growth boundary has been successfully defined and modelled using only kinetic data (Ratkowsky and Ross, 1995). In this study, the good fit to the kinetic data by the probability model is evident (Fig. 5.12) which demonstrates a success not only in incorporation of the kinetic data to generating a probability model, but also the ability of the probability model to describe accurately the conditions under which growth rate could not possibly be measured. This may represent an integration of the two extremes, kinetic and probabilistic aspects, of predictive microbiology. Consider the interpolation region described by Baranyi *et al.* (1996) as the so-called 'minimum convex polyhedron' (MCP), of the combinations tested in a kinetic study. The defined growth/ no growth boundaries, at 50% probability of growth, present in this study may be envisaged as a bigger multidimensional 'tent' covering the MCP where 100% probability of growth occurred. This 'tent' may provide a rational criteria for a modeller to design an experiment such that the MCP is maximized to cover all the possible growth domain, so that prediction by extrapolation can be avoided. In addition, the probability model may also provides an indication of the probability of growth which is useful when the kinetic model predictions are made for extreme conditions. Conversely, knowing the conditions where growth rate is very slow a no growth condition can be anticipated if the conditions are made slightly less favourable to growth.

The conditions of growth or no growth in kinetic and probabilistic studies are considered to be similar. In the kinetic study, the growth was considered unlikely to occur if the $\Delta\%T$ <25 of which the corresponding cell yield was <0.23 OD. In most instances, the no growth conditions, confirmed by ecometric method, coincided with conditions in which there was no increase in turbidity. Also, under the less optimum conditions, a smaller increase in turbidity was found. As previously discussed in Chapter 4, the energy diversion of the micro-organism to maintenance functions under stressful conditions, in particular acid stress, may result in reduced cell yield. This concept can explain those turbidity changes in this growth/no growth study caused by either acid stress or combinations of acid-low temperature stress and acid-osmotic stress. The notion of similarity of both predictive models is in accord with Presser (1995) who suggested that the difference is only in the approach of modelling the effect of the response, rather than a difference in the response itself in extreme conditions under which growth rate is unable to be measured and the growth/no growth boundary occurs.

It is noteworthy that although the growth/no growth interface was clearly defined, extreme growth variation near this growth limit is recognised (Ratkowsky et al., 1991). Under extreme conditions close to the limit of growth, Wijtzes (1996) assumed that the microbial population consisted of two sub-populations. The first group was considered to die immediately under the stress conditions, while the second group experiences a lag time, adapts to the stress condition, and can survive or grow under those extreme conditions. The level of each group in a microbial population may vary depending on the ability of microbes to repair and perform maintenance functions, which may be explain the variation in responses of microbial populations at near growth-limiting conditions. Different population densities (McClure et al., 1989) or incubation history of cultures (Patchett et al., 1996) were also reported to play a role in the different responses of microbial populations at the extreme conditions.

The variation from "highly likely to grow" conditions (P=0.9 or 90% likelihood of growth) to "highly unlikely to grow" conditions (P=0.1 or 10% likelihood of growth) was predicted from the studies of the potential effect of combinations of pH and temperatures (Fig. 5.1e), water activities (Fig. 5.8d), or concentrations of lactic acid (Fig. 5.11) to be within a narrow range of pH (~0.1-0.2 pH units). This demonstrates the abruptness of the transition between growth or no growth conditions influenced by pH. Similar findings were reported by Presser et al. (in press). Beyond these range of probabilities, the model predicts the probability of almost 100% or 0% probability of growth which indicates that the response is an absolute, i.e. growth or no growth is not time dependent.

Despite of the qualitative nature of the data, the growth no growth interfaces depicted in this chapter have proven to be a convenient means of judging the probability that *L. monocytogenes* would grow at the given conditions of the pH value and levels of lactic acid and NaCl, and storage temperature. The growth or no growth responses of *L. monocytogenes* Scott A and L5 to the combinations of controlling factors are discussed in the following sections (5.4.1-5.4.3).

A convergence of predictive microbiology and the 'hurdle concept' (Leistner, 1994) is also demonstrated in this study. The clearly defined growth/no growth boundaries may represent a quantification of the hurdle concept with a tangible understanding of the combined effect of controlling factors. This may provide a criteria for a product formulation so that only necessary levels of controlling factors will be applied in order to obtain a safe product at a reasonable cost, or to satisfy consumer preferences for the minimal level of processing which achieves the required safety and shelf-life.

5.4.1 TEMPERATURE-PH-LACTIC ACID RESPONSE

The interaction between temperature and acidity in both absence and presence of various levels of lactic acid demonstrated in Figs. 5.1 and 5.3, for strains Scott A and L5 respectively, suggests there is a synergistic effect between low temperature and pH on the limits to growth of *L. monocytogenes*. Similar influences of incubation temperature on the ability of *L. monocytogenes* to grow at low pH levels are also reported by several researchers (Ingram and Mackey, 1976; Sorrells *et al.*, 1989; McClure *et al.*, 1991). The addition of lactic acid enhanced the inactivation effect on *L. monocytogenes*, i.e. growth inhibited at a higher pH value, may be explained by the finding from Chapter 4 that increasing [UD] is more effective than [H$^+$] in lowering the cytoplasmic pH. Similar trend responses and predictions were found with the increasing lactic acid concentration from 0 to 50 mM. The combined effect of [UD] and [H$^+$] on growth limits, under the conditions tested, can be determined from Figs. 5.2 and 5.4. At any concentration of lactic acid, the pH at the growth/no growth interface dictates the amount of [UD] and [H$^+$] which, in turn, dictates the chance for *L. monocytogenes* to initiate growth. For example, the pH at the growth limit was always higher in the presence of highers level of lactic acid. This reinforces the finding in Chapter 4 of the dominant effect of [UD] in preventing growth at the higher concentration of lactic acid.

In these growth/no growth experiments, the lowest pH values which permitted growth of strains Scott A and L5 (4.36 and 4.35 respectively) was found at 20°C in the absence of lactic acid. These low pH values coincide with the findings from kinetic experiments

(Chapter 4) which also demonstrate the ability of *L. monocytogenes* to grow at lower pH than previous reports (George *et al.*, 1988; Sorrells *et al.*, 1989).

The optimum temperature for the growth of *L. monocytogenes* Scott A and L5 in these acid stress conditions, in the absence or presence of lactic acid, appeared to be ~20°C compared to the observation at 4°, 10°, and 30°C in this study. These observations of growth or no growth were merely the final results of a delicate balance of dynamic mechanisms in the bacterial cell. Based on Arrhenius plot of bacterial growth and the concept of a single growth rate limiting enzyme catalysed reaction, master reaction models (Sharpe and DeMichele, 1977; Schoolfield *et al.*, 1981; Ross, 1993) have been developed to describe the influence of temperature on the rate of microbial growth (Fig. 5.13). The proportion of master enzyme in the active conformation is constant over the growth permissible temperature range, but declines abruptly at critical high and low temperatures.
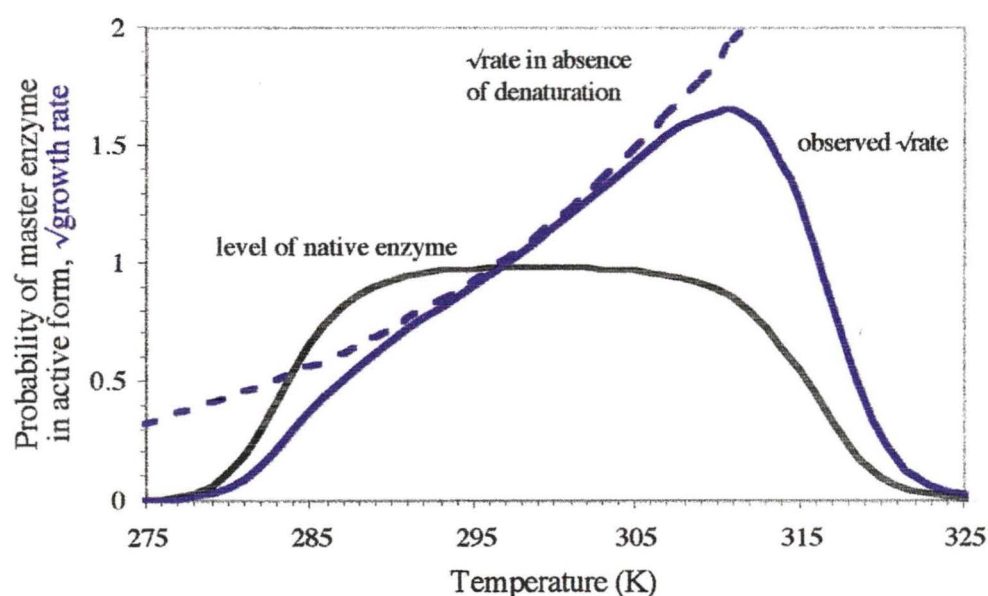
Figure 5.13 Master reaction model (McMeekin *et al.*, 1993; Ross, 1993) illustrating typical Arrhenius plot of bacterial growth rate in the absence of master enzyme denaturation (- -) bacterial growth in response to temperature (——), and the probability of the 'master enzyme' being in the active conformation (——). Rapid transitions occur as temperature approaches the high and low temperature limits for growth due to denaturation of the master enzyme.

This deviation appears to be analogous to the yield response previously reported in Chapter 4. The temperature which growth rate is maximal, ~37°C for *L. monocytogenes*, is not far apart from the maximal temperature where the growth rate and level of active enzyme decrease rapidly (Neidhardt *et al.*, 1990). In addition, at this optimum temperature for growth rate the decrease in yield was readily appreciable (Fig. 5.14). Thus, the temperature optimum for a growth rate may not be the optimum temperature for metabolic efficiency of microbial cells. Further investigation of this master reaction model and the above phenomena (T. Ross, unpublished) has revealed a predicted temperature for optimum metabolic efficiency to be in the middle of a temperature range in which yield is constant, referred to as 'normal physiological range' (Neidhardt *et al.*, 1990), which is ~21.3°C for *L. monocytogenes* (7°-35.5°C, Fig. 5.14). At this optimum temperature for metabolic efficiency, the maintenance energy is postulated to be minimised and metabolic coordination is optimised. Therefore, this optimum temperature permits the microbe to be able to grow at the most extreme value of a second constraint to growth.
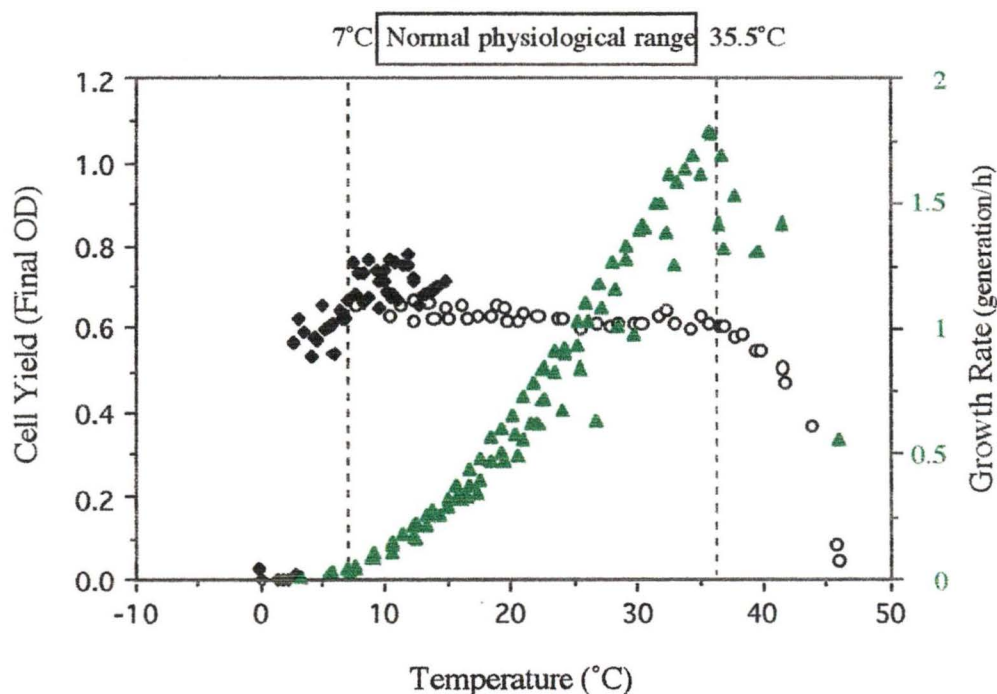
Figure 5.14 Change in cell yield (♦,O) (from Fig. 4.19) and growth rate (▲) of *L. monocytogenes* Scott A as a function of temperature. Rapid decline of yield occurred at temperatures approaching high and low temperature limits for growth. The normal physiological range of temperature for *L. monocytogenes* growth, 7°C to 35.5°C, were estimated from the constant range of cell yield. Thus, the middle of this range is the optimum temperature for metabolic efficiency which is 21.3°C. The yield data are reproduced from J. Kettlewell (unpublished). The growth rate data were from Ross (1993), J. Kettlewell, and this study (Chapter 4).

Several lines of experimental evidence lend support to this hypothesis. For some instances, Sorrells *et al.* (1989) reported growth of several strains of *L. monocytogenes* in TSB acidified by HCl, lactic acid or other acidulants occurred in lower pH at 25°C but not at 10° and 35°C. Several reports of the greatest antimicrobial effect of acidity on *L. monocytogenes* occurred at temperature of 35°C when compared to at 7°, 13°, and 21°C (Ahamad and Marth, 1989; 1990). In addition, temperature at 25°C was also reported (Salter, 1998) to provide an increase in osmotic tolerance for *E. coli* which its optimum temperature for growth rate is ~40°C.

5.4.2 WATER ACTIVITY-PH-LACTIC ACID RESPONSE

The growth limits for *L. monocytogenes* determined by the interaction between water activity, and pH, in the absence or presence of lactic acid, reveals a synergistic effect, especially at the low water activity levels. When HCl was the acidulant, the optimum water activity for growth was shown to be ~0.995, however, when lactic acid was added, this optimum a_w appeared to shift toward a lower value of water activity, between 0.95 to 0.995. At this range of a_w, the pH limit to growth appeared to be less sensitive to water activity as the lactic acid concentration increased. The increase in pH sensitivity by lactic acid was apparent at high a_w. While the physiological basis for this is unknown, Cole *et al.* (1990) reported that low concentrations of salt, 4-6% NaCl (a_w of 0.977-0.964), provided a slight protective effect against inactivation of *L. monocytogenes* at low pH values and 4-8% NaCl (a_w of 0.977-0.950) provided a rapid recovery for pH-injured cells than in the absence of salt. Other workers (de Martinis *et al.*, 1997) also reported a low level of salt (2-3.5% NaCl equal a_w of 0.989-0.980) to be an optimum level in supporting *L. monocytogenes* to tolerate other type of food preservatives including nisin.

Similar to the finding reported in the previous section that ~21°C is the optimum temperature for metabolic efficiency, in the combination effect of water activity and pH, *L. monocytogenes* was also found to tolerate low pH better at temperature of 20°C than at 30°C. This emphasizes the essential role of incubation temperature on the tolerance of *L. monocytogenes*, in particular in studies of the potential effect of a preservative on microbial growth or survival.

Reduced a_w enhanced *L. monocytogenes* inhibition as can be noticed from a steady decrease in amount of [H⁺] required at the growth/no growth interfaces (Figs. 5.7a and 5.10a) as a_w decreases. The decrease in slopes of [H⁺] when lactic acid was added results from the additional inhibitory effect of [UD].

The cause of the different results from kinetic and probability experiments at extreme a_w level (Figs. 5.3c,d and 5.9c,d) which occurred in both strains is not obvious. For these anomalies (8 conditions), growth occurred at a lower pH values in the probability experiments while did not occur in the kinetic studies. There were some variations in performing the experiments; 1) aeration: the kinetic experiment was processed on the TGI operating with shaking (~33±1 rpm) while the growth/no growth experiments were incubated statically. The increase in amount of oxygen was not reported to pose any more inhibition of *Listeria* (ter Steeg *et al.*, 1995), although there was no report on the effect of rocking, 2) amount of inoculum: McClure *et al.* (1989) reported on the effect of inoculum size on NaCl inhibition for *L. monocytogenes*, i.e. the higher inoculum size the higher probability for survival. In these studies, higher inoculum size (~9×10^7 cfu/ml) was used in kinetic studies to provide sufficient turbidity for the upper sensitivity of the spectrophotometer when compared to ~6×10^6 cfu/ml used in probability studies. Thus, this is not the reason for the no growth observed in kinetic studies, 3) amount of nutrient: 15 ml TSB-YE was prepared for kinetic experiments while 2 ml of similar broth (quadruplicate) was used in probability experiments. Again, this is not likely to be the reason, and 4) time for observation: in probability studies growth at those supporting conditions was always observed within 3 weeks which was the incubation time for kinetic studies,thus, sufficient time for any growth in the kinetic experiments to be observed. Viable counts of the broth cultures were also performed to confirm the no growth result. However, as several positive results were found from the probability studies, there may be some unknown factors in the kinetic studies which caused these erratic growth/no growth results. There may be fluctuation in temperature which at the very limit for growth, may be very significant. Apart from these anomalies, the data obtained from kinetic studies agree with the results from probability studies and, thus, the integration of data from both studies for generation of the probability model is supported.

To summarize; the increase in low temperature stress or osmotic stress caused an increase in the pH sensitivity which demonstrates a synergistic effect of both, especially at extreme conditions. Although the temperature and a_w units are different and can not be compared, the trends in pH-sensitivity in combination with lowered temperature or reduced a_w over the permissible range can be noticed. For pH-temperature stress, there is, in general a consistent pH value which prevents growth in the temperature range of 10-30°C with optimum temperature at ~20°C, both in the absence and presence of lactic acid. However, in the pH-a_w stress experiments, when HCl was the acidulant, there was a steady increase in the pH which prevented growth in the range of a_w from 0.995-0.95. The addition of lactic acid appeared to change the inhibitory characteristic in that less sensitivity to pH occurred in this range of a_w. The differences between the effects of pH-temperature stress (Figs. 5.2 and 5.4) and pH-a_w stress (Figs. 5.7 and 5.10) can be seen

more clearly with the trends effect of [H⁺] and [UD] discussed above. Thus, these observations suggest that temperature and a_w may exert their effects on cellular functions by different mechanism.

5.4.3 LACTIC ACID-PH RESPONSE

At a constant temperature (~21°C) and a_w (~0.96) the increase in pH sensitivity was observed with the increase in concentration of lactic acid (Figs. 5.11 and 5.12). The minimum pH_I at which growth occurred was found to increase as the lactic acid concentrations increased. For example, a 10-fold increase in lactic acid concentration (e.g. from 20 mM to 200 mM) resulted in an increase of ~0.6 pH unit at the growth limit. Similar findings that the rate of inactivation was dependent on pH, type and concentration of acidulant were reported for *L. monocytogenes* (Sorrells *et al.*, 1989; Buchanan and Golden, 1994), *Yersinia enterocolitica* (Adams *et al.*, 1991), and *Vibrio paraheamolyticus* (Miles, 1994).

Figs. 5.11b and 5.12b illustrate the predominant effect of [H⁺] in the absence and in the low level of lactic acid. This pH effect decreased rapidly with the addition of small amount of lactic acid. Above ~50 mM lactic acid, a steady decrease in the effect of [H⁺] was observed. This effect occurred correspondingly with the effect of [UD] in that, at low concentrations of lactic acid less [UD] was observed at the inhibitory conditions (Figs. 5.11c and 5.12c) as greater inhibitory effect was caused by [H⁺]. The [UD] effects became more profound with the increase of lactic acid concentration as shown by the rise of the growth/no growth interface. Above ~50 mM a consistent level of [UD], ~4.5 mM, was observed at the interface of 50% probability for growth. The combination effect of [H⁺] and [UD] on the inhibition of *L. monocytogenes* is in agreement with the finding in Chapter 4 where the rate of inactivation caused by each component was separately calculated (see section 4.4.1.3).

The decrease of [H⁺] and increase of [UD] at the growth/no growth interface following the increase of lactic acid concentration and the minimum pH_I for growth were clearly explained by the models underlying hypotheses (Figs. 5.11 and 5.12). This illustrates the good performance of the predicted growth/no growth interface obtained from the probability models (Eqns. 5.1 and 5.2) including the data from kinetic studies.

The water activity in the lactic acid concentration studies was ~0.96 which is a typical water activity of cold-smoked salmon. Figs. 5.11 and 5.12 demonstrate that at ~20°C (represents a temperature abuse) none of the levels of lactic acid tested (up to 450 mM) could inhibit growth of *L. monocytogenes* in cold-smoked salmon at its typical pH of ~6.0. At 5°C, a_w ~0.96 and pH ~6.0 (data not shown graphically, see Appendix G,

Table G. 6), ≥350 mM lactic acid was required to inhibit growth of *L. monocytogenes*. Although one of the main functions of lactic acid used in food products, apart from the preservation, is flavour enhancement, and the use of lactic acid in foods is not limited (see section 4.1), a change in organoleptic properties may be caused by the use of such a high level of lactic acid. Alternatively, the pH of cold-smoked salmon could be manipulated by lactic acid. This possibility was investigated, as a part of validation, described in Chapter 6. It is noteworthy that the high inoculum of *L. monocytogenes* used in preparing the data for the predictive models represents a worse case scenario. Naturally contaminated cold-smoked salmon was generally reported to have <10-100 cfu *L. monocytogenes* /g (see section 3.1.1.1).

5.4.4 INTER-STRAIN VARIABILITY

A high degree of similarity of the levels of pH, in combination with temperature, a_w, or lactic acid required to prevent growth of *L. monocytogenes* was found for the two strains investigated. Similar parameter estimates, a_{wmin}, pH_{min}, and U_{min}, were generated from both probability models (Eqns. 5.1 and 5.2). Good performance observed when fitting both models to the observed data indicate the validity of the methodology used in modelling and in predictions for both strains. Thus, this suggests there were no substantial differences between the strains of *L. monocytogenes* (Scott A and L5) employed in this study, and a single model may be sufficient for this species for the conditions tested in this study.

6 MODELS VALIDATION

6.1 INTRODUCTION

Predictive models, whether kinetic or probabilistic, (as demonstrated in Chapters 4 and 5 respectively) are developed typically from observations of growth and/or no growth of microbes in well-defined liquid substrates and under well-controlled environmental conditions. Although good fit of the models to the data used to generate them was demonstrated in the previous chapter, before the models can be used in practice it is necessary also to test their performance in foods, which are heterogeneous and ill-defined environments. This is the so-called 'validation' process (Ross, 1993).

As there are numerous types of foods available, it is well documented (WHO Working Group, 1988; Mackey and Bratchell, 1989) that *L. monocytogenes* can be eliminated by adequate cooking. Therefore, in this study the models were developed with intended application to ready-to-eat foods which are consumed without any subsequent heating. In particular, cold-smoked salmon, a lightly preserved RTE food which is sliced, reformed and handled without any listericidal process and, additionally, can support growth of *L. monocytogenes* (see review in section 3.1.1) was considered. In addition to temperature, water activity, and acidity as major factors controlling growth of microbes in foods, the models also contain lactic acid concentrations as variable which is of interest as another factor for non-thermal inactivation of *L. monocytogenes*.

A number of validation methods can be used to assess the predictive ability of a model (see details in McMeekin *et al.*, 1993, pp. 59-60). In this study challenge tests involving direct addition of different levels of lactic acid onto traditional cold-smoked salmon products were performed as an approach to non-thermal inhibition or inactivation of *L. monocytogenes* and also to test the performance of the models. In addition, to assess the potential for the model to be used generally, the models prediction to different types of foods supporting growth of different strains of *L. monocytogenes* reported in literature were also evaluated. The bias and accuracy factors described earlier (see section 4.1.1.3) were employed as an indication of the reliability of the models.

6.2 MATERIALS AND METHODS

6.2.1 MATERIALS

Details of consumables, reagents and media, and equipment used are presented in Appendix A.

6.2.2 METHODS FOR VALIDATION OF KINETIC MODELS

Predicted growth rates from the models developed in Chapter 4 were corrected, using Eqn. 4.6, for the systematic difference between the estimates from turbidity and viable count data (see section 4.1.1.1). The corrected growth rate was compared to independent growth rate data obtained from: 1) challenge test results for the traditional or lactic acid modified cold-smoked salmon, and 2) published data for the growth rates of *L. mono-cytogenes* in various foods, using bias (Eqn. 4.9) and accuracy (Eqn. 4.10) factors.

6.2.2.1 Validation using results from challenge tests on cold-smoked salmon

L. monocytogenes L5, a cold-smoked salmon wild type strain, was employed in a series of experiments on traditional cold-smoked salmon and that product modified by the addition of various concentration of L-lactic acid.

Preliminary tests

Two batches of thin sliced (~3 mm thickness) cold-smoked salmon (*Salmo salar*) produced in two different processing runs were obtained from a local commercial producer. The first batch of the product was used in the study of 'aerobic incubation without lactic acid treatment' described below. The second batch was used for all the other challenge tests, i.e. 'vacuum-packed with and without lactic acid treatment'. To ensure the absence of *Listeria* spp. in the products prior to the inoculation of *L. mono-cytogenes* L5, the product was tested using the method described in section 3.2.2.2.

Growth rate determinations on cold-smoked salmon

- Sample Preparation and Inoculation *L. monocytogenes* L5 was grown as described in section 4.2.2.1. To minimise changes in a_w, washed cells were suspended in 100 ml of chilled (4°C) 5.5% NaCl, to obtain approximately 1×10^5 cfu/ml. Ross (1993) found that 7% NaCl, had no effect on viability of cells during a similar inoculation process. The inoculum was kept in an ice water bath before and during the inoculation process.

Water activity in various parts of the product was measured, and sites with consistent a_w were cut into 4 cm × 4 cm pieces ready for the two sets of experiments described below. All sample preparation and inoculation procedures were performed in a laminar flow cabinet to prevent extraneous contaminants.

a) Aerobic incubation without lactic acid treatment:

The behaviour of *L. monocytogenes* on traditional commercial cold-smoked salmon incubated at 5°C, and 20°C in aerobic packages was determined. Each of 40 pieces was dipped into ~25 ml of prepared culture suspension for 15 sec, removed and placed onto a sterile stainless steel mesh to drain off excess liquid. After dipping ~20 pieces, the inoculum was replaced with a new culture suspension from cold stock. Each of the inoculated pieces was placed in a 100×160 mm stomacher bag (Disposable Products). Excess air was squeezed out by hand, and the bags were folded in half several times, and secured with adhesive tape. All samples were kept on ice before and after the inoculation, until incubations were commenced.

b) Anaerobic (i.e. vacuum-packed) incubation with and without lactic acid treatment:

With lactic acid treatment: In order to minimise changes in water activity of the product after immersion into lactic acid, each concentration of lactic acid, i.e. 200, 250, 300, 350, and 450 mM, was also prepared by adding filter sterilised lactic acid into sterile 5.5% NaCl to match the a_w of the product in a 100 ml volumetric flask. Each piece was dipped in the prepared concentration of lactic acid for 15 seconds and then left on a sterile stainless steal mesh to drain off excess liquid. After dipping ~15 pieces, a fresh lactic acid suspension was used. To mimic the oxygen permeability of the vacuum-packed retail product, each piece of sample was placed separately in a 172×253 mm plastic bag as used by the processor to package products for retail display and sale, and weighed. All samples were kept on ice before and after lactic acid application. To avoid changes in lactic acid concentration during sample preparation, the process was started from the lowest concentration of lactic acid, 200 mM, and the stainless steel mesh was dried with a sterile paper towel and sprayed with alcohol between each concentration of lactic acid. Samples were inoculated as described below:

Without lactic acid treatment: Each piece was kept separately in the retail package used by the processor, and weighed. The samples were inoculated as described below:

Approximately 1×10⁵ cfu/ml *L. monocytogenes* culture was prepared in the same manner as described above. 25 µl of the culture was spread onto each side of cold-smoked salmon piece to obtain approximately 10³ cfu/g or 10⁴ cfu/piece. a_w of the inoculated products were measured. The product was kept on ice, and immediately vacuum-packed

using a chamber vacuum packaging machine operated at vacuum (0.5 mbar) and 60% heat welding power.

Incubation of product

Batches of 20-36 samples individually packaged were then placed in water-tight plastic bags and immersed in 5°C or 20°C water baths. To ensure complete temperature control, all bags were weighted down so that samples were incubated below the level of water in the water bath.

Assessment of growth

Duplicate samples were withdrawn at 10 to 18 sampling intervals. For the trials with high levels of lactic acid where *L. monocytogenes* was anticipated to be inhibited or grow very slowly, monitoring was continued for up to 26 days which is the recommended shelf-life of the retail product at 5°C. Chilled diluent (0.1% peptone+0.85% NaCl) was added in the ratio of 4 mls or 9 mls per gram of product (preinoculation weight). The sample was stomached for ~2 min. Serial tenfold dilutions of the homogenate were then prepared in 0.1% peptone+0.85% NaCl (ambient temperature). Spread plates of three dilutions were performed on Listeria selective agar base with Listeria Selective Supplement (Oxford Formulation) (OXF, Oxoid), in duplicate, and on TSA-YE (replicated in some dilutions). The plates were incubated for 36-48 hr at 30°C. After completion of the enumeration process, the pH of the homogenate was measured. Two samples from each block of experiments were withheld at the commencement of incubations. These samples were immediately processed as described above to provide estimates of 'zero time' counts for incubations at all levels of lactic acid in that block. The number of *L. monocytogenes* L5 was determined from the number of typical colonies on OXF. Total viable counts (TVC) were determined from the numbers on TSA-YE. Colony counting methods are described in Appendix A, section A.2.2.

Growth rate estimation

Growth rate, estimated from colony counts, were calculated from the fitted parameters of Eqn. 4.1 using Eqn. 4.2. Generation time was calculated as the reciprocal of the growth rate.

6.2.2.2 Validation using Data from literature

Growth data of *L. monocytogenes* from a range of published challenge tests with cold-smoked salmon, fish products and various foods were compiled. Comparison of those generation times with the predictions calculated from four different models were made. In cases when there was no direct report of generation time in the literature, it was manually

calculated (see Fig. 4.1) from a plot of the growth curve data. In cases when the relevant values were reported as a range or were not documented, representative or estimated values appropriate to the product were used. Alternatively, values cited in other publications (Buchanan *et al.*, 1993; Ross, 1993; Dalgaard and Jørgensen, 1998) in which literature values were compared to model predictions, were adopted.

Natural accumulation of lactic acid in fish flesh generated from anaerobic conversion of its glycogen reserves during rigor mortis is reported (Partmann, 1965; Sikorski *et al.*, 1990). In fresh salmon muscle, values were reported to range from 0.6 to 1.0% (Partmann, 1965). Approximate 5000 ppm (0.5%) and 5000-10000 ppm (0.5-1%) lactate were also found in cold-smoked salmon produced in Denmark (Dalgaard, 1997) and Canadian cold-smoked salmon (Truelstrup Hansen *et al.*, 1995) respectively. The average level of 8,000 ppm water phase lactate (~89 mM) (Truelstrup Hansen *et al.*, 1995; Dalgaard and Jørgensen, 1998) was, therefore, also included in the comparisons of the models predictions for 'fish' in this study (Table 6.1-6.3).

Note that predictions were obtained only by interpolation within the ranges covered by the models (Chapter 4, Tables 4.4 and 4.5). Data for which one or more factors exceeded the range of the model, could result in the need to calculate the logarithm of a negative number, which is not possible. This is indicated as "not done" (ND) in the results.

6.2.2.3 Indices of bias and accuracy

The indices of goodness-of-fit of a kinetic model to the observed data introduced by Ross (1996) are the "bias" and "accuracy" factors. These indices are employed in the validation of kinetic models in this chapter to serve as an assessment of the models performance. To avoid reiteration (see section 4.1.1.3), only the equations are re-presented here.

$$\text{BIAS factor} = 10^{(\Sigma \log(GT_{observed}/GT_{predicted}))/n} \tag{4.9}$$

$$\text{ACCURACY factor} = 10^{(\Sigma |\log(GT_{observed}/GT_{predicted})|)/n} \tag{4.10}$$

where $GT_{observed}$ is the observed generation time (h), $GT_{predicted}$ is the predicted generation time (h), and n is the number of observations used in the calculation.

6.2.3 METHODS FOR VALIDATION OF PROBABILITY MODELS

Data reported in the literature were transformed to "growth" or "no growth" and compared to the probability models (Eqns. 5.1 and 5.2) predictions, using two methods: 1) a

graphical method; comparisons were made with the growth/no growth interfaces predicted by the models corresponding to the conditions reported in the literature, 2) a tabular method comparing percentage of probability for growth; the no growth conditions (0% probability for growth) from the published data were compared to the predicted percent probability for growth A predicted probability for growth of $\leq 50\%$ ($P \leq 0.5$) was considered a correct prediction of no growth. These comparisons were combined and presented as percentage correct predictions.

6.3 RESULTS

6.3.1 VALIDATION OF KINETIC MODELS

Table 6.1 presents comparisons of the generation times of $L.$ $monocytogenes$ L5 from the novel challenge tests on cold-smoked salmon and the predicted values from Eqns. 4.17a,b and 4.18a,b developed for $L.$ $monocytogenes$ Scott A and L5 respectively. Comparison of predicted generation times from those equations versus published generation times of various strains of $L.$ $monocytogenes$ on cold-smoked salmon and fish products is presented in Table 6.2. Reported generation times in laboratory media and food which contained lactic acid were also compared to the predictions (Table 6.3). Published generation times of $L.$ $monocytogenes$ Scott A, and other strains on various foods compared to predicted values are presented in Table 6.4, and 6.5(a-c) respectively. Table 6.6 compares those equations to generation times determined in naturally contaminated cold-smoked salmon. The bias and accuracy factors, indicating the models' performance, are also given in each Table and summarised in Table 6.7.

Table 6.1 Evaluation of Eqns. 4.17a,b and 4.18a,b for the growth of *L. monocytogenes* Scott A and L5 respectively by comparison to novel data on cold-smoked salmon.

Pack-aging[a]	Temp[b] (°C)	Water activity	pH	LAC[c] (mM)	Observed GT[d] (h)	Predicted Generation Time (h) Models Scott A: 4.17a	4.17b	Models L5: 4.18a	4.18b
Air	5	0.966	6.3	0	18.06	57.92	43.12	37.45	34.54
"	20	0.966	6.3	0	2.29	2.14	2.00	2.09	1.84
VP	20	0.974	6.3	0	1.69	1.79	1.68	1.74	1.54
"	5	0.971	6.3	0	36.4	51.67	38.59	33.19	30.78
"	5	0.973	6.0	200	59.34	90.39	69.95	50.81	55.17
"	5	0.976	5.93	250	96.39	113.32	87.00	58.19	65.29
"	5	0.976	5.85	300	164.46	265.12	193.11	94.01	108.86
"	5	0.974	5.8	350	302.04	ND	ND	278.48	312.04
"	5	0.970	5.58	450	NG	ND	ND	ND	ND
		Bias factor				0.69	0.87	1.09	1.09
		Accuracy factor				1.47	1.24	1.32	1.29
Including 89 mM Lactic acid[e]:		Bias factor				0.55	0.69	0.83	0.80
		Accuracy factor				1.82	1.48	1.26	1.30

[a] Packaging: Air, Air, Aerobic-packed; VP, Vacuum-packed. [b] Temperature. [c] Lactic acid. [d] Generation time. [e] Indices if approx. concentration of naturally occurring lactic acid was included in calculation (see section 6.2.2.2). ND = Not done, lactic acid or undissociated lactic acid range not in square-root equation. NG = No growth observed within the 26 days experiment.

Table 6.2 Evaluation of Eqns. 4.17a,b and 4.18a,b by comparison to published generation times of *L. monocytogenes* in cold-smoked salmon and fish products.

Ref[a]	Fish product	*L. mono-cytogenes* strain	Pack-aging[b]	Temp[c] (°C)	Water activity	pH	Observed GT[d] (h)	Predicted Generation Time (h) Models Scott A: 4.17a	4.17b	Models L5: 4.18a	4.18b
1	Cold-smoked salmon	Cocktail: NCTC 7973, L296, L419	VP	4	0.972	6.19	57.80[e]	97.63	66.71	52.71	50.97
			"	4	0.972	6.19	48.19[e]	97.63	66.71	52.71	50.97
			"	4	0.978	6.13	45.87	86.66	59.84	46.50	45.52
2	Cold-smoked salmon	Cocktail: NCTC 7973, L70	VP	5	0.974	6.1	19.30	48.58	37.21	31.09	29.56
			Air	5	0.974	6.1	21.20	48.58	37.21	31.09	29.56
			VP	10	0.974	6.1	8.10	8.43	7.59	7.21	6.51
			Air	10	0.974	6.1	6.70	8.43	7.59	7.21	6.51
3	Cured salmon (*Oncorhynchus keta*)	Scott A	VP	5	0.983	6.1	33.92[e]	41.40	31.82	26.29	25.16
			"	5	0.970	6.2	31.70[e]	52.47	39.58	33.72	31.56
			"	5	0.964	6.2	53.77[e]	61.31	46.05	39.79	36.94
			Air	5	0.983	6.0	38.93[e]	41.54	32.48	26.39	25.65
			"	5	0.970	6.2	35.03[e]	52.47	39.58	33.72	31.56
			"	5	0.964	6.2	NG[e]	61.31	46.05	39.79	36.94
			VP	10	0.983	6.1	11.12[e]	7.18	6.49	6.10	5.54
			"	10	0.970	6.1	9.64[e]	9.13	8.21	7.84	7.06
			"	10	0.964	6.1	21.23[e]	10.67	9.55	9.25	8.26
			Air	10	0.983	6.0	9.62[e]	7.21	6.63	6.12	5.65

(continued overleaf)

Table 6.2 (contd.) Evaluation of Eqns. 4.17a,b and 4.18a,b by comparison to published generation times of *L. monocytogenes* in cold-smoked salmon and fish products.

Ref[a]	Fish product	*L. mono- cytogenes* strain	Pack- aging[b]	Parameters: Temp[c] (°C)	Parameters: Water activity	Parameters: pH	Observed GT[d] (h)	Predicted Generation Time (h) Models Scott A: 4.17a	Predicted Generation Time (h) Models Scott A: 4.17b	Predicted Generation Time (h) Models L5: 4.18a	Predicted Generation Time (h) Models L5: 4.18b
3 (contd.)	Cured salmon	Scott A	Air	10	0.970	6.1	10.95[e]	9.13	8.21	7.84	7.06
			"	10	0.964	6.1	17.53[e]	10.67	9.55	9.25	8.26
			VP	5	0.983	5.9	24.70	41.28	33.36	26.51	26.30
			"	5	0.970	6.2	43.37	52.47	39.58	33.72	31.56
			"	5	0.964	6.2	62.07	61.31	46.05	39.79	36.94
			"	10	0.983	6.1	10.84	7.18	6.49	6.10	5.54
			"	10	0.970	6.1	12.05	9.13	8.21	7.84	7.06
			"	10	0.964	6.1	13.86	10.67	9.55	9.25	8.26
4	Minced mussels	NCTC 7973+L70	-	5	0.997	6.7	20.7	32.94	24.24	20.73	19.14
			-	10	0.997	6.7	7.3	5.72	4.95	4.81	4.22
5	Crawfish tail meat	cocktail	Air	0	0.997	6.6	72.2	ND	ND	ND	ND
			"	6	0.997	6.6	17	20.12	15.77	14.05	12.78
			"	12	0.997	6.6	6.9	3.76	3.34	3.30	2.89
6	Smoked salmon	ATCC 19115	-	4	0.975	6.2	171.89	93.85	64.11	50.56	48.92
			-	8	0.975	6.2	12.85	14.36	12.30	11.49	10.37
7	Smoked salmon	SLCC 2755	-	4	0.945	6.1	45.13	228.11	152.78	132.89	121.80
			-	10	0.945	6.1	29.2	20.52	17.87	18.98	16.04
		wild type	-	4	0.945	6.1	41.52	228.11	152.78	132.89	121.80
			-	10	0.945	6.1	19.65	20.52	17.87	18.98	16.04
8	Smoked salmon	1A1	Air	22	0.995	6.6	0.92	1.02	0.95	0.98	0.87
			"	22	0.995	6.6	1.25	1.02	0.95	0.98	0.87
			"	30	0.995	6.6	0.40	0.53	0.50	0.53	0.51
			"	30	0.995	6.6	0.50	0.53	0.50	0.53	0.51
9	Comminuted salmon	Scott A	VP	5	0.980	6.2	61.45[e]	41.14	31.20	26.12	24.70
			"	10	0.980	6.2	12.05[e]	7.14	6.37	6.06	5.44
			Air	5	0.980	6.2	24.10[e]	41.14	31.20	26.12	24.70
			"	10	0.980	6.2	12.05[e]	7.14	6.37	6.06	5.44
		Bias factor						0.90	1.10	1.21	1.32
		Accuracy factor						1.52	1.46	1.46	1.54
Including 89 mM lactic acid[f]:		Bias factor						0.80	0.98	1.10	1.14
		Accuracy factor						1.55	1.43	1.42	1.45

[a] Reference: 1 Rorvik *et al.*, 1991; 2 Hudson and Mott, 1993a; 3 Peterson *et al.*, 1993. 4 Hudson, 1994; 5 Dorsa *et al.*, 1993; 6 Rosso *et al.*, 1996; 7 Guyer and Jemmi, 1991; 8 McCarthy, 1997; 9 Pelroy *et al.*, 1994. [b] Packaging: VP, Vacuum-packed; Air, Aerobic-packed. [c] Temperature. [d] Generation time. [e] The *L. monocytogenes* inoculum was ≤10 cfu/g. [f] Indices if approx. concentration of naturally occurring lactic acid was included in calculation (see section 6.2.2.2). NG; No growth. ND, Not done; temperature range not in the fitted Square-root models.

Table 6.3 Comparison of predictions of Eqns. 4.17a,b and 4.18a,b to published generation times of various strains of *L. monocytogenes* in laboratory broth media and food contained lactic acid.

Ref[a]	Broth	*L. mono-cytogenes* strain	Temp[b] (°C)	Water activity	pH	Lac[c] (mM)	Observed GT[d] (h)	Models Scott A 4.17a	Models Scott A 4.17b	Models L5 4.18a	Models L5 4.18b
1	TSB + Yeast extract and Glucose	F6861	20	0.994	4.70	19.5	7.87	5.71	ND	4.14	ND
			20	0.994	7.20	195.4	2.22	1.30	1.19	1.23	1.20
			16	0.994	5.50	24.4	4.27	2.55	3.04	2.29	2.59
			8	0.994	7.00	73.3	11.11	10.28	8.50	8.08	7.40
			4	0.994	6.10	195.4	47.62	95.98	66.22	47.36	52.33
2	TSB	Scott A	19.5	0.990	7.60	200	1.96	1.43	1.30	1.36	1.32
			19.5	0.990	7.45	200	1.87	1.44	1.31	1.37	1.33
			19.5	0.990	7.25	200	1.86	1.45	1.32	1.38	1.34
			19.5	0.990	6.90	200	2.00	1.50	1.37	1.41	1.38
			19.5	0.990	6.55	200	1.85	1.60	1.48	1.49	1.47
			19.5	0.990	6.20	200	2.07	1.90	1.80	1.71	1.73
			19.5	0.990	6.00	200	2.41	2.36	2.28	2.03	2.09
			19.5	0.990	5.85	200	3.41	3.21	3.18	2.53	2.68
			19.5	0.990	5.80	200	5.80	3.77	3.76	2.82	3.02
			19.5	0.990	5.65	200	10.60	10.74	10.27	4.88	5.43
		MurrayB	19.5	0.990	7.60	200	1.88	1.43	1.30	1.36	1.32
			19.5	0.990	7.50	200	1.87	1.44	1.31	1.37	1.33
			19.5	0.990	7.30	200	1.83	1.45	1.32	1.37	1.34
			19.5	0.990	6.90	200	1.90	1.50	1.37	1.41	1.38
			19.5	0.990	6.55	200	1.97	1.60	1.48	1.49	1.47
			19.5	0.990	6.10	200	2.13	2.08	1.99	1.84	1.88
			19.5	0.990	5.90	200	2.27	2.83	2.78	2.32	2.43
			19.5	0.990	5.80	200	2.98	3.77	3.76	2.82	3.02
			19.5	0.990	5.75	200	4.35	4.68	4.68	3.22	3.50
			19.5	0.990	5.70	200	8.23	6.39	6.35	3.84	4.23
3	Comminuted salmon	Scott A	10	0.983	6.20	222	19.28[e]	9.82	8.68	7.82	7.96
			10	0.989	6.20	278	21.08[e]	9.84	8.68	7.62	8.05
			10	0.986	6.20	278	18.07[e]	10.32	9.10	8.01	8.45
			10	0.983	6.20	278	24.10[e]	10.85	9.55	8.44	8.89
			10	0.989	6.20	333	19.88[e]	10.96	9.63	8.26	9.04
			10	0.986	6.20	333	29.52[e]	11.50	10.10	8.68	9.48
	Bias Factor							1.30	1.39	1.59	1.56
	Accuracy Factor							1.39	1.46	1.59	1.57
Including 89 mM lactic acid	Bias Factor							1.26	1.36	1.56	1.51
in fish data (Ref. 4)[f]:	Accuracy Factor							1.35	1.42	1.56	1.53

[a] Reference: 1 George *et al.*, 1996; 2 Ross (1993). 3 Pelroy *et al.*, 1994. [b] Temperature. [c] Lactic acid [d] Generation time. [e] The *L. monocytogenes* inoculum was ≤10 cfu/g. [f] Indices if approx. concentration of naturally occurring lactic acid was included in calculation (see section 6.2.2.2). ND, Not done; pH range not in the fitted Square-root models.

Table 6.4 Comparison of predictions of Eqns. 4.17a,b and 4.18a,b to published generation times of *L. monocytogenes* Scott A on foods.

Ref[a]	Food	pack-aging[b]	Temp[c] (°C)	Water activity	pH	Observed GT[d] (h)	Models Scott A 4.17a	4.17b	Models L5 4.18a	4.18b
1	Whole milk	Air	10	0.995	6.4	10	5.90	5.18	4.97	4.41
		"	10	0.995	6.4	7	5.90	5.18	4.97	4.41
2	Skim milk	Air	4	0.995	6.5	32.3	65.49	43.94	34.73	33.28
		"	8	0.995	6.5	12.6	10.02	8.43	7.89	7.05
		"	13	0.995	6.5	6.13	3.23	2.91	2.89	2.53
	Whole milk	"	4	0.995	6.5	31	65.49	43.94	34.73	33.28
		"	8	0.995	6.5	13.1	10.02	8.43	7.89	7.05
		"	13	0.995	6.5	5.83	3.23	2.91	2.89	2.53
	Chocolate milk	"	4	0.995	6.5	31.1	65.49	43.94	34.73	33.28
		"	8	0.995	6.5	10.75	10.02	8.43	7.89	7.05
		"	13	0.995	6.5	4.6	3.23	2.91	2.89	2.53
	Cream	"	4	0.995	6.5	32	65.49	43.94	34.73	33.28
		"	8	0.995	6.5	12.25	10.02	8.43	7.89	7.05
		"	13	0.995	6.5	5.83	3.23	2.91	2.89	2.53
3	Uncultured whey	Air	6	0.995	5.6	28.9	21.52	20.61	15.07	16.43
		"	6	0.995	6.2	21.1	20.82	16.82	14.56	13.60
		"	6	0.995	6.8	18	20.66	16.07	14.44	13.04
	Cultured whey	"	6	0.995	5.6	19.4	21.52	20.61	15.07	16.43
		"	6	0.995	6.2	10.3	20.82	16.82	14.56	13.60
		"	6	0.995	6.8	9.5	20.66	16.07	14.44	13.04
4	Baby food	-	12	0.990	5.4	4.9	4.45	5.70	3.93	4.78
		-	12	0.990	6.8	3.1	4.16	3.66	3.66	3.17
		-	12	0.976	5.3	4.9	5.76	8.50	5.15	7.05
		-	12	0.976	6.8	3.9	5.29	4.64	4.71	4.05
		-	20	0.997	5.9	1.8	1.24	1.25	1.18	1.12
		-	20	0.976	5.6	1.6	1.79	2.01	1.73	1.80
		-	25	0.983	6.0	1.35	0.95	0.94	0.93	0.89
		-	30	0.990	5.3	0.6	0.62	0.98	0.62	0.94
		-	30	0.990	6.9	0.6	0.57	0.53	0.57	0.54
		-	30	0.976	5.3	1	0.79	1.24	0.80	1.20
		-	30	0.976	6.8	0.7	0.72	0.68	0.73	0.69
		-	35	0.997	5.9	0.6	0.38	0.42	0.40	0.45
		-	35	0.976	5.6	0.7	0.55	0.67	0.59	0.73
5	Asparagus	Air	4	0.980	5.9	46	84.59	61.06	45.34	46.24
		"	15	0.980	5.9	5.41	3.03	2.98	2.81	2.62
	Broccoli	"	4	0.980	6.5	79.9	83.24	55.60	44.58	42.36
		"	15	0.980	6.5	9.33	2.99	2.71	2.76	2.40
	Cauliflower	"	4	0.980	5.6	55.60	86.46	70.24	46.39	52.65
		"	15	0.980	5.6	7.2	3.10	3.43	2.87	2.98
6	Raw chicken	-	10	0.997	6.8	4.06	5.71	4.93	4.80	4.20
		-	10	0.997	6.8	3.98	5.71	4.93	4.80	4.20
	Bias factor						1.02	1.12	1.28	1.31
	Accuracy factor						1.47	1.45	1.42	1.45

[a] Reference: 1 Marshall and Schmidt, 1988; 2 Rosenow and Marth, 1987; 3 Ryser and Marth, 1988; 4 Walls and Scott, 1997; 5 Berrang *et al.*, 1989; 6 Wimpfheimer *et al.*, 1990. [b] Packaging: Air, Aerobic-packed. [c] Temperature. [d] Generation time.

Table 6.5a Comparison of predictions of Eqns. 4.17a,b and 4.18a,b to published generation times of *L. monocytogenes* Murray B on beef fat. The samples were stored aerobically. Data of Grau and Vanderlinde (1993).

Temp[a] (°C)	Water activity	pH	Observed GT[b] (h)	Models Scott A 4.17a	4.17b	Models L5 4.18a	4.18b
0	0.993	5.7	62.89	ND	ND	ND	ND
0	0.993	5.7	67.57	ND	ND	ND	ND
2.5	0.993	5.7	29.94	400.23	199.66	106.53	128.70
4.7	0.993	5.7	17.12	42.86	35.87	25.96	27.72
4.8	0.993	5.7	18.45	40.36	34.07	24.80	26.42
7.5	0.993	5.7	10.25	12.44	11.94	9.60	9.81
10.1	0.993	5.7	6.49	6.10	6.16	5.16	5.19
10.1	0.993	5.7	6.67	6.10	6.16	5.16	5.19
14.9	0.993	5.7	3.04	2.53	2.66	2.32	2.31
15.0	0.993	5.7	3.24	2.49	2.62	2.28	2.28
19.8	0.993	5.7	1.86	1.36	1.46	1.30	1.31
19.9	0.993	5.7	2.08	1.34	1.45	1.28	1.29
22.0	0.993	5.7	1.52	1.08	1.17	1.05	1.06
24.8	0.993	5.7	1.05	0.84	0.91	0.82	0.85
25.0	0.993	5.7	1.15	0.83	0.90	0.81	0.84
27.4	0.993	5.7	0.88	0.68	0.74	0.67	0.71
30.6	0.993	5.7	0.69	0.54	0.60	0.54	0.60
30.6	0.993	5.7	0.81	0.54	0.60	0.54	0.60
Bias factor				0.92	0.94	1.13	1.08
Accuracy factor				1.61	1.43	1.45	1.44

[a] Temperature. [b] Generation time. ND, Not done; temperature range not in the fitted Square-root models.

Table 6.5b Comparison of predictions of Eqns. 4.17a,b and 4.18a,b to published generation times of *L. monocytogenes* Murray B on beef lean. The samples were stored aerobically. Data of Grau and Vanderlinde (1993).

Parameters			Observed	Predicted Generation Time (h)			
Temp[a]	Water	pH	GT[b]	Models Scott A		Models L5	
(°C)	activity		(h)	4.17a	4.17b	4.18a	4.18b
0	0.993	5.61	NG	ND	ND	ND	ND
5.1	0.993	5.56	26.46	51.73	48.22	33.11	37.50
5.5	0.993	5.51	31.94	42.31	41.98	28.34	32.90
10.1	0.993	5.61	10.67	9.22	9.74	7.80	8.18
15.5	0.993	5.55	5	3.52	4.05	3.25	3.50
22.3	0.993	5.56	2.32	1.60	1.87	1.55	1.69
22.6	0.993	5.59	2.22	1.55	1.78	1.50	1.61
24.9	0.993	5.55	1.87	1.27	1.50	1.24	1.39
25	0.993	5.59	1.83	1.25	1.44	1.22	1.34
25.4	0.993	5.6	1.68	1.21	1.39	1.18	1.29
27.3	0.993	5.56	1.52	1.04	1.23	1.03	1.17
27.4	0.993	5.59	1.56	1.03	1.19	1.02	1.14
29.8	0.993	5.57	1.29	0.86	1.02	0.86	1.01
29.8	0.993	5.6	1.3	0.86	1.00	0.86	0.99
14.8	0.993	5.73	4.33	3.84	3.99	3.51	3.47
35	0.993	5.73	0.81	0.61	0.71	0.65	0.76
0	0.993	6.06	81.3	ND	ND	ND	ND
4.8	0.993	6.09	18.41	59.31	45.08	36.41	35.26
10	0.993	6.09	6.71	9.17	8.32	7.73	7.08
14.4	0.993	6.1	3.6	4.00	3.78	3.65	3.31
14.9	0.993	6.11	3.42	3.71	3.51	3.40	3.08
15.7	0.993	6.08	3.11	3.31	3.16	3.06	2.78
19.8	0.993	6.11	1.94	1.99	1.93	1.90	1.74
19.9	0.993	6.11	1.9	1.97	1.91	1.88	1.72
25.1	0.993	6.08	1.22	1.20	1.18	1.18	1.11
25.4	0.993	6.11	1.12	1.17	1.15	1.15	1.08
30	0.993	6.08	0.85	0.83	0.82	0.82	0.83
30.1	0.993	6.11	0.79	0.82	0.81	0.81	0.82
35	0.993	6.08	0.67	0.60	0.64	0.64	0.69
5	0.993	6.34	24.63	52.53	39.41	33.13	31.11
15.5	0.993	6.32	3.17	3.38	3.13	3.12	2.76
25.1	0.993	6.34	1.03	1.19	1.14	1.17	1.07
0	0.993	6.68	66.67	ND	ND	ND	ND
4.9	0.993	6.7	15.85	55.36	40.39	34.45	31.83
5.2	0.993	6.98	13.55	46.82	34.67	30.26	27.59
10	0.993	6.71	5.88	9.08	7.84	7.65	6.70
14.8	0.993	6.68	3	3.73	3.37	3.41	2.96
15	0.993	6.98	2.79	3.61	3.24	3.31	2.86
20	0.993	6.71	1.79	1.93	1.78	1.84	1.62
26	0.993	6.68	0.96	1.10	1.04	1.08	0.99
		Bias factor		0.92	0.94	1.04	1.04
		Accuracy factor		1.38	1.24	1.27	1.22

[a] Temperature. [b] Generation time. NG; No growth. ND, Not done; temperature range not in the fitted Square-root models.

Table 6.5c Comparison of predictions of Eqns. 4.17a,b and 4.18a,b to published generation times of various strains of *L. monocytogenes* on foods.

Ref[a]	Food	*L. mono-cytogenes* strain	pack-aging[b]	Temp[c] (°C)	Water activity	pH	Observed GT[d] (h)	Predicted Generation Time (h) Models Scott A 4.17a	4.17b	Models L5 4.18a	4.18b
1	Uncultured Whey	OH	Air	6	0.995	5.6	25.2	21.52	20.61	15.07	16.43
		V7	"	6	0.995	6.2	14.8	20.82	16.82	14.56	13.60
			"	6	0.995	6.8	14	20.66	16.07	14.44	13.04
	Cultured Whey	OH	"	6	0.995	5.6	16.5	21.52	20.61	15.07	16.43
			"	6	0.995	6.8	7.3	20.66	16.07	14.44	13.04
2	Camembert	OH	Air	6	0.986	6.1	21.69	23.95	19.55	16.83	15.84
3	Pâté	NCTC 7973	Air	4	0.995	6.1	68.7	66.03	46.03	35.03	34.76
			"	10	0.995	6.1	14.3	5.94	5.38	5.00	4.58
		L70	"	4	0.995	6.1	69	66.03	46.03	35.03	34.76
			"	10	0.995	6.1	14.4	5.94	5.38	5.00	4.58
4	Chicken breast	NCTC 11994	"	6	0.993	5.8	16.9	21.79	19.08	15.26	15.34
				15	0.993	5.8	4.52	2.47	2.51	2.27	2.19
5	Cooked beef	Cocktail:	-	5	0.997	5.8	18.6	33.73	27.87	21.25	21.80
		NCTC 7973,	-	10	0.997	5.8	9	5.85	5.69	4.93	4.80
6	Heated whole egg	Brie-1	-	20	0.980	7	2.1	1.59	1.45	1.53	1.32
	Heated egg yolk		-	20	0.980	6.2	1.76	1.60	1.52	1.54	1.39
7	Skim milk	V7	Air	4	0.995	6.5	37.8	65.49	43.94	34.73	33.28
			"	8	0.995	6.5	9.81	10.02	8.43	7.89	7.05
			"	13	0.995	6.5	4.88	3.23	2.91	2.89	2.53
	Whole milk		"	4	0.995	6.5	36.5	65.49	43.94	34.73	33.28
			"	8	0.995	6.5	10.8	10.02	8.43	7.89	7.05
			"	13	0.995	6.5	5.0	3.23	2.91	2.89	2.53
	Chocolate milk		"	4	0.995	6.5	41.5	65.49	43.94	34.73	33.28
			"	8	0.995	6.5	8.88	10.02	8.43	7.89	7.05
			"	13	0.995	6.5	4.5	3.23	2.91	2.89	2.53
	Cream		"	4	0.995	6.5	46	65.49	43.94	34.73	33.28
			"	8	0.995	6.5	10.25	10.02	8.43	7.89	7.05
			"	13	0.995	6.5	4.75	3.23	2.91	2.89	2.53
8	Minced meat	17a	Air	8	0.997	5.8	10.05	9.96	9.33	7.84	7.73
9	Roast beef	CRA 198	"	5	0.990	6	18.86	36.97	28.97	23.37	22.81
	and gravy		"	10	0.990	6	9.43	6.42	5.91	5.42	5.03
		CRA 433	"	5	0.990	6	17.24	36.97	28.97	23.37	22.81
				10	0.990	6	8.33	6.42	5.91	5.42	5.03
		Bias Factor						0.997	1.17	1.34	1.42
		Accuracy Factor						1.45	1.41	1.45	1.53
Combined results of Table 6.5a, b, and c:		Bias Factor						0.94	1.02	1.16	1.18
		Accuracy Factor						1.45	1.33	1.37	1.37

[a] Reference: 1 Ryser and Marth, 1988; 2 Ryser and Marth, 1987; 3 Hudson and Mott, 1993b; 4 Hart *et al.*, 1991, 5 Hudson, 1994; 6 Sionkowski and Shelef, 1990; 7 Rosenow and Marth, 1987; 8 Schillinger *et al.*, 1991; 9 Grant *et al.*, 1993. [b] Packaging: Air, Aerobic-packed. [c] Temperature. [d] Generation time.

Table 6.6 Comparison of predictions of Eqns. 4.17a,b and 4.18a,b to the growth of *L. monocytogenes* in naturally contaminated cold-smoked salmon stored under vacuum condition. Reproduced from Dalgaard and Jørgensen, 1998.

Initial LM[a] (Log MPN/g)	Parameters: Temp[b] (°C)	Water activity	pH	Lac[c] (mM)	Observed GT[d] (h)	Predicted Generation Time (h) Models Scott A 4.17a	4.17b	Models L5 4.18a	4.18b
0.6	5	0.97	6.2	74.44	72.29	58.26	43.83	36.82	35.86
<0	5	0.98	6.2	104.44	126.5	49.76	37.57	30.88	30.85
<0	5	0.977	6.2	86.67	126.5	51.32	38.73	32.09	31.67
0.9	5	0.97	6.1	97.78	101.2	62.53	47.59	39.01	38.94
0.8	5	0.975	6.1	91.1	253	55.21	42.15	34.31	34.30
<0	5	0.976	6.2	78.89	72.3	51.77	39.06	32.46	31.87
0.6	5	0.978	6.1	115.56	506	55.35	42.2	34.02	34.53
<0	5	0.968	6.2	94.44	506	63.23	47.5	39.88	39.17
0.9	5	0.969	6.3	103.3	168.7	60.34	44.9	38.11	37.33
<0	5	0.944	6.3	97.78	0	139.66	100.4	95.87	87.31
<0	5	0.974	6.2	102.2	0	55.86	42.1	34.89	34.64
<0	5	0.979	6.2	123.3	0	52.25	39.4	32.28	32.57
<0	5	0.965	6.2	83.3	253	65.95	49.4	41.87	40.74
Bias Factor						3.02	4.00	4.82	4.88
Accuracy Factor						3.02	4.00	4.82	4.88

[a] *L. monocytogenes.* [b] Temperature. [c] Lactic acid. [d] Generation time.

Table 6.7 Summary of Bias and Accuracy indices for Eqns. 4.17a,b and 4.18a,b.

Table No.	Bias/ Accuracy	Bias and Accuracy values[a] for Equation No. 4.17a	4.17b	4.18a	4.18b
6.1	Bias	0.69 (0.55)	0.87 (0.69)	1.10 (0.83)	1.09 (0.80)
	Accuracy	1.47 (1.82)	1.24 (1.48)	1.32 (1.26)	1.29 (1.30)
6.2	Bias	0.90 (0.80)	1.10 (0.98)	1.21 (1.10)	1.32 (1.14)
	Accuracy	1.52 (1.55)	1.46 (1.43)	1.46 (1.42)	1.54 (1.45)
6.3	Bias	1.30 (1.26)	1.39 (1.36)	1.59 (1.56)	1.56 (1.51)
	Accuracy	1.39 (1.35)	1.46 (1.42)	1.59 (1.56)	1.57 (1.53)
6.4	Bias	1.02	1.12	1.28	1.31
	Accuracy	1.47	1.45	1.42	1.45
6.5a	Bias	0.92	0.94	1.13	1.08
	Accuracy	1.61	1.43	1.45	1.44
6.5b	Bias	0.92	0.94	1.04	1.04
	Accuracy	1.38	1.24	1.27	1.22
6.5c	Bias	0.997	1.17	1.34	1.42
	Accuracy	1.45	1.41	1.45	1.53
Combined 6.5	Bias	0.94	1.02	1.16	1.18
	Accuracy	1.45	1.34	1.37	1.37
6.6	Bias	3.02	4.00	4.82	4.88
	Accuracy	3.02	4.00	4.82	4.88

[a] Value in the bracket shows the effect of lactic acid (89 mM) included in the models prediction.

6.3.2 VALIDATION OF PROBABILITY MODELS

The performance of the probability models, Eqns. 5.1 and 5.2 developed for *L. monocytogenes* Scott A and L5 respectively, evaluated by fitting the observed data (both growth and no growth) from the literature, with the predicted growth/no growth interfaces at *P*=0.1, 0.5, and 0.9 respectively are shown in Figs. 6.1 and 6.2. The no growth conditions from published data including the data presented in Figs. 6.1 and 6.2, compared to the predicted percent probability for growth, are presented in Table 6.8.

Table 6.8 Comparison of probability predictions by Eqns. 5.1 and 5.2 to reported no growth conditions of *L. monocytogenes* in laboratory media and food.

Ref[a]	Strain	Substrate/Food	Temperature	aw	pH	Lactic acid (mM)	Eqn. 5.1	Eqn. 5.2
1	NCTC 10357	TSB+	4	0.995	5.03	0	0.133	0.806
		1%glucose	7	0.995	4.63	0	0.345	0.560
		+0.3%YE	10	0.995	4.63	0	0.929	0.980
			10	0.995	4.43	0	0.181	0.039
			20	0.995	4.23	0	0.097	0.001
			30	0.995	4.43	0	0.905	0.724
			30	0.995	4.23	0	0.260	0.002
	Scott A		4	0.995	5.03	0	0.133	0.806
			7	0.995	4.61	0	0.268	0.405
			10	0.995	4.79	0	0.990	0.999
			10	0.995	4.59	0	0.871	0.941
			10	0.995	4.39	0	0.068	0.006
			20	0.995	4.2	0	0.038	0.000
			30	0.995	4.2	0	0.150	0.001
	F6868		4	0.995	5.03	0	0.133	0.806
			7	0.995	4.62	0	0.306	0.483
			10	0.995	4.42	0	0.145	0.025
			20	0.995	4.23	0	0.097	0.001
			30	0.995	4.21	0	0.183	0.001
			30	0.995	4.21	0	0.183	0.001
	F7059		4	0.995	5.03	0	0.133	0.806
			7	0.995	4.62	0	0.306	0.483
			10	0.995	4.79	0	0.990	0.999
			10	0.995	4.59	0	0.871	0.941
			10	0.995	4.39	0	0.068	0.006
			20	0.995	4.2	0	0.038	0.000
			30	0.995	4.2	0	0.150	0.001
2	NCTC9863	BHIB	25	0.977	4.5	0	0.784	0.632
			25	0.970	4.5	0	0.493	0.299
			25	0.964	4.5	0	0.191	0.096
			25	0.957	4.5	0	0.028	0.015
			25	0.950	4.5	0	0.002	0.001
			25	0.943	4.5	0	0.000	0.000
			25	0.935	4.5	0	0.000	0.000
			25	0.994	4.0	0	0.000	0.000
			25	0.989	4.0	0	0.000	0.000
			25	0.983	4.0	0	0.000	0.000
			25	0.977	4.0	0	0.000	0.000
			25	0.970	4.0	0	0.000	0.000
			25	0.964	4.0	0	0.000	0.000

Table 6.8 (contd.) Comparison of probability predictions by Eqns. 5.1 and 5.2 to reported no growth conditions of *L. monocytogenes* in laboratory media and food.

Ref[a]	Strain	Substrate/ Food	Tem- perature	Water activity	pH	Lactic acid (mM)	Eqn. 5.1	Eqn. 5.2
2 (cont.)	NCTC9863	BHIB	25	0.957	4.0	0	0.000	0.000
			25	0.950	4.0	0	0.000	0.000
			25	0.943	4.0	0	0.000	0.000
			25	0.935	4.0	0	0.000	0.000
3	Murray B	lean beef	0	0.993	5.6	0	4.56E-12	6.43E-12
4	Wild-types (serotype 1+4)	BHIB	5	0.997	5.5	222	0.000	0.000
			5	0.997	5.5	333	ND	ND
			5	0.997	5.8	333	0.019	0.016
			5	0.997	6.0	333	0.418	0.927
			10	0.997	5.5	222	0.009	0.003
			10	0.997	5.8	222	0.989	0.998
			10	0.997	5.5	333	ND	ND
			10	0.997	5.8	333	0.789	0.977
			10	0.997	6.0	333	0.993	0.999
5	ScottA	Crayfish	4	0.995	6.2	222	0.499	0.985
			4	0.995	6.2	222	0.499	0.985
			4	0.995	6.6	167	0.584	0.992
6	Scott A	Comminuted salmon	5	0.983	6.1	333	0.892	0.979
			5	0.983	6.1	222	0.958	0.997
Accepted ≤ 0.500							77.6%	63.8%

[a] Reference: 1 George *et al.*, 1988; 2 McClure *et al.*, 1989; 3 Grau and Vanderlinde, 1993; 4 Qvist *et al.*, 1994; 5 Pothuri *et al.*, 1996; 6 Pelroy *et al.*, 1994. ND, Not done; pH range not in growth/no growth interface models.

a) Eqn. 5.1 VS Strain NCTC 10357
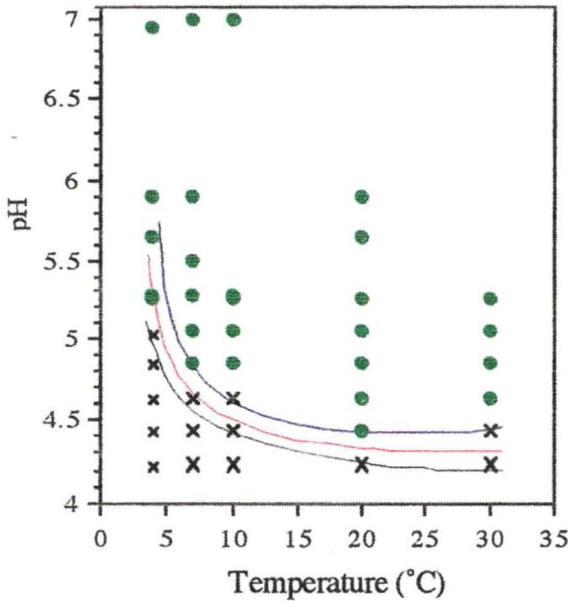
b) Eqn. 5.1 VS Strain Scott A
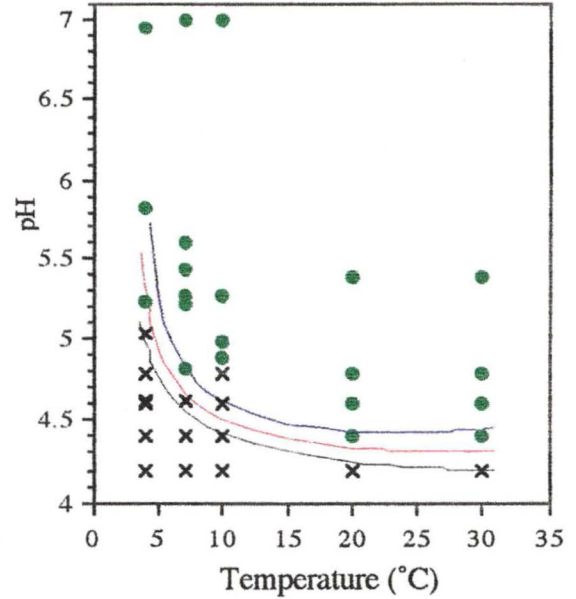
c) Eqn. 5.2 VS Strain NCTC 10357
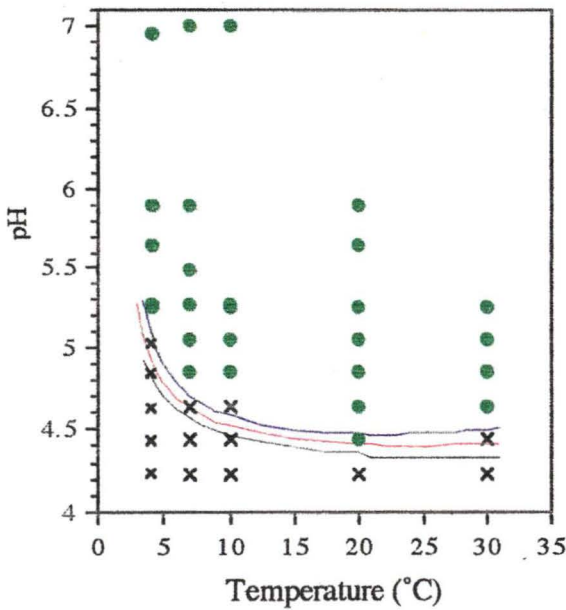
d) Eqn. 5.2 VS Strain Scott A
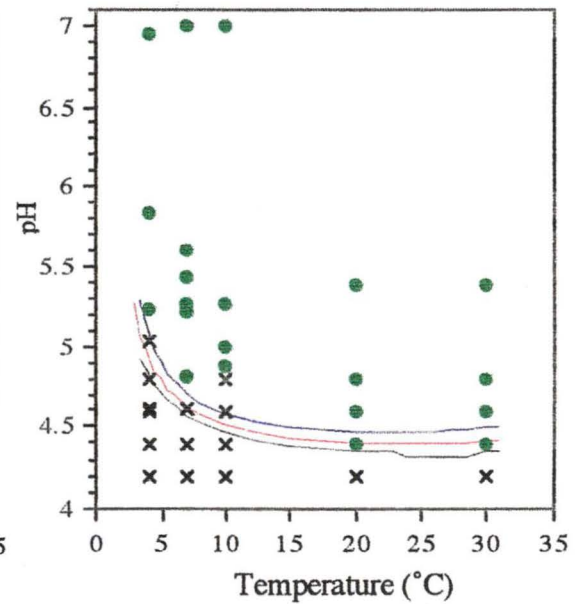
Figure 6.1 Evaluation of the probability models. Data of George *et al.* (1988) for the effect of temperature on the growth (●) and no growth (×) of *L. monocytogenes* NCTC 10357 and Scott A in TSB+1% glucose+0.3% yeast extract (a_w ~0.995) using micro-well plates are shown. The growth/no growth interfaces at P=0.9, 0.5, and 0.1 predicted from Eqn. 5.1 (Figs. a, b) and Eqn. 5.2 (Figs. c,d) are shown as blue, red and black lines respectively. The abruptness of the transition from high (P=0.9) to low (P=0.1) probability of growth is illustrated.

a) Eqn. 5.1 VS NCTC 9863
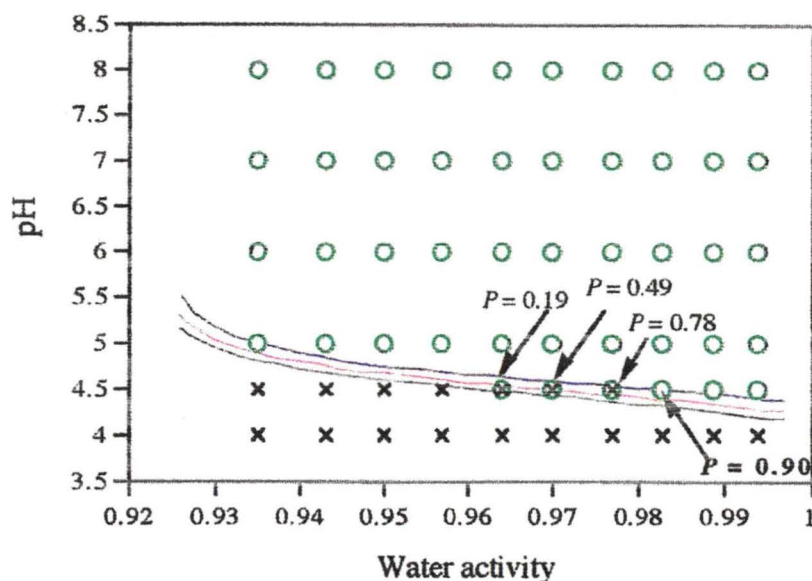
b) Eqn. 5.2 VS NCTC 9863
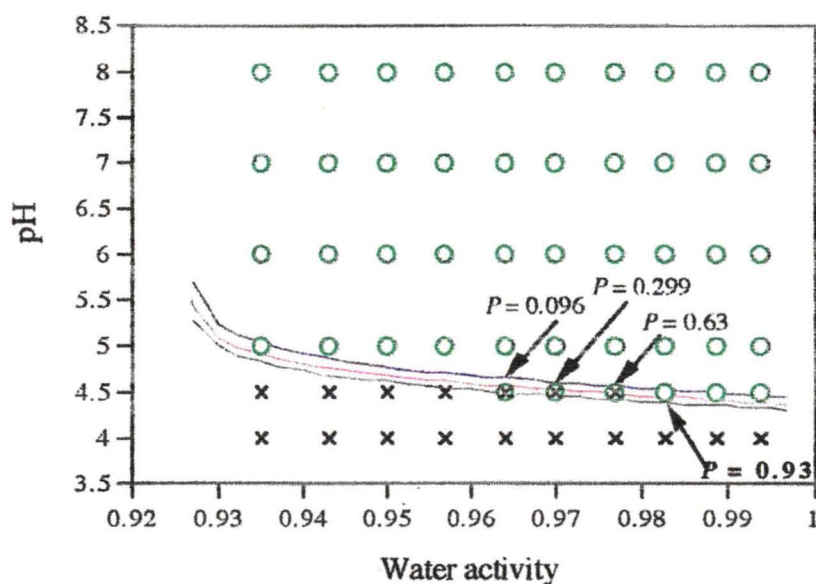
Figure 6.2 Effect of water activity on the growth (○) and no growth (×) of *L. mono-cytogenes* NCTC 9863 in TSB at 25°C using micro-well plates. Data of McClure *et al.* (1989). The growth/no growth interfaces predicted by a) Eqn. 5.1 and b) Eqn. 5.2 at *P*=0.9, 0.5, and 0.1 are shown as blue, red and black lines respectively. The abruptness of the transition from high (*P*=0.9) to low (*P*=0.1) probability of growth is illustrated. The probability values of others no growth conditions are given in Table 6.8.

6.4 DISCUSSION

The usefulness of the four kinetic models (Eqns 4.17a,b and 4.18a,b), and two probabilistic models (Eqns. 5.1 and 5.2) generated in Chapters 4 and 5 respectively are ultimately dependent on validating their ability to describe microbial growth or stasis in actual food systems.

The two batches of cold-smoked salmon used in the challenge tests were free from detectable *Listeria* spp. Vacuum-packed cold-smoked salmon stored at chill temperature was reported to contain several types of microflora dominated by lactic acid bacteria and low level of *Enterobacteriaceae*, Gram-negative bacteria and yeasts (Cann *et al.*, 1984; Truelstrup Hansen *et al.*, 1995; Gram and Huss, 1996). Growth of *L. monocytogenes* in cold-smoked salmon products was found to be inhibited by simultaneous growth of high levels of lactic acid bacteria (Carminati *et al.*, 1989; Harris *et al.*, 1989; Campanini *et al.*, 1993). The initial level of microflora in the cold-smoked salmon used in this study was found to be low ($<1\times10^3$ cfu/g). This level was less than the typical acceptable limit of 10^5 cfu/g for total viable count in the sliced, vacuum-packed product (Truelstrup Hansen *et al.*, 1995; Kelly *et al.*, 1996). Extreme care was also taken to avoid post-processing contamination in the sample preparation and inoculation process.

The study of responses of *L. monocytogenes* to different environmental conditions in defined media reported in Chapters 4 and 5 demonstrated several combination conditions of levels of lactic acid, pH, water activity and temperature that prevent growth of *L. monocytogenes*. However, for cold-smoked salmon, which is the product of interest in this study, the pH is typically ~6.0 and water activity ~0.97 (Dillon *et al.*, 1992). Additionally, there is increasing consumer demand to minimse salt concentration and other stability enhancing processes on the product. Approximately 3% NaCl (a_w after smoked process ~0.97) is the normal level of salt added to cold-smoked salmon (R. Skinner, pers. comm.). Additionally, Jakobsen *et al.* (1988), cited in Dalgaard (1997), reported levels of 4.5-5.0% water phase salt for the optimal taste of salmon. Results from Chapters 4 and 5 suggested that water activity of 0.96, and pH 6.0, does not appear to exert much inhibitory effect on *L. monocytogenes*. In addition, the intrinsic properties of fish flesh in relation to the very high post-mortem pH (>6.0) (Gram and Huss, 1996) and its buffering capacity (Cutting, 1953) have been documented. To formulate this product so that the growth of *L. monocytogenes* is inhibited, it appeared that a high level of lactic acid, i.e. at >350 mM must be employed to reduce the product pH and consequently increase the effect of lactic acid. The increasing concentration of undissociated lactic acid at the conditions studied in cold-smoked salmon (Table 6.1) is presented in Table 6.9. Note that the U_{min} estimated from the kinetic models (Chapter 4) are ~3.8 mM for strain Scott A and ~4.6 mM for strain L5. High levels of lactic acid

Table 6.9 Comparison of the amount of undissociated lactic acid and [H⁺] in cold-smoked salmon studied in the challenge tests (Table 6.1) at different concentrations of lactic acid.

Lactic acid (mM)	Initial pH[a]	Undissociated lactic acid (mM)	Hydrogen ion (μM)
200	6.00	1.44	1.0
250	5.93	2.11	1.17
300	5.85	3.04	1.41
350	5.80	3.97	1.58
450	5.58	8.41	2.63

[a] Average pH value from 2-3 fish samples measured at the beginning of the experiments. The pH observed over the course of each experiment was in a narrow range of ±0.1 to 0.2 pH unit (data not shown).

were found to prolong the lag phase (data not shown) and decrease the growth rate of *L. monocytogenes* in cold-smoked salmon (Table 6.1). At 450 mM lactic acid, no growth occurred and a decrease in the level of *L. monocytogenes* was observed over the course of experiment (26 days).

It is noteworthy that the numbers of *L. monocytogenes* obtained from both agar media used in the study, OXF and TSA-YE, were consistent even from samples containing high levels of lactic acid (data not shown). This indicates the injured cells were able to recover on OXF as well as on TSA-YE. Interestingly, at 5°C when the growth of *L. mono-cytogenes* in the vacuum-packed cold-smoked salmon was suppressed by those high levels of lactic acid, growth of other psychrotrophic micro-organisms, especially very large, Gram-positive yeast-like cells was observed. No attempt was made to identify these microbes. The anti-microbial effect of lactic acid on several micro-organisms is well documented, however, lactic acid resistance by some yeasts and moulds is also reported (Lueck, 1980; Houstma, 1996). This finding may suggest the requirement for further investigation for one or more additional 'hurdles' which may help to reduce the amount of lactic acid needed for complete growth inactivation.

6.4.1 VALIDATION OF KINETIC MODELS

It is useful to reiterate that the models 4.17a and 4.18a were developed from the full data sets and cover a wider range of pH conditions than the models 4.17b and 4.18b. Eqn. 4.17a contains a higher, anomalous, T_{min} of 1.4°C, while the T_{min} of the other models are in the range from 0.3-0.9°C.

The effect of atmosphere (packaging method) is not included in the models. *L. monocytogenes* is a facultative anaerobic micro-organism, and Buchanan *et al.* (1989a) reported generally equivalent growth rates of *L. monocytogenes* Scott A in response to either condition in laboratory broth media. At low temperature (5°C), those authors found a tendency of anaerobic incubation to favor growth of *L. monocytogenes*. However, in Bologna-type sausages, Houstma (1996) found that *L. monocytogenes* preferred aerobic to anaerobic conditions for growth. Similar results were found in the limited tests on aerobic and anaerobic (vacuum) packaged cold-smoked salmon performed in this study, i.e. faster growth of *L. monocytogenes* occurred in aerobic condition (in the absence of lactic acid). Variation of the effects of oxygen on growth of *L. monocytogenes* in meat procucts are reported (Garcia de Fernando *et al.*, 1995). The models performance for the anaerobic conditions presented in this study, however, appeared to be as satisfactory as for the aerobic conditions (Table 6.2).

A relatively high inoculum of *L. monocytogenes* Scott A and L5 (10^6-10^7 cfu/ml) was used in the model generation to mimic "worse case" circumstances. However, only strain L5 which is a wild-type strain isolated from cold-smoked salmon was used as the challenge organism. Comparisons of model predictions on the basis of generation times of *L. monocytogenes* L5 (10^3 cfu/g or 10^4 cfu/piece) in cold-smoked salmon under well-controlled conditions (Table 6.1), indicate small bias for both the Eqns 4.18a and 4.18b. The accuracy of the models is ±30%. The Eqns. 4.17a and 4.17b, models for strain Scott A, are seen to over-predict generation times in cold-smoked salmon especially for the extrapolated predictions at level of lactic acid >200 mM.

The limitations in model validation using data from published reports is recognised (Ross, 1993). It is not always possible to obtain all the relevant information from literature to enable an appropriate prediction from the models. Additionally, a full range of the modelled parameters especially a_w and pH are not always available in published reports. The validations presented in this chapter attempted to cover as wide a range of the controlling factors as possible, e.g. temperature from 0 to 35°C (Table 6.5b), a_w from 0.945 to 0.997 (Table 6.2), pH from 4.7 to 7.6 and lactic acid from 19.5 to 333 mM (Table 6.3). Various single or mixtures of strains of *L. monocytogenes* growing in a variety of foods such as vegetables, and fish, meat and dairy products were included in the validation process. In addition to comparing the models predictions to the artificial inoculation tests, growth of a naturally occurring *Listeria* contaminant on cold-smoked salmon, reported by Dalgaard and Jørgensen (1998), was also evaluated (Table 6.6).

The candidate is aware of the practical limitations of applying the proposed equations within the range of the present experimental data, the so-called 'interpolation region' or 'minimum convex polyhedron' (McMeekin *et al.*, 1993; Baranyi *et al.*, 1996). However,

there is no resource readily available for calculation for the precise MCP of the models developed. Validations of the models prediction in this chapter for each combination, however, may be estimated from the variable combinations diagrams present in Fig. G.1, Appendix G. Some comparisons between the combinations reported and the models prediction presented here were extrapolated beyond the previously defined limits.

The summary of the models prediction to various strains of *L. monocytogenes* and various foods on the basis of bias and accuracy factors presented in Table 6.7 indicates a resonably good accuracy performance range from ±24 to ±61% for models 4.17a and 4.17b, and from 22 to 59% for models 4.18a and 4.18b (Table 6.6 is not included). In agreement with these findings, Ross (1993) suggested there may be a limitation of the accuracy of model predictions to independent data especially in heterogeneous and ill-defined environments such as foods. The highest degree of accuracy found in that study when the models were applied to well-controlled challenge tests and published data were reported to be ~25% and ~35% respectively.

In most instances, however, the models developed in this study conservatively predicted as 'fail safe'. While the models correctly predicted the combined effects of temperature-a_w-pH-lactic acid concentrations, some discrepancies between the reported values and the predictions were found at the conditions close to the growth boundaries, i.e. minimum temperature, minimum a_w, minimum pH and minimum [UD]. These may be caused by the problem of detection of growth itself at conditions near growth extremes as lag time increases and growth rate decreases. Another possible reason is that microbial responses at the conditions close to the minimum theoretical value(s) are highly variable (Ratkowsky *et al.*, 1991). The influence of an anomalous T_{min}, in Eqn. 4.17a in particular, on reducing the models performance is noticed for model predictions at temperatures close to T_{min}, e.g. the prediction at 2.5°C shown in Table 6.5a, which caused a high difference in predicted generation time.

The inclusion of a 89 mM lactic acid (the suggested average level of natural occurrence of lactic acid in fish) in the models prediction (Tables 6.1-6.3) generally improved the performance of the models. It is noteworthy that, in the range of foods of pH ≥6.0 reported in Tables 6.1-6.3, only small amount of undissociated lactic acid (≥0.64 mM) and hydrogen ion (≥1 μM) were calculated from the 89 mM lactic acid, which caused only slightly reduced predicted growth rate. Nontheless the models always predicted faster growth rate than the observation in foods.

Large differences between the observed and predicted generation times with very high bias and accuracy factors were only obtained when the models were applied to naturally contaminated cold-smoked salmon with a low number of initial contaminations (<0 to 0.9

Log MPN/g) (Table 6.6). In most instances, the models predicted a faster growth rate than was observed. Similar results of over estimation of growth rates were also obtained from the 'Food MicroModel' predictions with lactate, i.e. bias and accuracy factors of 5.2 (Dalgaard and Jørgensen, 1998). The models developed in this study performed well when applied to the reported challenge tests in vacuum-packed cold-smoked salmon using very low inoculum, i.e. 6-10 cfu *L. monocytogenes*/g (Table 6.2). Thus, the discrepancies between the observed and predicted generation times in Table 6.6 may reflect the effect of factors not included in the predictive model, e.g. smoke component, microbial interaction.

While both L5 models (4.18a,b) gave similar predictions, the models 4.17a and 4.17b appeared to perform differently. This, again, may be caused by the high T_{min} in Eqn. 4.17a as discussed above. The overall performance of the models presented here suggest the models 4.18a and 4.18b always predicted a faster growth than the models 4.17a and 4.17b especially at the extereme conditions of temperature or [UD]. Generally, good performances with similar predictions were found in the optimum growth conditions.

6.4.2 VALIDATION OF PROBABILITY MODELS

Probability models provide predictions of the chance that *L. monocytogenes* would be able to proliferate in various conditions in foods, without considering time. The abruptness of the transitions between the occurrence of growth or no growth over a narrow change of pH (0.1 to 0.2 pH units) was shown and discussed in Chapter 5. In other word, the conditions which resulted in the probability of growth at 90% or 10% are actually not "far apart". If the conditions are made slightly less favourable to growth the probability of growth could rapidly drop from likely (>90%) to highly unlkiely (<10%). Presentation of the models evaluation in terms of percent probabilities of growth may confuse the analysis of the influences of those controlling factors and the models performance and value. Thus, in this chapter, the evaluation of probability models is presented as both percentage agreement (Table 6.8) and by graphical (Figs. 6.1 and 6.2) methods.

Published reports on the growth limits of *L. monocytogenes* covering a range of temperatures (George *et al.*, 1988), or water activities (McClure *et al.*, 1989) were compared to the growth/no growth interface predicted by the models 5.1 and 5.2. Sixteen different strains of *L. monocytogenes* were studied by George *et al.* (1988) and 4 representative strains were reported. The growth/no growth interfaces predicted by both equations show a good fit to the published observations (strains NCTC 10357 and Scott A) (Figs. 6.1a-d). However, it should be noted that at low temperature (<5°C) and

especially at $P=0.9$, Eqn. 5.1 (Scott A model) generated an erratic prediction for both reported strains (Figs. 6.1a,b). The cause of this is unclear. Nontheless, good performance was obtained from the model prediction at $P=0.1$ and 0.5 which are more of relevance to the analyst or food industry. Eqn. 5.2 appeared to perform better for both strains even though the model was generated from a different strain (L5). Variation of the responses of various strains of *L. monocytogenes* especially at the conditions close to the growth/no growth interface can be discerned in Fig. 6.1, e.g. at 30°C strain Scott A initiated growth at pH 4.39 but strain NCTC 10357 could not. A similar predicted probability for growth for strains F6868 and F7059 was reported in Table 6.8 (Ref. 1).

The abruptness of the fall in predicted probability of growth can be seen in both Figs. 6.1 and 6.2. As previouly discussed in Chapter 5 (section 5.4), an extreme variation in microbial growth occurrs especially at the conditions close to the growth/no growth boundary (Ratkowsky *et al.*, 1991). Additionally, a higher population density is anticipated to exhibit a higher probability for growth under the extreme conditions (see section 5.4). This notion is supported by the results of McClure *et al.* (1989) who reported the variation in the growth response of *L. monocytogenes* was influenced by the inoculum size. This effect is in accord with the explaination by the predicted probability values presented in Fig. 6.2 (indicated by the arrows) and Table 6.8 (Ref. 2). For example using Eqn. 5.1 (Fig. 6.2a), at the condition with a probability for growth of $P \geq 0.90$, growth was observed from all of the tested inoculum sizes, i.e. low, medium and high concentrations (5.2×10^3, 5.2×10^4 and 5.2×10^5 cells/ml respectively). At the predicted lower probability for growth, i.e. $P=0.78$ and 0.49, growth was detected from the medium and high concentrations but no growth was found in the low inoculum broth. At the probability for growth of 0.19, only growth from the high concentration inoculum was observed. No growth was observed in any of the inocula levels tested when the predicted probability was lower than 0.19 (Table 6.8, Ref. 2).

Very low probabilities for growth were predicted by both Eqns. 5.1 and 5.2 (see Table 6.8, Ref. 3) for the observed no growth data (within 13 weeks at 0°C, pH 5.6) reported by Grau and Vanderlinde (1993). However, some of the no growth conditions reported in broth media and foods in the presence of lactic acid (Refs. 4-6) show a likelihood ($P>0.9$) for growth to occur. This may be a result of insufficient time in observation (20 days to 46 days) or other factors such as microbial interaction which may suppress the growth of *L. monocytogenes* in those studies.

To generate a 'fail-safe' prediction, the growth limits of *L. monocytogenes* may be defined by the probability for growth of $P \leq 0.05$ (i.e. 95% confidence). The growth/no growth interface models presented here may help to design safety into product by manipulation of the controlling factor(s) such as temperature, pH, lactic acid

concentration etc. to conditions unfavourable for growth of *L. monocytogenes*. Using the same approach, other 'hurdles' such as nisin, monolaurin, Glucono-delta-lactone etc. can be further studied and included in the models. From this, appropriate combinations of condition(s) for each type of food product, which maintain the appearance and organoleptic acceptability of the products, but which inhibit or inactivate *L. mono-cytogenes*, may be derived.

7 SUMMARY AND CONCLUSIONS

Recognition of *L. monocytogenes* as a foodborne pathogen has raised concerns about the possible sources and routes of contamination in food processing factories and foods, and spawned the search for strategies to control or prevent its growth in food products.

L. monocytogenes is widely distributed in the environment and has been isolated from a variety of sources. However, few studies have considered the aquatic environment and its relationship to contamination of fish and seafood. In Chapter 2, a high recovery rate of *Listeria* spp. including *L. monocytogenes* in various aquatic habitats, and in particular rivers and effluents was reported. However, *L. monocytogenes* appeared to "die-off" when it reached the estuarine environment, which indicates that the estuarine water column may not serve as a natural habitat for the organisms. Nonetheless, estuarine sediment and shellfish appeared to serve as better reservoir for *Listeria* spp. than estuarine water.

Statistical analysis using a logistic method suggested the sanitary indicators, i.e. faecal coliforms and *E. coli* , and recent rainfall were the most significant variables related to the occurrence of *Listeria* spp. and *L. monocytogenes* in estuarine water. Multilocus enzyme electrophoresis of the 113 *L. monocytogenes* isolates from the North West Bay study revealed that wide range of electrophoretic types (ETs) present in the natural environment. Additionally, the distribution throughout the aquatic system studied and revealed the transmission of the organism to shellfish growing in those contaminated waters.

The microbial quality of fish depends on the quality of the ambient environment (i.e. marine farm), which could be a source of contamination of the processing lines and finished products. In an investigation (Chapter 3) of consecutive stages in the production of cold-smoked salmon, i.e. from harvesting to packaging, including the environment outside the processing factory, *L. monocytogenes* was recovered only from the environmental samples. This suggests the possibility to be able to control and prevent recurrence of earlier *L. monocytogenes* contamination in the factory and food products. The identification, using rep-PCR, of the 19 *L. monocytogenes* isolates collected from the previous contamination event indicated a single clone consistently contaminated the processing lines, equipment and products. Further comparisons of this clone with the isolates from the factory's environments, and some of the ETs more frequently isolated in the North West Bay study, demonstrated different genomic fingerprints in all of the

isolates. There was insufficient information to reveal the source and route of that contamination incident.

L. monocytogenes is notable for its ability to withstand adverse environmental conditions. These characteristics have made it challenging to control its survival and growth in many foods, especially in minimally thermal processed refrigerated products. The predictive microbiology approach taken in this study has revealed the ecology and physiological responses of *L. monocytogenes* to various controlling factors including temperature, water activity, pH, and lactic acid. Several combinations of those environmental factors could be used as a non-thermal treatment to prevent growth of *L. monocytogenes*. Results in this study indicate that it is possible to suppress growth of *L. monocytogenes* in chilled cold-smoked salmon by high amounts of lactic acid combined with lowering of pH. Further study of the appearance and organoleptic acceptability of the modified product is, however, required. The addition of one or more "hurdles" such as nisin, monolaurin etc. may be further studied and incorporated if required.

The studies of the effect of lactic acid on *L. monocytogenes* growth rate inhibition revealed the co-operative effects of hydrogen ions and undissociated lactic acid. The predominant effect of hydrogen ion was found at low lactic acid concentrations, with the undissociated acid effects becoming more profound as concentrations increased. Synergistic effects among the variables, i.e. pH-temperature, and pH-a_w were described in this study (sections 4.4.2 and 5.4.1-2).

The development of kinetic, square-root type models, using PROC NLIN for the combined effects of temperature-a_w-pH-lactic acid was also succesfully demonstrated in this study. Although, the puzzle of the sigmoid pH response remains to be solved (section 4.4.1.3), good performance of the models when validated with "real foods" were achieved.

Integration of the kinetic and probability modelling approaches, and modelling using NLIN procedure were demonstrated in this study. The novel "growth/no growth interface" models for *L. monocytogenes* Scott A and L5 demonstrated their practical uses as they were able to accurately predict the growth/no growth interface for other *L. monocytogenes* strains (section 6.3.2). The abruptness of the transition between the conditions of "highly likely to grow" (90% probability for growth) and the "highly unlikely to grow" (10% probability for growth) was discussed in Chapter 5 and supported by independent data (Chapter 6). For this type of model to be applied to food safety problems, conditions that lead to a probability of growth of 5% or less may be required to ensure that growth of *L. monocytogenes* does not occur throughout the shelf-life of the product.

Predictive microbiology is not "the sole answer to foodborne illness" but it is a promising tool providing rational understanding and strategies to enable those problems to be identified and finally eliminated. An understanding of the ecology of pathogens both in the natural, factory and food environments would add substantially to a farm-to-table approach for microbial food safety.

REFERENCES

Adams, C.J., T.E. Neff, and L.L. Jackson. 1979. Induction of *Listeria monocytogenes* infection by the consumption of ponderosa pine needles. *Infect. Immune.* **2 5**:117-120.

Adams, M.R., C.L. Little, and M.C. Easter. 1991. Modelling the effect of pH, acidulant and temperature on the growth rate of *Yersinia enterocolitica*. *J. Appl. Bacteriol.* **7 1**:65-71.

Ahamad, N., and E.H. Marth. 1989. Behaviour of *Listeria monocytogenes* at 7, 13, 21, and 35°C in tryptose broth acidified with acetic, citric, or lactic acid. *J. Food Prot.* **5 2**:688-695.

Ahamad, N., and E.H. Marth. 1990. Acid-injury of *Listeria monocytogenes*. *J. Food Prot.* **5 3**:26-29.

Al-Ghazali, M.R., and S.K. Al-Azawi. 1986. Detection an enumeration of *Listeria monocytogenes* in a sewage treatment plant in Iraq. *J. Appl. Bacteriol.* **6 0**:251-254.

Al-Ghazali, M.R., and S.K. Al-Azawi. 1988a. Effect of sewage treatment on the removal of *Listeria monocytogenes*. *J. Appl. Bacteriol.* **6 5**:203-208.

Al-Ghazali, M.R., and S.K. Al-Azawi. 1988b. Storage effects of sewage sludge cake on the survival of *Listeria monocytogenes*. *J. Appl. Bacteriol.* **6 5**:209-213.

Albritton, W.L., G.L. Wiggins, W.E. DeWitt, and J.C. Feeley. 1980. *Listeria monocytogenes. In* E.H. Lennette, A. Balows, W.J. Hausler, Jr., and J.P. Truant (Eds.), *Manual of Clinical Microbiology*, 3rd edition, American Society for Microbiology, Washington, D.C., pp. 139-142.

Anonymous. 1993a. Cheese, seafood subject to Class I recalls due to *Listeria* contamination. *Food Chem. News.* **3 5**:13, 16-17.

Anonymous. 1993b. FDA places import alert on cooked seafood, surimi products. *Food Chem. News.* **3 5**:10, 51-52.

Anonymous. 1993c. *Listeria* check on NZ seafood. *The Daily Telegraph Mirror*, 13 August 1993, Sydney, NSW, p. 17.

Anonymous. 1994. For pregnant women dietary advice on listeriosis. National Food Authority, Australia. *Pamphlet.*

Anonymous. 1995. Environmental Health Guide, *Listeria* infection and pregnancy. Health Department of Western Australia, Western Australia. *Pamphlet.*

ANZECC (Australian and New Zealand Environment and Conservartion Council). 1992. National Water Quality Guidelines for Fresh and Marine Waters. Department of Arts, Sport, Environment, Tourism and Territories, Canberra.

Arnold, G.J., and J. Coble. 1995. Incidence of *Listeria* species in foods in NSW. *Food Australia.* **47**:71-75.

Australian Standard. 1995. Water Microbiology Method 5: Coliforms-Membrane Filtration Method. Standards Australia. NSW, AS 4276.5.

Avoyne, C., M. Butin, J. Delaval, and J-L. Bind. 1997. Detection of *Listeria* spp. in food samples by imunomagnetic capture: ListerScreen method. *J. Food Prot.* **60**:377-384.

Baird-Parker, A.C. 1980. Organic acids. *In* J.H. Siliker, R.P. Elliott, A.C. Baird-Parker, F.L. Bryan, J.H.B. Christian, D.S. Clark, J.C. Olson, Jr., and T.A. Roberts (Eds.), *Microbial Ecology of Foods, Vol.I, Factors Affecting the Life and Death of Microorganisms,* Academic Press, London, pp. 126-135.

Bajard, S., L. Rosso, G. Fardel, and J.P. Flandrois. 1996. The particular behaviour of *Listeria monocytogenes* under sub-optimal conditions. *Int. J. Food Microbiol.* **29**:201-211.

Baker, D., C. Genigeorgis, J. Glover, and V. Razavilar. 1990. Growth and toxigenesis of *C. botulinum* type E in fishes packaged under modified atmospheres. *Int. J. Food Microbiol.* **10**:269-290.

Baker, M., M. Brett, P. Short, L. Calder, and R. Thornton. 1993. Listeriosis and mussels. *CDNZ.* **93**:13-14.

Baranyi, J., and T.A. Roberts. 1995. Mathematics of predictive food microbiology. *Int. J. Food Microbiol.* **26**:199-218.

Baranyi, J., T. Ross, T.A. McMeekin, and T.A. Roberts. 1996. Effects of parameterization on the performance of empirical models used in 'predictive microbiology'. *Food Microbiol.* **13**:83-91.

Baranyi, J., T.A., Roberts, and P. McClure. 1993. A non-autonomous differential equation to model bacterial growth. *Food Microbiol.* **10**:43-59.

Baxter, F., F. Wright, R.M. Chalmers, J.C. Low, and W. Donachie. 1993. Characterization by multilocus enzyme electrophoresis of *Listeria monocytogenes* isolates involved in ovine listeriosis outbreaks in Scotland from 1989 to 1991. *Appl. Env. Microbiol.* **59**:3126-3129.

Ben Embarek, P.K. 1994. Presence, detection and growth of *Listeria monocytogenes* in seafoods: a review. *Int. J. Food Microbiol.* **23**:17-34.

Ben Embarek, P.K., and H.H. Huss. 1992. Growth of *Listeria monocytogenes* in lightly preserved fish products. *In* H.H. Huss, M. Jakobsen, and J. Liston (Eds.), *Quality Assurance in the Fish Industry*, Elsivier Science Publishers B.V., Amsterdam, pp. 293-303.

Benedict, R.C. 1990. *Listeria monocytogenes*: physiology and metabolism. *In* A.L. Miller, J.L. Smith, and G.A. Somkuti (Eds.), *Foodborne Listeriosis*. Elsevier Science Publishers B.V., Amsterdam, pp. 13-22.

Berrang, M.E., R.E. Brackett, and L.R. Beuchat. 1989. Growth of *Listeria monocytogenes* on fresh vegetables stored under controlled atmosphere. *J. Food Prot.* 5 2:702-705.

Bessesen, M.T., Q. Luo, H.A. Rotbart, M.J. Blaser, and R.T.Ellison III. 1990. Detection of *Listeria monocytogenes* by using the polymerase chain reaction. *Appl. Environ. Microbiol.* 5 6:2930-2932.

Bibb, W.F., B.G. Gellin, R. Weaver, B. Schwartz, B.D. Plikaytis, M.W. Reeves, R.W. Pinner, and C.V. Broome. 1990. Analysis of clinical and food-boarne isolates of *Listeria monocytogenes* in the United States by multilocus enzyme electrophoresis and application of the method to epidemiologic investigations. *Appl. Env. Microbiol.* 5 6:2133-2141.

Bille, J. 1990. Epidemiology of human listeriosis in Europe, with special reference to the Swiss outbreak. *In* A.L. Miller, J.L. Smith, and G.A. Somkuti (Eds.), *Foodborne Listeriosis*, Elsevier Science Publishers B.V., Amsterdam, pp. 71-74.

Boerlin, P., and J.C. Piffaretti. 1991. Typing of human, animal, food, and environmental isolates of *Listeria monocytogenes* by multilocus enzyme electrophoresis. *Appl. Environ. Microbiol.* 5 7:1624-1629.

Boerlin, P., F. Boerlin-Petzold, E. Bannerman, J. Bille, and T. Jemmi. 1997. Typing *Listeria monocytogenes* isolates from fish products and human listeriosis cases. *Appl. Environ. Microbiol.* 6 3:1338-1343.

Boerlin, P., J. Rocourt, and J-C. Piffaretti. 1991. Taxonomy of the genus *Listeria* by using multilocus enzyme electrophoresis. *Int. J. Syst. Bacteriol.* 4 1:59-64.

Bojsen-Moller, J. 1972. Human listeriosis. Diagnostic, epidemiological and clinical studies. *Acta Path. Microbiol. Scand. Sec.* B Suppl. 2 2 9:72-155.

Booth, I.R. 1985. Regulation of cytoplkasmic pH in bacteria. *Microbiol. Reviews* 4 9:359-378.

Botzler, R.G., A.B. Cowan, and T.F. Wetzler. 1974. Survival of *Listeria monocytogenes* in soil and water. *J. Wildlife Dis.* 1 0:204-212.

Box, G.E.P., and N.R. Draper. 1987. Least Squares for Response Surface Work: Empirical Model-Building and Response Surfaces. John Wiley, New York.

Brackett, R.E. 1988. Presence and persistence of *Listeria monocytogenes* in food and water. *Food Technol.* **42**:162-164.

Breed, R.S., EG.D. Murray, and A.P. Hitchens. 1948. Bergey's Manual of Determinative Bacteriology, 6th edition. Williams and Wilkins, Baltimore.

Breed, R.S., EG.D. Murray, and N.R. Smith. 1957. Bergey's Manual of Determinative Bacteriology, 7th edition. Williams and Wilkins, Baltimore.

Britten, R.J., and D.E. Kohne. 1968. Repeated sequences in DNA. *Science.* **161**:529-540.

Broome C.V. 1993. Listeriosis: Can we prevent it? New monitoring techniques are helping to prevent this relatively rare but frequently fatal food-borne disease. *ASM News.* **59**:444-446.

Brosch, R., J. Chen, and J.B. Luchansky. 1994. Pulsed-field fingerprinting of Listeriae: identification of genomic divisions for *Listeria monocytogenes* and their correlation with serovar. *Appl. Environ. Microbiol.* **60**:2584-2592.

Brown, M.H., and I.R. Booth. 1991. Acidulants and low pH. *In* N.J. Russell, and G.W. Gould (Eds.), *Food Preservatives*, Blackie and Son, New York, pp. 22-43.

Buchanan, R., M.H. Golden, and R.C. Whiting. 1993. Differentiation of the effects of pH and lactic or acetic acid concentration on the kinetics of *Listeria monocytogenes* inactivation. *J. Food Prot.* **56**:474-478.

Buchanan, R.E., and N.E. Gibbons. 1974. Burgey's Manual of Determinative Bacteriology, Williams and Wilkins, Baltimore.

Buchanan, R.L., 1993. Developing and distributing user-friendly application software. *J. Industrial Microbiol.* **12**:251-255.

Buchanan, R.L., and J.G. Phillips. 1990. Response surface model for predicting the effects of temperature pH:, sodium chloride content, sodium nitrite concentration and atmosphere on the growth of *Listeria monocytogenes*. *J. Food Prot.* **53**:370-376.

Buchanan, R.L., and M.H. Golden. 1994. Interaction of citric acid concentration and pH on the kinetics of *Listeria monocytogenes* inactivation. *J. Food Prot.* **57**:567-570.

Buchanan, R.L., and M.H. Golden. 1995. Model for the non-thermal inactivation of *Listeria monocytogenes* in a reduced oxygen environment. *Food Microbiol.* **12**:203-212.

Buchanan, R.L., H.G. Stahl, and R.C. Whiting. 1989a. Effects and interactions of temperature, pH, atmosphere, sodium chloride, and sodium nitrite on the growth of *Listeria monocytogenes*. *J. Food Prot.* **52**:844-851.

Buchanan, R.L., H.G. Stahl, M.M. Bencivengo, and F.D. Corral. 1989b. Comparison of lithium chloride-phenylethanol-moxalactam and modified Vogel Johnson agars for detection of *Listeria* spp. in retail-level meats, poultry, and seafood. *Appl. Environ. Microbiol.* **5 5**:599-603.

Buchanan, R.L., W.G. Damert, R.C. Whiting, and M. van Schothorst. 1997. Use of epidemiologic and food survey data to estimate a purposefully dose-response relationship for *Listeria monocytogenes* levels and incidence of listeriosis. *J. Food Prot.* **6 0**:918-922.

Burn, C.G. 1936. Clinical and pathological features of an infection caused by a new pathogen of the genus *Listerella. Amer. J. Pathol.* **1 2**:341-348.

Campanini, M., I. Pedrazzoni, S. Barbuti, and P. Baldini. 1993. Behaviour of *Listeria monocytogenes* during the maturation of naturally and artificially contaminated salami: effect of lactic-acid bacteria starter cultures. *Int. J. Food Microbiol.* **2 0**:169-175.

Cann, D.C., N.C. Houston, L.Y. Taylor, G.L. Smith, A.B. Smith, and A. Craig. 1984. Studies of Salmonids Packed and Stored under a Modified Atmosphere. Torry Research Station, Ministry of Agriculture, Fisheries and Food, Aberdeen, Scotland.

Carminati, D., G. Giraffa, and M.G. Bossi. 1989. Bacteriocin-like inhibitors of *Streptococcus lactis* against *Listeria monocytogenes. J. Food Prot.* **5 2**:614-617.

Carosella, J.M. 1990. Occurrence of *Listeria monocytogenes* in meat and poultry. *In* A.L Miller, J.L. Smith, and G.A. Somkuti (Eds.), *Foodborne Listeriosis.* Elsevier Science Publishers B.V., Amsterdam, pp. 165-173.

Chai, T.J., T.J. Han, and R.R. Cockey. 1994. Microbiological quality of shellfish-growing waters in Chesapeake bay. *J. Food Prot.* **5 7**:229-234.

Christie, R., N.E. Atkins, and E. Munch-Petersen. 1944. A note on a lytic phenomenon shown by group b streptococci. *Aust. J. Exp. Biol. Med. Sci.* **2 2**:197-200.

Colburn, K.G., C.A. Kaysner, C. Abeyta, and M.M. Wejekk. 1990. *Listeria* species in a California coast estuarine environment. *Appl. Env. Microbiol.* **5 6**:2007-2011.

Cole, M.B., M.V. Jones, and C. Holyoak. 1990. The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes. J. Appl. Bacteriol.* **6 9**:63-72.

Conner, D.E., V.N. Scott, and D.T. Bernard. 1990. Growth, inhibition, and survival of *Listeria monocytogenes* as affected by acidic conditions. *J. Food Prot.* **5 3**:652-655.

Corlett, D.A., and M.H. Brown. 1980. pH and acidity. *In* J.H. Siliker, R.P. Elliott, A.C. Baird-Parker, F.L. Bryan, J.H.B. Christian, D.S. Clark, J.C. Olson, Jr., and T.A. Roberts (Eds), *Microbial Ecology of Foods, Vol.I, Factors Affecting the Life and Death of Microorganisms,* Academic Press, London, pp. 92-111.

Cortesi, M.L., T. Sarli, A. Santoro, N. Murru, and T. Pepe. 1997. Distribution and behaviour of *Listeria monocytogenes* in three lots of naturally-contaminated vacuum-packaged smoked salmon stored at 2 and 10°C. *Int. J. Food Microbiol.* 3 7:209-214.

Cossart, P., M.F.Vicente, J. Mengaud, F. Baquero, J.C. Perez-Diaz, and P. Berche. 1989. Listeriolysin O is essential for virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation. *Infect. Immun.* 5 7:3629-3636.

Curry, G.I., R.M. Feldman, and P.J.H. Sharpe. 1978. Foundations of stochastic development. *J. Theor. Biol.* 7 4:397-410.

Cutting, C.L. 1953. Changes in the pH and buffering capacity of fish during spoilage. *J. Sci. Food Agric.* 4:597-603.

Czajka, J., N. Bsat, M. Pani, W. Russ, K. Sultana, M. Wiedmann, R. Whitaker, and C.A. Batt. 1993. Differentiation of *Listeria monocytogenes* and *Listeria innocua* by 16S rRNA and intraspecies discrimination of *Listeria monocytogenes* strains by random amplified polymorphic DNA polymorphisms. *Appl. Environ. Microbiol.* 5 9:304-308.

Dalgaard, P. 1997. Predictive microbiological modelling and seafood quality. *In* J. Luten, T. Børresen, and J. Oehlenschläger (Eds.), *Proceedings of the International (WEFTA) Seafood Conference on Seafood from Producer to Consumer, Integrated Approach to Quality*, Noord-wijkerhout, The Netherlands, 13-16 November, pp. 431-433.

Dalgaard, P., and L.V. Jørgensen. 1998. Predicted and observed growth of *Listeria monocytogenes* in seafood challenge testes and in naturally contaminated cold-smoked salmon. *Int. J. Food Microbiol.* 4 0:105-115.

Dalgaard, P., T. Ross, L. Kamperman, K. Neumeyer, and T.A. McMeekin. 1994. Estimation of bacterial growth rates from turbidimetric and viable count data. *Int. J. Food Microbiol.* 2 3:391-404.

Datta, A.R., B.A. Wentz, and W.E. Hill. 1987. Detection of hemolytic *Listeria monocytogenes* by using DNA colony hybridization. *Appl. Environ. Microbiol.* 5 3:2256-2259.

Daughtry, B.J., K.R. Davey, and K.D. King. 1997. Temperature dependence of growth kinetics of food bacteria. *Food Microbiol.* 1 4:21-30.

Davey, K.R. 1989. A predictive model for combined temperature and water activity on microbial growth during the growth phase. *J. Appl. Bacteriol.* 6 7:483-488.

de Bruijn, F.J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Appl. Environ. Microbiol.* 5 8:2180-2187.

de Martinis, E.C.P., A.D. Crandall, A.S. Mazzotta, and T.J. Montville. 1997. Influence of pH, salt, and temperature on nisin resistance in *Listeria monocytogenes*. *J. Food Prot.* 6 0:420-423.

de Wit, J.C. and F.M., Rombouts. 1990. Antimicrobial activity of sodium lactate. *Food Microbiol.* 7:113-120.

Decastelli, L., C. Ercolini, S. Fisiichella, C. Bianchi. 1993. Incidenza di *Listeria* spp. in mitili (*Mytilus galloprovincialis*) di allevamento. *Microbiologie-Aliments-Nutrition.* 1 1:51-56.

Delignette-Muller, M.L., L. Rosso, and J.P. Flandrois. 1995. Accuracy of microbial growth predictions with square root and polynomial models. *Int. J. Food Microbiol.* 2 7:139-146

Dennis, M., and W.H. Lee. 1989. FSIS Method for the Isolation and Identification of *Listeria monocytogenes* from Processed Meat and Poultry Products. Laboratory Communication No.57. USDA, FSIS, Microbiology Division, Beltsville.

Difco. 1984. Difco Manual: Dehydrated Culture Media and Reagents, Bacto FA *Listeria* Poly. Difco Laboratories, Detroit, Michigan.

Dijkstra, R.G. 1975. Recent experiences of the survival times of *Listeria* bacteria in suspension of brain tissue, silage, faeces and in milk. *In* M. Woodbine (Ed.), *Problems of Listeriosis*, Leicester University Press, Leicester, pp. 71-74.

Dijkstra, R.G. 1982. The occurrence of *Listeria monocytogenes* in surface water of canals and lakes, in ditches of one big polder and in the effluents of canals of a sewage treatment plant. Zentbl. Bakt. Parasite. *Infekt. Hyg. Abts. 1. Orig. B.* 1 7 6:202-205.

Dillon, R., T. Patel, S. Ratnam. 1994. Occurrence of *Listeria* in hot and cold smoked seafood products. *Int. J. Food Microbiol.* 2 2:73-77.

Dillon, R.M., and T.R. Patel. 1992. *Listeria* in seafoods: a review. *J. Food Prot.* 5 5:1009-1015.

Dillon, R.M., T.R. Patel, and S. Ratnam. 1992. Prevalence of *Listeria* in smoked fish. *J. Food Prot.* 5 5:866-870.

Donelly, C.W., and G.J. Baigent. 1986. Methods for flow cytometric detection of *Listeria monocytogenes* in milk. *Appl. Environ. Microbiol.* 5 2:689-695.

Donnelly, C.W. 1988. Historical perspectives on methodology to detect *Listeria monocytogenes*. *J. Assoc. Off. Anal. Chem.* 7 1:644-646.

Dorsa, W.J., D.L. Marshall, M.W. Moody, and C.R. Hackney. 1993. Low temperature growth and thermal inactivation of *Listeria monocytogenes* in precooked crawfish tail meat. *J. Food Prot.* 5 6:106-109.

Doyle, M.P., and J.L. Schoeni. 1987. Comparison of soft, surface-ripened cheese. *J. Food Prot.* **5 0**:4-6.

Doyle, M.P., K.A. Glass, J.T. Beery, G.A. Garcia, D.J. Pollard, and R.D. Schultz. 1987. Survival of Listeria monocytogenes in milk during high-temperature, short-time pasteurization. *Appl. Environ. Microbiol.* **5 3**:1433-1438.

Duh, Yeu-Hsin, and D.W. Schaffner. 1993. Modelling the effect of temperature on the growth rate and lag time of *Listeria innocua* and *Listeria monocytogenes*. *J. Food Prot.* **5 6**:205-210.

Eklund, M.W., F.T. Poysky, R.N. Paranjpye, L.C. Lashbrook, M.E. Peterson, and G.A. Pelroy. 1995. Incidence and sources of *Listeria monocytogenes* in cold-smoked fishery products and processing plants. *J. Food Prot.* **5 8**:502-508.

Eklund, T. 1983. The antimicrobial effect of dissociated and undissociated sorbic acid at different pH levels. *J. Appl. Bacteriol.* **5 4**:383-389.

Eklund, T. 1989. Organic acids and esters. *In* G.W. Gould (Ed.), *Mechanisms of Action of Food Preservation Procedures*, Elsivier Science Publishers, New York, pp. 161-200.

Ericsson, H., A. Eklow, M.L. Danielsson-tham, S. Loncarevic, L.O. Mentzing, I. Persson, H. Unnerstad, and W. Tham. 1997. An outbreak of listeriosis suspected to have been caused by rinbow trout. *J. Clin. Microbiol.* **3 5**:2904-2907.

FAO. 1994. The Use of Hazard Analysis Critical Control Point (HACCP) Principles in Food Control. Food and Nutrition paper 58, Report of an FAO Expert Technical Meeting, Vancouver, Canada, 12-16 December.

Farber, J.M. 1991. *Listeria monocytogenes* in fish products. *J. Food Prot.* **54**:922-934.

Farber, J.M. 1993. Current research on *Listeria monocytogenes* in foods: an overview. *J. Food Prot.* **5 6**:640-643.

Farber, J.M. 1996. An introduction to the hows and whys of molecular typing. *J. Food Prot.* **5 9**:1091-1101.

Farber, J.M., and P.I. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Reviews.* **5 5**:476-511.

Farber, J.M., F. Coates, and E. Daley. 1992. Minimum water activity requirements for the growth of *Listeria monocytogenes*. *Lett. Appl. Microbiol.* **1 5**:103-105.

Farber, J.M., G.W. Sanders, and M. A. Johnston. 1989a. A survey of various foods for the presence of *Listeria* species. *J. Food Prot.* **5 2**:456-458.

Farber, J.M., G.W. Sanders, and S. A. Malcom. 1988. The presence of *Listeria* spp. in raw milk in Ontario. *Canadian J. Microbiol.* 34:95-100.

Farber, J.M., G.W. Sanders, S. Dunfield, and R. Prescott. 1989b. The effect of various acidulants on the growth of *Listeria monocytogenes*. *Lett. Appl. Microbiol.* **9**:181-183.

Farber, J.M., W.H. Ross, and J. Harwig. 1996. Health risk assessment of *L. monocytogenes* in Canada. *Int. J. Food Microbiol.* **3 0**:145-146.

Farmiloe, F.J., S.J. Cornford, J.B.M. Copock, and M. Ingram. 1954. The survival of *Bacillus subtilis* spores in the baking of bread. *J. Science Food and Agriculture* **5**:292-304.

Faud, A.T., S.D. Weagant, M.M. Wekell, and J. Liston. 1989. Survival of *Listeria monocytogenes* in the estuarine environment. *In Annu. Meet. Am. Soc. Microbiol.* Abstr. Q243.

Fenlon, D.R. 1985. Wild birds and silage as reservoirs of *Listeria* in the agricultural environment. *J. Appl. Bacteriol.* **5 9**:537-543.

Fenlon, D.R. 1986. Rapid quantitative assessment of the distribution of *Listeria* in silage implicated in a suspected outbreak of listeriosis in calves. *Vet. Rec.* **11 8**:240-242.

Fenlon, D.R., and J. Wilson. 1989. The incidence of *Listeria monocytogenes* in raw milk from farm bulk tanks in North-East Scotland. *J. Appl. Bacteriol.* **6 6**:191-196.

Fenlon, D.R., J. Wilson, and W. Donachie. 1996. The incidence and level of *Listeria monocytogenes* contamination of food sources at primary production and initial processing. *J. Appl. Bacteriol.* **8 1**:641-650.

Fenlon, D.R., T. Stewart, and W. Donachie. 1995. The incidence, numbers and types of *Listeria monocytogenes* isolated from farm bulk tank milks. *Lett. Appl. Microbiol.* **2 0**:57-60.

Fernandez Garayzabal, J.F., L. Domonguez Rodriguez, J.A. Vazquez Boland, E.F. Rodriguez Ferri, V. Briones Dieste, J.L. Blanco Cancelo, and G. Suarez Fernandez. 1987. Survival of *Listeria monocytogenes* in raw milk treated in a pilot plant pasteurizer. *J. Appl. Bacteriol.* **6 3**:533-537.

Fitter, S., M. Heuzenroder, and C.J. Thomas. 1992. A combined PCR and selective enrichment method for rapid detection of *Listeria monocytogenes*. *J. Appl. Bacteriol.* **7 3**:53-59.

Fleming, D.W., S.L. Cochi, K.L. MacDonald, J. Brondum, P.S. Hayes, B.D. Plikaytis, M.B. Holmes, A. Audurier, C.V. Broome, and A.L. Reingold. 1985. Pasteurised milk as a vehicle of infection in an outbreak of listeriosis. *N. Engl. J. Med.* **3 1 2**:404-407.

Fluit, A.C., R. Torensma, M.J.C. Visser, C.J.M. Aarsman, M.J.J.G. Poppelier, B.H.I. Keller, P. Klapwijk, and J. Verhoef. 1993. Detection of *Listeria monocytogenes* in cheese with the magnetic imuno-polymerase chain reaction assay. *Appl. Environ. Microbiol.* **5 9**:1289-1293.

Fraser, G. 1964. The effect of animal erythrocytes of combinations of diffusible substances produced by bacteria. *J. pathol. bacteriol.* **8**8:43-53.

Freese, E., C.W. Sheu, and E. Galliers. 1973. Function of lipophilic acids as antimicrobial food additives. *Nature.* **241**:321-325.

Fuchs, R.S., and L. Nicolaides. 1994. Incidence of *Listeria* in hot- and cold-smoked fish. *Lett. Appl. Microbiol.* **1**9:394-396.

Fuchs, R.S., and P.J.A. Reilly. 1992. The incidence and significance of *Listeria monocytogenes* in seafoods. *In* H.H. Huss, M. Jakobsen, and J. Liston (Eds.), *Quality Assurance in the Fish Industry*, Elsevier Science Publishers B.V., Amsterdam, pp. 217-229.

Fuchs, R.S., and P.K. Surendran. 1989. Incidence of *Listeria* in tropical fish and fishery products. *Lett. Appl. Microbiol.* **9**:49-51.

Gameson, A. L. H. 1975. Discharge of sewage from sea outfalls. *In Proceedings of International Symposium Progress in Water Technology*, Church House, London, 27 August to 2 September 1974.

García de Fernando, G.D., G.J.E. Nychas, M.W. Peck, J.A. Ordóñez. 1995. Growth/survival of psychrotrophic pathogens on meat packed under modified atmospheres. *Int. J. Food Microbiol.* **2**8:221-231.

Garland, C.D. 1995. Microbiological quality of aquaculture products with special reference to *Listeria monocytogenes* in Atlantic salmon. *Food Aust.* **47**:559-563.

Gay, M., O. Cerf, and K.R. Davey. 1996. Significance of pre-incubation temperature and inoculum concentration on subsequent growth of *Listeria monocytogenes* at 14°C. *J. Appl. Bacteriol.* **8**1:433-438.

Genigeorgis, C., S. Martin, C.E. Franti, and H. Riemann. 1971. Initiation of Staphylococcal growth in laboratory media. *Appl. Microbiol.* **2**1:934-939.

Genigeorgis, C.A., D. Dutulescu, and J.F. Garayzabal. 1989. Prevalence of *Listeria* spp. in poultry meat at the supermarket and slaughterhouse level. *J. Food Prot.* **5**2:618-624.

George, S.M., B.M., Lund, and T.F. Brocklehurst. 1988. The effect of pH and temperature on initiation of growth of *Listeria monocytogenes*. *Lett. Appl. Microbiol.* **6**:153-156.

George, S.M., L.C.C. Richardson, and M.W. Peck. 1996. Predictive models of the effect of temperature, pH and acetic and lactic acids on the growth of *Listeria monocytogenes*. *Int. J. Food Microbiol.* **3**2:73-90.

Gerner-Smidt, P., P. Boerlin, F. Ischer, and J. Schmidt. 1996. High-frequency endonuclease (REA) typing: results from the WHO collaborative study group on subtyping of *Listeria monocytogenes*. *Int. J. Food Microbiol.* **32**:313-324.

Geunich, H-H., H.E. Müller. 1984. Isolation and quantitative determination of *Listeria monocytogenes* in raw and biologically treated wasted water. *Zbl. Bakt. Hyg. I. Abt. Orig. B.* **179**:266-273.

Gibson, A.M., and T.A. Roberts. 1989. Predicting microbial growth: development of a mathematical model to predict bacterial growth responses. *Food Aust.* **12**:1075-1079.

Gibson, A.M., N. Bratchell, and T.A. Roberts. 1987. The effects of sodium chloride and temperature on the rate and extent of growth of *Clostridium botulinum* type A in pasteurized pork slurry. *J. Appl. Bacteriol.* **62**:479-490.

Gibson, D.M. 1992. Pathogenic microorganisms of importance in seafood. *In* H.H. Huss, M. Jakobsen, and J. Liston (Eds.), *Quality Assurance in the Fish Industry*. Elsevier Science Publishers B.V., Amsterdam, pp.197-209.

Gilbert, R.J. 1995. Zero tolerance for *Listeria monocytogenes* in foods-is it necessary or realistic? *In Proceedings of the XII International Symposium on Problems of Listeriosis*, Perth, Australia, 2-6 October, pp. 351-356.

Gill, C.O. 1986. Temperature function integration for hygiene evaluation of food processing procedures. *Food Technol. Aust.* **38**:203-204.

Gill, C.O., G.G. Greer, and B.D. Dilts. 1997. The aerobic growth of *Aeromonas hydrophila* and *Listeria monocytogenes* in broths and on pork. *Int. J. Food Microbiol.* **35**:67-74.

Gill, D.A. 1931. Circling disease of sheep in New Zealand. *Vet. J.* **87**:60-74.

Gill, D.A. 1937. Ovine bacterial encephalitis (Circling Disease) and the bacterial genus Listerella. *Aust. Vet. J.* **13**:46-56.

Gilot, P., and P. Andre. 1995. Characterization of five esterases from *Listeria monocytogenes* and use of their electrophoretic polymorphism for strain typing. *Appl. Env. Microbiol.* **61**:1661-1665.

Gilson, E., J.M. Clément, D. Brutlag, and M. Hofnung. 1984. A family of dispersed repetitive extragenic palindromic DNA sequences in *E. coli*. *EMBO J.* **3**:1417-1421.

Gitter, M., R.StJ. Stebbings, J.A. Morris, D. Hannam, and C. Harris. 1986. Relationship between ovine listeriosis and silage feeding. *Vet. Rec.* **118**:207-208.

Gouet, P., J.P. Girardeau, and Y. Riou. 1977. Inhibition of *Listeria monocytogenes* by defined lactic microflora in gnotobiotic silages of lucerne, fescue, ryegrass and maize-influence of dry matter and temperature. *Anim. Feed. Sci. Technol.* **2**:297-305.

Gould, G.W. 1989. Mechanisms of action of food preservation procedures. Elsivier Science Publishers, New York.

Gould, G.W., and M.V. Jones. 1989. Combination and synergistic effects. *In* G.W., Gould (Ed.), *Mechanisms of Action of Food Preservation Procedures*, Elsivier Science Publishers, New York, pp. 401-421.

Gram, L., and H.H. Huss. 1996. Microbiological spoilage of fish and fish products. *Int. J. Food Microbiol.* **3 3**:121-137.

Grant, I.R., C.R. Nixon, and M.F. Patterson. 1993. Comparison of the growth of *Listeria monocytogenes* in unirradiated and irradiated cook-chill roast beef and gravy at refrigeration temperatures. *Lett. Appl. Microbiol.* **1 7**:55-57.

Grau, F.H., and P.B. Vanderlinde. 1993. Aerobic growth of *Listeria monocytogenes* on beef lean and fatty tissue: equations describing the effects of temperature and pH. *J. Food Prot.* **5 6**:96-101.

Gray, M.L. 1960a. A possible link in the relationship between silage feeding and listeriosis. *J. Amer. Vet. Med. Assoc.* **1 3 6**:205-208.

Gray, M.L. 1960b. Isolation of *Listeria monocytogenes* from oat silage. *Science.* **1 3 2**:1767-1768.

Gray, M.L., and A.H. Killinger. 1966. *Listeria monocytogenes* and Listeric infections. *Bacteriol. Rev.* **3 0**:309-382.

Gray, M.L., H.J. Stafseth, and F.JR. Thorp. 1950. Note: the use of potassium tellurite, sodium azide, and acetic acid in a selective medium for the isolation of *Listeria monocytogenes*. *J. Bacteriol.* **5 9**:443-444.

Gray, M.L., H.J. Stafseth, F. Thorp Jr. , L.B. Sholl, and W.F. Riley Jr. 1948. A new technique for isolating listerellae from the bovine brain. *J. Bacteriol.* **5 5**:471-476.

Grønstøl, H. 1979. Listeriosis in sheep- *Listeria monocytogenes* excretion and immuno-logical state in sheep in flocks with clinical listeriosis. *Acta Vet. Scand.* **2 0**:417-428.

Groves, R.D., and H.J. Welshimer. 1977. Separation of pathogenic from apathogenic *Listeria monocytogenes* by three in vitro reactions. *J. Clin. Microbiol.* **5**:559-563.

Guyer, S., and T. Jemmi. 1990. Betriebsuntersuchungen zum Vorkommen von *Listeria monocytogenes* in geräuchertem Lachs. (Analysis of the occurrence of *Listeria monocytogenes* in smoked salmon from a processing plant). *Arch. Lebensmittelhyg* **4 1**:144-146.

Guyer, S., and T. Jemmi. 1991. Behavior of *Listeria monocytogenes* during fabrication and storage of experimentally contaminated smoked salmon. *Appl. Environ. Microbiol.* **5 7**:1523-1527.

Hansen, L.T., T. Gill, and H.H. Huss. 1995. Effects of salt and storage temperature on chemical, microbiological and sensory changes in cold-smoked salmon. *Food Res. Int.* **2 8**:123-130.

Harris, L.J., M.A. Daeschel, M.E. Stiles, and T.R. Klaenhammer. 1989. Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes*. *J. Food Prot.* 5 2:384-387.

Hart, C.D., G.C. Mead, and A.P. Norris. 1991. Effects of gaseous environment and temperature on the storge behaviour of *Listeria monocytogenes* on chicken breast meat. *J. Appl. Bacteriol.* 7 0:40-46.

Hartemink, R., and F. Georgsson. 1991. Incidence of *Listeria* species in seafood and seafood salads. *Int. J. Food Microbiol.* 12:189-196.

Harvey, J. and A. Gilmour. 1994. Application of multilocus enzyme electrophoresis and restricton fragment length polymorphism analysis to the typing of *Listeria monocytogenes* strains isolated from raw milk, nondairy foods, and clinical and veterinary sources. *Appl. Env. Microbiol.* 6 0:1547-1553.

Harvey, J., and A. Gilmour. 1993. Occurrence and characteristics of *Listeria* in foods produced in Northern Ireland. *Int. J. Food Microbiol.* 1 9:193-205.

Hayes, P.S., J.C. Feeley, L.M. Graves, G.W. Ajello, and D.W. Fleming. 1986. Isolation of *Listeria monocytogenes* from raw milk. *Appl. Environ. Microbiol.* 5 1:438-440.

Hayes, P.S., L.M. Graves, B. Swaminathan, G.W. Ajello, G.B. Malcolm, R.E. Weaver, R. Ransom, K. Deaver, B.D. Plikaytis, A. Schuchat, J. D. Wener, R.W. Pinner, C.V. Broome, and The Listeria study group. 1992. Comparison of three selective enrichment methods for the isolation of *Listeria monocytogenes* from naturally contaminated foods. *J. Food Prot.* 5 5:952-959.

Heisick, J.E., D.E. Wagner, M.L. Nierman, and J.T. Peeler. 1989. *Listeria* spp. found on fresh market produce. *Appl. Env. Microbiol.* 5 5:1925-1927.

Heisick, J.E., L.I. Rosas-Marty, and S.R. Tatini. 1995. Enumeration of vialble *Listeria* species and *Listeria monocytogenes* in foods. *J. Food Prot.* 5 8:733-736.

Henry, B.S. 1933. Dissociation in the genus Brucella. *J. Infect. Diseases* 5 2:374-402.

Herman, L.M.F., J.H.G. Block, and R.J.B. Moermans. 1995. Direct detection of *Listeria monocytogenes* in 25 milliliters of raw milk by a two-step PCR with nested primers. *Appl. Environ. Microbiol.* 6 1:817-819.

Higgins, C.F., G.F. Ames, W.M. Barnes, J.M. Clement, and M. Hofnung. 1982. A novel intercistronic regulatory element of prokaryotic operons. *Nature.* 29 8:760-762.

Hird, D.W. 1987. Review of evidence for zoonotic Listeriosis. *J. Food Prot.* 5 0:429-433.

Ho, J.L., K.N. Shands, G. Friedland, P. Ecklind, and D.W. Fraser. 1986. An outbreak of type 4b *Listeria monocytogenes* infection involving patients from eight Boston hospitals. *Arch. Intern. Med.* 1 46:520-524.

Holt, J.G. 1977. The Shorter Bergey's Manual of Determinative Bacteriology, Part 16: Non-Sporing, Rod-Shaped Bacteria. Williams and Wilkins, Baltimore.

Holt, J.G., N.R. Krieg, P.H.A. Sneath, J.T. Staley, and S.T. Williams. 1994. Bergey's Manual of Determinative Bacteriology, Group 19: Regular, Nonsporing Gram-Positive Rods. Williams and Wilkins, Baltimore.

Horner, B. 1992. Fish smóking: ancient and modern. *Food Sci. Technol. Today.* **6**:166-171.

Houtsma, P.C. 1996. The antimicrobial activity of sodium lactate. PhD. Thesis, Agricultural University, Wageningen, The Netherlands.

Hudson, J. A., S.J. Mott, K.M. Delacy, and A.L. Edridge. 1992. Incidence and coincidence of *Listeria* spp., motile aeromonads and Yersinia enterocolitica on ready-to-eat fleshfoods. *Int. J. Food Microbiol.* **1 6**:99-108.

Hudson, J.A. 1994. Comparison of response surface models for *Listeria monocytogenes* strains under aerobic conditions. *Food Res. Int.* **2 7**:53-59.

Hudson, J.A., and S.J. Mott. 1993a. Growth of *Listeria monocytogenes, Aeromonas hydrophila* and *Yersinia enterocolitica* on cold-smoked salmon under refrigeration and mild temperature abuse. *Food Microbiol.* **1 0**:61-68.

Hudson, J.A., and S.J. Mott. 1993b. Growth of *Listeria monocytogenes, Aeromonas hydrophila* and *Yersinia enterocolitica* in pâté and a comparison with predictive models. *Int. J. Food Microbiol.* **2 0**:1-11.

Hulton, C.S.J., C.F Higgins, and P.M. Sharp. 1991. ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli, Salmonella typhimurium* and other enterobacteria. *Mol. Micorbiol.* **5**:825-834.

Huss, H.H., P. Karim Ben Embarek, and V. From Jeppesen. 1995. Control of biological hazards in cold smoked salmon production. *Food Control.* **6**:335-340.

ICMSF. 1988. Microorganisms in Food 4: Application of the Hazard Analysis Critical Control Point (HACCP) System to Ensure Microbiological Safety and Quality. Blackwell Scientific Publ., Oxford.

Ingram, M., and B.M. Mackey. 1976. Inactivation by cold. *In* F.A. Skinner, and W.B. Hugo (Eds.), *Inhibition and Inactivation of Vagetative Microbes,* Academic Press, New York, pp. 111-151.

Inoue, S., T. Tanikawa, J. Kawaguchi, T. Iida, and C. Morita. 1992. Prevalence of *Listeria* (spp.) in wild rats captured in the Kanto area of Japan. *J. Vet. Med. Sci.* **5 4**:461-463.

Ita, P.S., and R.W. Hutkins. 1991. Intracellular pH and survival of *Listeria monocytogenes* Scott A in tryptic soy broth containing acetic, lactic, citric, and hydrochloric acids. *J. Food Prot.* **5 4**:15-19.

Jemmi, T. 1993. *Listeria monocytogenes* in smoked fish: an overview. *Arch. Lebensmittelhyg* **44**:10-13.

Jemmi, T., and A. Keusch. 1994. Occurrence of *Listeria monocytogenes* in freshwater fish farms and fish-smoking plants. *Food Microbiol.* **11**:309-316.

Jepras, R.I., J. Carter, S.C., F.E. Paul,.and M.J. Wilkinson. 1995. Development of a robust flow cytometric assay for determining numbers of viable bacteria. *Appl. Environ. Microbiol.* **61**:2696-2701.

Jersek, B., E. Tcherneva, N. Tijpens, and L. Herman. 1996. Repetitive element sequence-based PCR for species and strain discrimination in the genus *Listeria*. *Lett. Appl. Microbiol.* **23**:55-60.

Jones, D. 1990. Foodborne listeriosis. *Lancet* **336**:1171-1174.

Junttila, J.T., S.I. Niemela, and J. Hirn. 1988. Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic listeria. *J. Appl. Bacteriol.* **65**:321-327.

Kampelmacher, E.H., and L.M. van Noorle Jansen. 1969. Isolation of *Listeria monocytogenes* from faeces of clinically healthy humans and animals. *Zbl. Bakteriol. Abt. I. Orig.* **211**:353-359.

Kampelmacher, E.H., and L.M. van Noorle Jansen. 1975. Occurrence of *Listeria monocytogenes* in effluents. *In* M. Woodbine (Ed.), *Problems of Listeriosis*, Leicester University Press, Leicester, England.

Kaplan, M.M. 1945. Listerellosis. *N. Eng. J. Med.* **232**:755-759.

Kaufmann, S.H.E. 1988. Listeriosis: new findings-current concerns. *Microb. Pathogenesis.* **5**:225-231.

Kawamura, K., and M. Kaneko. 1986. Microbial quality of human wastes and treatment plant effluent. *Water Science and Technology.* **18**:257-265.

Kelly, W.J., R.V. Asmundson, and C.M. Huang. 1996. Isolation and characterization of bacteriocin-producing lactic acid bacteria from ready-to-eat food products. *Int. J. Food Microbiol.* **33**:209-218.

Klausner, R.B., and C.W. Donnelly. 1991. Environmental sources of *Listeria* and *Yersinia* in Vermont dairy plants. *J. Food Prot.* **54**:607-611.

Koch, A.L. 1981. Growth measurement. *In* P. Gerhardt, R.G.E. Murray, and R.N. Costello *et al.* (Eds.) *Methods for General Bacteriology*, American Society for Microbiology, Washington, pp. 179-207.

Kramer, P.A., and D. Jones. 1969. Media selective for *Listeria monocytogenes*. *J. Appl. Bacteriol.* **32**:381-394.

Krist, K. 1997. Description and mechanisms of bacterial growth responses to water activity and compatible solutes. PhD. Thesis, University of Tasmania, Hobart, Australia.

Lawrence, L.M., and A. Gilmour. 1995. Characterization of *Listeria monocytogenes* isolated from poultry products and from the poultry-processing environment by random amplification of polymorphic DNA and multilocus enzyme electrophoresis. *Appl. Environ. Microbiol.* **61**:2139-2144.

Lehnert, C. 1960. Die Tenezitat von *Listeria monocytogenes* in der Aussenwelt. *Zentralbl. Bakt. ParasitKde. Abt. I. Originale.* **180**:350-356.

Leistner, L. 1985. Hurdle technology applied to meat products of the shelf stable and intermediate moisture food types. *In* D. Simatos, and J.L. Multon (Eds.), *Properties of Water in Foods in Relation to Quality and Stability*, Martinus Nijhoff Publishers, Dorrecht, The Netherlands, pp. 309-329.

Leistner, L. 1994. Further developments in the utilization of hurdle technology for food preservation. *J. Food Engineering.* **22**:421-432.

Lemeshow, S., and J-R Le Gall. 1994. Modeling the severity of illness of ICU patients. *JAMA.* **272**:1049-1055.

Leung, C-K., Y-W. Huang, and O.C. Pancorbo. 1992. Bacterial pathogens and indicators in catfish and pond environments. *J. Food Prot.* **55**:424-427.

Liewen, M.B., and M.W. Plautz. 1988. Occurrence of *Listeria monocytogenes* in raw milk in Nebraska. *J. Food Prot.* **52**:618-624.

Linnan, M., L. Mascola, X.D. Low, B. Goulet, S. May, C. Salminen, D. Hird, L. Yonekura, P. Hayes, R. Weaver, A. Andurier, B.D. Plikaytis, S.L. Fannin, A. Kleks, and C.V. Broome. 1988. Epidemic listeriosis associated with Mexican-style cheese. *N. Eng. J. Med.* **319**:823-828.

Loncarevic, S., W. Tham, and M.-L. Danielsson-Tham. 1996. Prevalence of *Listeria monocytogenes* and other *Listeria* spp. in smoked and "gravad" fish. *Acta Vet. Scand.* **37**:13-18.

Louws, F.J., F.W. Fulbright, C.T. Stephens, and F.J. de Bruijn. 1994. Specific genomic fingerprints of phytopatogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Appl. Environ. Microbiol.* **60**:2286-2295.

Lovett, J. 1988. Isolation and identification of *Listeria monocytogenes* in dairy products. *J. Assoc. Off. Anal. Chem.* **71**:658-660.

Lovett, J. 1989. *Listeria monocytogenes*. *In* M.P. Doyle (Ed.), *Foodborne Bacterial Pathogens*, Marcel Dekker, New York, pp. 283-310.

Lovett, J. 1990. Taxonomy and general characteristics of *Listeria* spp. *In* A.L. Miller, J.L. Smith, and G.A. Somkuti (Eds.), *Foodborne Listeriosis*, Elsevier Science Publishers B.V., Amsterdam, pp. 9-12.

Lovett, J., D.W. Francis, and J.M. Hunt. 1987. *Listeria monocytogenes* in raw milk: detection, incidence, and pathogenicity. *J. Food Prot.* **50**:188-192.

Low, J.C., and C.P. Renton. 1985. Septicemia, encephalitis and abortions in a housed flock of sheep caused by *Listeria monocytogenes* type 1/2. *Vet. Rec.* **116**:147-150.

Lueck, E. 1980. Antimicrobial Food Additives: Characteristics, Uses, Effects. Springer-Verlag, Berlin.

Lupski, J.R., and G.M. Weinstock. 1992. Short, interspersed repetitive DNA sequences in prokaryotic genomes. *J. Bacteriol.* **174**:4525-4529.

Mackey, B.M., and N. Bratchell. 1989. The heat resistance of *Listeria monocytogenes*. *Lett. Appl. Microbiol.* **9**:89-94.

Marmur, J., and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. Mol. Biol.* **5**:109-118.

Marshall, D.L., and R.H. Schmidt. 1988. Growth of *Listeria monocytogenes* at 10°C in milk preincubated with selected Pseudomonads. *J. Food Prot.* **51**:277-282.

Martin, B., O. Humbert, M. Camara, E. guenzi, H. Walker, T. Mitchell, P. Andrew, M. Prudhomme, G. Alloing, R. Hakenbeck, D.A. Morrison, G.J. Boulois, and J.-P. Clavrys. 1992. A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. *Nucl. Acids Res.* **20**:3479-3483.

Matthews, H.H., and A. Volframs. 1978. Investigating the physical aspects of bays and estuaries: a study of the hydrology and hydrodynamics of North West Bay. Board of Environmental Studies, University of Tasmania, Hobart, Australia.

McCarthy, S.A. 1997. Incidence and survival of *Listeria monocytogenes* in ready-to-eat seafood products. *J. Food Prot.* **60**:372-376.

McClure, P.J., C. de W. Blackburn, M.B. Cole, P.S. Curtis, J.E. Jones, J.D. Legan, I.D. Ogden, M.W. Peck, T.A. Roberts, J.P. Sutherland, and S.J. Walker. 1994. Modelling the growth, survival and death of microorganisms in foods: the UK Food Micromodel approach. *Int. J. Food Microbiol.* **23**:265-275.

McClure, P.J., T.A. Robert, and P. Otto Oguru. 1989. Comparison of the effects of sodium chloride, pH and temperature on the growth of *Listeria monocytogenes* on gradient plates and in liquid medium. *Lett. Appl. Microbiol.* **9**:95-99.

McClure, P.J., T.M. Kelly, and T.A. Roberts. 1991. The effects of temperature, pH, sodium chloride and sodium nitrite on the growth of *Listeria monocytogenes*. *Int. J. Food Microbiol.* **14**:77-92.

McDonald, P. 1970.Trends in silage making. *In* F.A. Skinner, and J.G. Carr (Eds.), *Microbiology in Agriculture, Fisheries and Food*, Academic Press, London, pp. 109-123.

McKay, A.M. 1993. The effect of temperature on culturability and detection of *Listeria innocua* in water. *Lett. Appl. Microbiol.* **17**:185-187.

McKellar, R.C. 1994a. Identification of the *Listeria monocytogenes* virulence factors involved in the CAMP reaction. *Lett. Appl. Microbiol.* **18**:79-81.

McKellar, R.C. 1994b. Minireview: use of the CAMP test for identification of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **60**:4219-4225.

McLauchlin, J. 1995. What is the infective dose for human listeriosis?. *In Proceedings of the XII International Symposium on Problems of Listeriosis*, Perth, Australia, 2-6 October, pp. 365-369.

McLauchlin, J. 1996. Molecular conventional typing methods for *Listeria monocytogenes*: the UK approach. *J. Food Prot.* **59**:1102-1105.

McLauchlin, J., A. Audurier, and A.G. Taylor. 1986. The evaluation of a phage-typing system for *Listeria monocytogenes* for use in epidemiological studies. *J. Med. Microbiol.* **22**:357-365.

McLauchlin, J., and L. Newton. 1995. Human listeriosis in England, Wales and northern Ireland: a changing pattern of infection. *In Proceedings of the XII International Symposium on Problems of Listeriosis*, Perth, Australia, 2-6 October, pp.177-179.

McLauchlin, J., S.M. Hall, S.K. Velani, and R.J. Gilbert. 1991. Human listeriosis and paté: a possible association. *Brit Med J.* **303**:773-775.

McMeekin, T.A., and T. Ross. 1996. Shelf life prediction: status and future possibilities. *Int. J. Food Microbiol.* **33**:65-83.

McMeekin, T.A., J. Brown, K. Krist, D. Miles, K. Neumeyer, D.S. Nichols, J. Olley, K. Presser, D.A. Ratkowsky, T. Ross, M. Salter, and S. Soontranon. 1997. Quantitative microbiology: a basis for food safety. *Emerging Infectious Diseases.* **3**:541-549.

McMeekin, T.A., J.N. Olley, T. Ross, and D.A. Ratkowsky. 1993. Predictive Microbiology: Theory and Application. Research Studies Press, Taunton, UK.

McMeekin, T.A., R.E. Chandler, P.E. Koe, C.D. Garland, J. Olley, S. Putro, and D.A. Ratkowsky. 1987. Model for the combined effect of temperature and water activity on the growth rate of *Staphylococcus xylosus*. *J. Appl. Bacteriol.* **62**:543-550.

Miles, D. 1994. Predicting the growth of *Vibrio parahaemolyticus*. B.Sc.(Hons.) Thesis, University of Tasmania, Hobart, Australia.

Miller, A.J., J.L. Smith, and G.A. Somkuti. 1990. Foodborne listeriosis. Elsevier Science Publishers B.V., Amsterdam.

Mitchell, R. 1974. *Introduction to environmental microbiology*. Prentice-Hall, Inc., New Jersey.

Monfort, P., J. Minet, J. Rocourt, G. Piclet, and M. Cormier. 1998. Incidence of *Listeria* spp. in Breton live shellfish. *Lett. Appl. Microbiol.* **26**:205-208.

Montville, T.J. 1997. Principles which influence microbial growth, survival, and death in foods. *In* M.P. Doyle, L.T. Beuchat, and T.J. Montville (Eds.), *Food Microbilogy: Fundamentals and Frontiers*, ASM Press,Washington, D.C., pp. 13-29.

Mossel, D.A.A., F.V. Rossem, M. Doopmans, M. Hendriks, M. Verouden and I. Eelderink. 1980. Quality control of solid culture media: a comparison of the classic and the so-called ecometric technique. *J. Appl. Bacteriol.* **49**:439-454.

Mossel, D.A.A., M. Jansma and J. De Waart. 1981. Growth potential of 114 strains of epidemiologically most common Salmonellae and Arizonae between 3 and 17°C. *In* T.A. Roberts, G. Hobbs, J.H.B. Christian, and N. Skovgaard (Eds), *Growth Potential of 114 Sstrains of Epidemiologically Most Common Salmonellae and Arizonae between 3 and 17°C*, Aalborg, Denmark, pp. 29-37.

Mossel, D.A.A., T.M.G.B-V. Laarhoven, A.M.Th. Ligtenberg-Merdus, and M.E.B. Werdler. 1983. Quality asssurance of selective culture media for bacteria, moulds and, yeasts: an attempt at standardization at the international level. *J. Appl. Bacteriol.* **54**:313-327.

Motes, M.L., Jr. 1991. Incidence of *Listeria* spp. in shrimp, oysters, and estuarine waters. *J. Food Prot.* **54**:170-173.

Munday, B.L., F.B. Ryan, S.J. King, and A. Corbould. 1966. Preparturient infections and other causes of foetal loss in sheep and cattle in Tasmania. *Aus. Vet. J.* **42**:189-193.

Murray, EG.D., R.A. Webb, and M.B.R. Swann. 1926. A disease of rabbits character-ised by a large mononuclear leucocytosis, caused by a *hitherto* undescribed bacillus *Bacterium monocytogenes* (n.sp.). *J. Pathol. Bacteriol.* **29**:407-439.

Nasim R. B., and R. Vahidy. 1993. Human Listerial meningitis: reported from Karachi, Pakistan. *J. Islamic Academy Sciences.* **6**:253-258.

National Food Authority. 1994. Food Standards Code Incorporating Amendments up to and Including Amendment 22 October 1994. The Australian Government Publishing Service, Canberra.

Neidhardt, F.C., J.L. Ingraham, and M. Schaechter. 1990. Physiology of the Bacterial Cell. Sinauer Associates Inc., Sunderland., USA.

Neumeyer, K., T. Ross, and T.A. McMeekin. 1997a. Development of a predictive model to describe the effects of temperature and water activity on the growth of spoilage pseudomonads. *Int. J. Food Microbiol.* **38**:45-54.

Neumeyer, K., T. Ross, G. Thomson, and T.A. McMeekin. 1997b. Validation of a model describing the effects of temperature and water activity on the growth of psychrotrophic pseudomonads. *Int. J. Food Microbiol.* **38**:55-63.

Newbury, S.F., N.H. Smith, E.C. Robinson, I.D. Hiles, and C.f. Higgins. 1987. Stabilization of translationally active mRNA by prokaryotic REP sequences. *Cell,* **48**:297-310.

Ng, S., and J.R.L. Forsyth. 1993. Listeriosis in Victoria. *Comm. Dis. Intell.* **1 7**:422-423.

Nolan, D.A., D.C. Chamblin, and J.A. Troller. 1992. Minimal water activity levels for growth and survival of *Listeria monocytogenes* and *Listeria innocua. Int. J. Food Microbiol.* **1 6**:323-335.

Nørrung, B., and N. Skovgaard. 1993. Application of multilocus enzyme electrophoresis in studies of the epidemiology of *Listeria monocytogenes* in Denmark. *Appl. Environ. Microbiol.* **5 9**:2817-2822.

Odegard, B., R. Grelland, and R.L. Hendrikwen. 1952. A case of *Listeria*-infection in man, transmitted from sheep. *Acta. Med. Scand.* **1 4 2**:231-238.

Oladepo, D.K., A.A.G. Candlish, and W.J. Stimson. 1992. Detection of *Listeria monocytogenes* using polyclonal antibody. *Lett. Appl. Microbiol.* **1 4**:26-29.

Olafson, P. 1940. *Listerella encephalitis* (circling disease) in sheep, cattle, and goats. *Cornell Bet.* **3 0**:141-150.

Owen, C.R., A. Meis, J.W. Jackson, and H.G. Stoenner. 1960. A case of primary cutaneous listeriosis. *N. Eng. J. Med.* **2 6 2**:1026-1028.

Partmann, W. 1965. Changes in proteins, nucleotides and carbohydrates during rigor mortis. *In the Technology of Fish Utilization, International Symposium,* Husum (Schleswig-Holstein) May 1964, Fishing News, London.

Patchett, R.A., N. Watson, P.S. Fernandez, and R.G. Kroll. 1996. The effect of temperature and growth rate on the susceptibility of *Listeria monocytogenes* to environmental stress conditions. *Lett. Appl. Microbiol.* **2 2**:121-124.

Pelroy, G.A., M.E. Peterson, P.J. Holland, and M.W. Eklund. 1994. Inhibition of *Listeria monocytogenes* in cold-process (smoked) salmon by sodium lactate. *J. Food Prot.* **5 7**:108-113.

Perry, C.M., and C.W. Donnelly. 1990. Incidence of *Listeria monocytogenes* in silage and its subsequent control by specific and nonspecific antagonism. *J. Food Prot.* **5 3**:642-647.

Peterson, M.E., G.A. Pelroy, R.N. Paranjpye, F.T. Poysky, J.S. Almond, and M.W. Eklund. 1993. Parameters for control of *Listeria monocytogenes* in smoked fishery produucts: sodium chloride and packaging method. *J. Food Prot.* **5 6**:938-943.

Petran, R.L., and E.A. Zottola. 1989. A study of factors affecting growth and recovery of *Listeria monocytogenes* Scott A. *J. Food Sci.* **5 4**:458-460.

Petran, R.L., and K.M.J. Swanson. 1993. Simultaneous growth of *Listeria mono-cytogenes* and *Listeria innocua*. *J. Food Prot.* **5 6**:616-618.

Phillips, C. 1993. Factors effecting water quality on oyster leases. B.Sc.(Hons.) Thesis, University of Tasmania, Hobart, Australia.

Piffaretti, J-C., H. Kressebuch, M. Aeschbacher, J. Bille, E. Bannerman, J.M. Musser, R.K. Selander, and J. Rocourt. 1989. Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease. *Proc. Natl. Acad. Sci. USA.* **8 6**:3818-3822.

Pirie, J.H.H. 1927. A new disease of veld rodents "Tiger Tiver Disease". *Publications of the South Africa Institute for Medical Research.* **3**:163-186.

Pirie, J.H.H. 1940. Listeria: change of name for a genus of bacteria. *Nature.* **145**:264.

Portnoy, D.A., T. Chakraborty, W. Goebel, and P. Cossart. 1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. *Infect. Immun.* **6 0**:1263-1267.

Pothuri, P., D.L. Marshall, and K.W. McMillin. 1996. Combined effects of packaging atmosphere and lactic acid on growth and survival of *Listeria monocytogenes* in crayfish tail meat at 4oC. *J. Food Prot.* **5 9**:253-256.

Prentice, G.A., and P. Neaves. 1992. The identification of *Listeria* species. *In* R.G. Board, D. Jones, and F.A. Skinner (Eds.), *Identification Methods in Applied and Environmental Microbiology*, Blackwell scientific Publications, Melbourne, pp. 283-296.

Presser, K.A. 1995. Modelling the growth response of *E. coli* to pH and lactic acid. B.Sc.(Hons.) Thesis, University of Tasmania, Hobart, Australia.

Presser, K.A., D.A. Ratkowsky, and T. Ross. 1997a. Modelling the growth rate of *Escherichia coli* as a function of pH and lactic acid concentration. *Appl. Environ. Microbiol.* **6 3**:2355-2360.

Presser, K.A., D.A. Ratkowsky, M.A. Salter, and S. Soontranon. 1997b. A new approach for modelling the interface between growth and no growht for *E. coli* and *Listeria monocytogenes* using generalised nonlinear regression. *Poster presented at the 9th Annual Australian Food Microbiology Conference, Sydney 20-23 July 1997.*

Presser, K.A., T. Ross, and D.A. Ratkowsky. 1998. Modelling the growth limits (growth/no growth interface) of *Escherichia coli* as a function of temperature, pH, lactic acid concentration, and water activity. *Appl. Environ. Microbiol.* **64(5)**:in press.

Price, T.J., and J.S. Lee. 1970. Inhibition of *Pseudomonas* species by hydrogen peroxide producing lactobacilli. *J. Milk Food Technol.* **3**:13-18.

Proctor, M.E., R. Brosch, H.W. Mellen, L.A. Garrett, C.W. Kaspar, and J.B. Luchansky. 1995. Use of pulsed-field gel electrophoresis to link sporadic cases of invasive listeriosis with recalled chocolate milk. *Appl. Environ. Microbiol.* **6**1:3177-3179.

Qvist, S., K. Sehested, and P. Zeuthen. 1994. Growth suppression of *Listeria monocytogenes* in a meat product. *Int. J. Food Microbiol.* **2**4:283-293.

Racz, P., K. Tenner, and E. Mérö. 1972. Experimental Listeria enteritis. I. An electron microscopic study of the epithelial phase in experimental Listeria infection. *Lab Invest.* **2**6:694-700.

Ratkowsky, D.A., and T. Ross. 1995. Modelling the bacterial growth/no growth interface. *Lett. Appl. Microbiol.* **2**0:29-33.

Ratkowsky, D.A., J. Olley, T.A. McMeekin, and A. Ball. 1982. Relationship between temperature and growth rate of bacterial cultures. *J. Bacteriol.* **149**:1-5.

Ratkowsky, D.A., R.K. Lowry, T.A. McMeekin, A.N. Stokes, and R.E. Chandler. 1983. Model for bacterial culture growth rate throughout the entire biokinetic temperature range. *J. Bacteriol.* **154**:1222-1226.

Ratkowsky, D.A., T. Ross, T.A. McMeekin, and J. Olley. 1991. Comparison of Arrhenius-type and Belehrádek-type models for prediction of bacterial growth in foods. *J. Appl. Bacteriol.* **7**1:452-459.

Ray, B., 1996. Fundamental Food Microbiology. CRC Press, New York.

Roberts, T.A. 1997. Microbial growth and survival: developments in predictive modeling. *Food Technol.* **5**1:88-90.

Roberts, T.A., A.C. Baird-Parker, and R.B. Tompkin. 1996. Micro-organisms in Foods 5: Microbiological Specifications of Food Pathogens. Blackie Academic and Professional, London.

Rocourt, J., and P.A.D. Grimont. 1983. *Listeria welshimeri* sp. nov. *and Listeria seeligeri* sp. nov. *Int. J. Syst. Bacteriol.* **3**3:866-869.

Rocourt, J., U. Wehmeyer, P. Cossart, and E. Stackebrandt. 1987a. Transfer of *Listeria denitrificans* to a new genus, *Jonesia* gen. nov., as *Jonesia denitrificans* comb. nov. *Int. J. Syst. Bacteriol.* **3**7:266-270.

Rocourt, J., U. Wehmeyer, P. Cossart, and E. Stackebrandt. 1987b. Proposal to retain *Listeria murrayi and Listeria grayi* in the genus *Listeria. Int. J. Syst. Bacteriol.* **3**7:298-300.

Rørvik, L.M., and M. Yndestad. 1991. *Listeria monocytogenes* in foods in Norway. *Int. J. Food Microbiol.* **1**3:97-104.

Rørvik, L.M., D.A. Caugant, and M. Yndestad. 1995. Contamination pattern of *Listeria monocytogenes* and other *Listeria* spp. in a salmon slaughterhouse and smoked salmon processing plant. *Int. J. Food Microbiol.* 25:19-27.

Rørvik, L.M., E. Skjerve, B.R. Knudsen, and M. Yndestad. 1997. Risk factors for contamination of smoked salmon with *Listeria monocytogenes* during processing. *Int. J. Food Microbiol.* 37:215-219.

Rørvik, L.M., M. Yndestad, and E. Skjerve. 1991. Growth of *Listeria monocytogenes* in vacuum-packed, smoked salmon, during storage at 4°C. *Int. J. Food Microbiol.* 14:111-118.

Rosenow, E.M., and E.H. Marth. 1987. Growth of *Listeria monocytogenes* in skim, whole and chocolate milk, and in whipping cream during incubation at 4, 8, 13, 21 and 35°C. *J. Food Prot.* 50:452-459.

Ross, T. 1993. A philosophy for development of kinetic models in predictive microbiology. PhD. Thesis, University of Tasmania, Hobart, Australia.

Ross, T. 1996. Indices for performance evaluation of predictive models in food microbiology. *J. Appl. Bacteriol.* 81:501-508.

Ross, T., and T.A. McMeekin. 1991. Pedictive microbiology: Applications of a square root model. *Food Australia* 43:202-207.

Ross, T., and T.A. McMeekin. 1994. Review paper: predictive microbiology. *Int. J. Food Microbiol.* 23:241-264.

Rosso, L., S. Bajard, J.P. Flandrois, C. Lahellec, J. Fournaud, and P. Vett. 1996. Differential growth of *Listeria monocytogenes* at 4 and 8°C: consequences for the shelf life of chilled products. *J. Food Prot.* 59:944-949.

Roszak, D.B., and R.R. Colwell. 1987. Survival strategies of bacteria in the natural environment. *Microbiol. Rev.* 51:365-379.

Russell, N.J., and G.M. Cook. 1995. Energetic of bacterial growth: balance of anabolic and catabolic reactions. *Microbiol. Reviews.* 59:48-62.

Ryser, E.T. 1995. Newly developed automated ribotyping system for *Listeria*. *In Proceedings of the XII International Symposium on Problems of Listeriosis: Listeria Methods Workshop Manual: Detection, Identification and Typing of Listeria monocytogenes in Foods*, Perth, Australia, 1-2 October, pp. 71-74.

Ryser, E.T., and E.H. Marth. 1988. Growth of *Listeria monocytogenes* at different pH values in uncultured whey or whey cultured with Penicillium camemberti. *Can. J. Microbiol.* 34:730-734.

Ryser, E.T., and E.H. Marth. 1991. *Listeria,* Listeriosis, and food safety. Marcel Dekker, New York.

Ryser, E.T., E.H. Marth, and M.P. Doyle. 1985. Survival of *Listeria monocytogenes* during manufacture and storage of cottage cheese. *J. Food Prot.* **4 8**:746-750,753.

Salter, M. 1998. Effects of temperature and water activity on *Escherichia coli* in relation to beef carcasses. PhD. Thesis, University of Tasmania, Hobart, Australia.

Sanders, B., P. Manoel, K. Levey, G. Klamus, C. Bell, and A. Ford. 1991. Review of Effluent Disposal Practices. Australian Water Resources Council Water Management Series No. 20, Department of Primary Industries and Energy, Australian Government Publishing Service, Canberra.

Schilling, U., M. Kaya, and F.-K. Lücke. 1991. Behaviour of *Listeria monocytogenes* in meat and its control by a bacteriocin-producing strain of *Lactobacillus sake*. *J. Appl. Bacteriol.* **7 0**:473-478.

Schlech, W.F., P.M. Lavigne, T.A. Bortolussi, A.C. Allen, E.V. Haldane, A.J. Wort, A.W. Heightower, S.E. Johson, S.H. King, E.S. Nicholls, and C.V. Broome. 1983. Epidemic listeriosis_evidence for transmission by food. *N. Eng. J. Med.* **3 0 8**:203-206.

Schoolfield, R.M., P.J.H. Sharpe, and C.E. Magnuson. 1981. Non-linear regression of biological temperature-dependent rate models based on absolute reaction rate theory. *J. Theor. Biol.* **8 8**:719-731.

Schuchat, A., B. Swaminathan, and C.V. Broome. 1991a. Epidemiology of human listeriosis. *Clin. Microbiol. Rev.* **4**:169-183.

Schuchat, A., B. Swaminathan, and C.V. Broome. 1991b. *Listeria monocytogenes* CAMP reaction. *Clin. Microbiol. Rev.* **4**:396.

Schwartz, B., C.V. Broome, G.R. Brown, A.W. Lightower, C.A. Ciesielski, S. Gaventa, B.G. Gellin, and L. Mascola. 1988. Association of sporadic listeriosis with consumption of uncooked hot dogs and undercooked chicken. *Lancet.* **I I**:779-782.

Seeliger, H.P.R. 1961. Listeriosis. Hafner Publishing Co., New York.

Seeliger, H.P.R. 1981. Apathogenic *Listeria: L. innocua* sp. n. (Seeliger and Schoop, 1977). Zbl. Bakt. Hyg., I. Abt. Orig. A **2 4 9**:487-493.

Seeliger, H.P.R. and K. Höhne. 1979. Serotyping of *Listeria monocytogenes* and related species. Academic Press, New York.

Seeliger, H.P.R., and D. Jones. 1986. Genus *Listeria*. *In* P.H.A. Sneath, N.S. Mair, M.E. Sharpe, and J.G. Holt (Eds.), *Bergey's Manual of Systemic Bacteriology, vol. 2*. Williams and Wilkins Co., Baltimore, pp. 1235-1245.

Seeliger, H.P.R., and H. Finger. 1983. Listeriosis, *In* J.S. Remington, and J.O. Klein (Eds.), *Infectious Diseases of the Fetus and Newborn Infant*. W.B. Saunders Co., Philadelphia, pp. 264-289.

Seeliger, H.P.R., J. Rocourt, A. Schrettenbrunner, P.A.D. Grimont, and D. Jones. 1984. *Listeria ivanovii* sp. nov. *Int. J. Syst. Bacteriol.* **3 4**:336-337.

Selander, R.K., D.A. Caugant, H. Ochman, J.M. Musser, M.N. Gilmour, and T.S. Whittam. 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl. Environ. Microbiol.* **5 1**:873-884.

Sergeant, E.S.G., S.C.J. Love, and A. McInnes. 1991. Abortions in sheep due to *Listeria ivanovii*. *Aust. Vet. J.* **6 8**:39.

Sharpe, P.J.H., and D.W. DeMichele. 1977. Reaction kinetics and poikilotherm development. *J. Theoretical Biol.* **6 4**:649-970.

Sharples, G.J., and R.G. Lloyd. 1990. A novel repeated DNA sequence located in the intergenic regions of bacterial chromosomes. *Nucl. Acids Res.*, **18**:6503-6508.

Shelef, L.A. 1994. Antimicrobial effects of lactates: a review. *J. Food Prot.* **5 7**:445-450.

Sikorski, Z.E., A. Kolakowska, and J.R. Burt. 1990. Postharvest biochemical and microbial changes. *In* Z.E. Skikorski (Ed.), *Seafood: Resurces, Nutritional Composition, and Preservation*, CRC Press Inc., Florida, pp. 55-75.

Sionkowski, P.J., and L.A. Shelef. 1990. Viability of *Listeria monocytogenes* strain Brie-1 in the avian egg. *J. Food Prot.* **5 3**:15-17.

Sizmur, K., and C.W. Walker. 1988. Listeria in prepacked salads. *Lancet*. **2 1**:1167.

Skalka, B., J. Smola, and K. Elischerova. 1982. Routine test for in vitro differentiation of pathogenic and apathogenic *Listeria monocytogenes* strains. *J. clin. Microbiol.* **1 5**:503-507.

Smith, L.T. 1996. Role of osmolytes in adaptation of osmotically stressed and chill-stressed *Listeria monocytogenes* growth in liquid media and on processed meat surfaces. *Appl. Environ. Microbiol.* **6 2**:3088-3093.

Smola, J. 1989. Possiblilties of differentiation of listerial hemolysins by synergistic hemolytic reactions (CAMP reactions). *Int. J. Food Microbiol.* **8**:265-267.

Sonnenwirth, A.C. 1973. Other pathogenic bacteria. *In* B.D. Davis, R. Dulbecco, H.N. Eisen, H.S. Ginsberg, W.B. Wood, and M. McCarty (Eds.), *Microbiology*, 2nd edition, Harper and Row Publishers, New York, pp. 946-947.

Sorrells, K.M., D.C. Enigl, and J.R. Hatfield. 1989. Effect of pH, acidulant, time, and temperature on the growth and survival of *Listeria monocytogenes*. *J. Food Prot.* **5 2**:571-573.

Souëf, P.N.L., and B.N.J. Walters. 1981. Neonatal listeriosis a summer outbreak. *Med. J. Aust.* **2**:188-191.

Stern, M.J., G.F-L. Smes, N.H. Smith, E.C. Robinson, and C.F. Higgins. 1984. Repetitive extragenic palindromic sequences: a major component of the bacterial genome. *Cell* **37**:1015-1026.

Stuart, S.E., and H.J. Welshimer. 1973. Intrageneric relatedness of *Listeria* Pirie. *Int. J. Syst. bacteriol.* **23**:8-14.

Stuart, S.E., and H.J. Welshimer. 1974. Taxonomic reexamination of *Listeria* Pirie and transfer of *Listeria grayi* and *Listeria murrayi* to a new genus, *Murraya. Int. J. Syst. bacteriol.* **24**:177-185.

Sutherland, P.S. 1989. *Listeria monocytogenes. In* K.A. Buckle, J.A. Davey, M.J. Eyles, A.D. Hocking, K.G. Newton, and E.J. Stuttard (Eds.), *Foodborne Micro-organisms of Public Health Significance*, 4th edition, J.M. Executive Printing Service, Australia, pp. 287-311.

Swaminathan, B., and G.M. Matar. 1993. Molecular typing methods. *In* D.H. Persing, T.F. Smith, F.C. Tenover, and T.J. White (Eds.), *Diagnostic Molecular Microbiology Principles and Applications*, ASM Press, Washington, D.C., pp. 26-50.

Tapia de Daza, M.S., Y. Villegas, and A. Martinez. 1991. Minimal water activity for growth of *Listeria monocytogenes* as affected by solute and temperature. *Int. J. Food Microbiol.* **14**:333-337.

ter Steeg, P.F., F.H., Pieterman, and J.C. Hellemons. 1995. Effects of air/nitrogen, temperature and pH on energy-dependent growth and survival of *Listeria innocua* in continuous culture and water-in-oil emulsions. *Food Microbiol.* **12**:471-485.

Teufel, P. 1994. European perspectives on *Listeria monocytogenes. Dairy, food and environmental sanitation.* **14**:212-214.

Thomas, C.J. 1995. PCR, RAPD and PFGE methods for screening isolates of *Listeria monocytogenes. In Proceedings of the XII International Symposium on Problems of Listeriosis: Listeria Methods Workshop Manual: Detection, Identification and Typing of Listeria monocytogenes in Foods*, Perth, Australia, 1-2 October, pp. 84-100.

Towner, K.J., and A. Cockayne. 1993. Molecular Methods for Microbial Identification and Typing. Chapan and Hall, London.

Truelstrup Hansen, L. 1995. Quality of chilled, vacuum packed cold-smoked salmon. Industrial PhD. Thesis, Technical University, Denmark.

Truelstrup Hansen, L., T. Gill, and H.H. Huss. 1995. Effects of salt and storage temperature on chemical, microbiological and sensory changes in cold-smoked salmon. *Food Res. Int.* **28**:123-130.

Vandegraaff, R., N.A. Borland, and J.W. Browning. 1981. An outbreak of listerial meningo-encephalitis in sheep. *Aust. Vet. J.* **57**:94-96.

Verheul, A., F.M. Rombouts, R.R. Beumer, and T. Abee. 1995. An ATP-dependent L-Carnitine transporter in *Listeria monocytogenes* Scott A is involved in osmoprotection. *J. Bacteriol.* **177**:3205-3212.

Versalovic, J., T. Koeuth, and J.R. Lupski. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucl. Acids Res.* **19**:6823-6831.

Vizcaino, L.L., M.-J. Cubero, and A. Contreras. 1988. Listeric abortions in ewes and cows associated to orange peel and artichoke silage feeding. *In the 10th International Symposium on Listeriosis*, Pecs, Hungary, 22-26 August, Abstr. 29.

Vlahovic, M.S., I. Kovincic, M. Mrdjan, L.J. Maslovaric, V. Pupavac, S. Bobos, and B. Trbic. 1988. The possibility of identification of *Listeria monocytogenes* in naturally infected cow's milk using fluorescent technic. *In the 10th International Symposium on Listeriosis*, Pecs, Hungary, 22-26 August, Abstr. p. 71.

Vorster, S.M., R.P. Greebe, and G.L. Nortjé. 1993. The incidence of *Listeria* in processed meats in South Africa. *J. Food Prot.* **56**:169-172.

Wagner, M., A. Maderner, and E. Brandl. 1996. Random amplification of polymorphic DNA for tracing and molecular epidemiology of *Listeria* contamination in a cheese plant. *J. Food Prot.* **59**:384-389.

Walker, S.J., P. Archer, and J.G. Banks. 1990. Growth of *Listeria monocytogenes* at refrigeration temperatures. *J. Appl. Bacteriol.* **68**:157-162.

Walls, I., and V.N. Scott. 1997. Validation of predictive mathematical models describing the growth of *Listeria monocytogenes*. *J. Food Prot.* **60**:1142-1145.

Wang, Guang-Hua, Ke-Ting Yan, Xiao-Ming Feng, Su-Ming Chen, Ai-Ping Lui, and Yataro Kodubo. 1992. Isolation and identification of *Listeria monocytogenes* from retail meats in Beijing. *J. Food Prot.* **55**:56-58.

Warburton, D.W., J.M. Farber, A. Armstrong, R. Caldeira, T. Hunt, S. Messier, R. Plante, N.P. Tiwari, and J. Vinet. 1991. A comparative study of the 'FDA' and 'USDA' methods for the detection of *Listeria monocytogenes* in foods. *Int. J. Food Microbiol.* **13**:105-118.

Watkins, J., and K.P. Sleath. 1981. Isolation and enumeration of *Listeria monocytogenes* from sewage, sewage sludge and river water. *J. Appl. Bacteriol.* **50**:1-9.

Watson, C., M. Jackson, H. Kelly, K. Ott, S. Knowles, and J. Wells. 1990. *Listeria* outbreak in Western Australia. *Comm. Dis. Intell.* **24**:9-12.

Watson, D.C. 1985. Potential risks to human and animal health arising from land disposal of sewage sludge. *J. Appl. Bacteriol.* **59**:95s-103s.

Weagant, S.D., P.N. Sado, K.G. Colburn, J.D. Torkelson, F.A. Stanley, M.H. Krane, S.C. Shields, and C.F. Thayer. 1988. The incidence of *Listeria* species in frozen seafood products. *J. Food Prot.* **51**:655-657.

Weis, J., and H.P.R. Seeliger. 1975. Incidence of *Listeria monocytogenes* in nature. *Appl. Microbiol.* 3 0:29-32.

Welsh, J., and M. McClelland. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucl. Acids Res.* 18:7213-7218.

Welshimer, H.J. 1960. Survival of *Listeria monocytogenes* in soil. *J. Bacteriol.* 8 0:316-320.

Welshimer, H.J. 1968. Isolation of *Listeria monocytogenes* from vegetation. *J. Bacteriol.* 9 5:300-303.

Welshimer, H.J., and J. Donker-Voet. 1971. *Listeria monocytogenes* in nature. *Appl. Microbiol.* 2 1:516-519.

Wesley, I.V., and F. Ashton. 1991. Restriction enzyme analysis of *Listeria monocytogenes* strains associated with foodborne epidemics. *Appl. Environ. Microbiol.* 5 7:969-975

Whiting, R.C., and J.C. Oriente. 1997. Time-to-turbidity model for non-proteolytic type B Clostridium botulinum. *Int. J. Food Microbiol.* 3 5:49-60.

Whiting, R.C., and M. Cygnarowicz-Provost. 1992. A quantitative model for bacterial growth and decline. *Food Microbiol.* 9:269-277.

Whiting, R.C., and R.L.B. Buchanan. 1997. Predictive modeling. *In* M.P. Doyle, L.R. Beuchat, and T.J. Montville (Eds.), *Food Microbiology Fundamentals and Frontiers*, American Society for Microbiology Press, Washington, D.C., pp. 728-739.

WHO Working Group. 1988. Foodborne Listeriosis. *Bulletin of the World Health Organization.* 6 6:421-428.

Wijtzes, T. 1996. Modelling the microbial quality and safety of foods. PhD. Thesis, Agricultural University, Department of Food Science, Wageningen, The Netherlands.

Wijtzes, T., P.J. McClure, M.H. Zwietering, and T.A. Roberts. 1993. Modelling bacterial growth of *Listeria monocytogenes* as a function of water activity, pH and temperature. *Int. J. Food Microbiol.* 1 8:139-149.

Wilesmith, H.W., and M. Gitter. 1986. Epidemiology of ovine listeriosis in Great Britain. *Vet. Rec.* 1 1 9:467-470.

Wilkins, P.O., R. Bourgeois, and R.G.E. Murray. 1972. Psychrotrophic properties of *Listeria monocytogenes*. *Can. J. Microbiol.* 1 8:543-551.

Wimpfheimer, L., N.S. Altman, and J.H. Hotchkiss. 1990. Growth of *Listeria monocytogenes* Scott A, serotype 4 and competitive spoilage organisms in raw chicken packaged under modified atmospheres and in air. *Int. J. Food Microbiol.* 1 1:205-214.

Wong, H-C, W-L Chao, and S-J Lee. 1990. Incidence and characterization of *Listeria monocytogenes* in foods available in Taiwan. *Appl. Environ. Microbiol.* **5 6**:3101-3104.

Woods, C.R., J. Versalovic, T. Koeuth, and J.R. Lupski. 1993. Whole-cell repetitive element sequence-based polymerase chain reaction allows rapid assessment of clonal relationships of bacterial isolates. *J. Clin. Microbiol* **3 1**:1927-1931.

Young, K.M., and P.M. Foegeding. 1993. Acetic, lactic and citric acids and pH inhibition of *Listeria monocytogenes* Scott A and the effect on intracellular pH. *J. Appl. Bacteriol.* **7 4**:515-520.

Zottola, E.A., and L.B. Smith. 1991. The microbiology of foodborne disease outbreaks: an update. *J. Food Safety.* **1 1**:13-29.

A COMMMON MATERIALS AND METHODS

A.1 MATERIALS

A.1.1 REAGENTS

Catalase reagent: Hydrogen peroxide (Sigma; Code: H 6520) 3% w/w aqueous solution

Kovacs reagent:

Paradimethylaminobenzaldehyde (Sigma; Code: D 5263)	5.0 g
Isoamyl alcohol (pH < 6.0) (Sigma; Code: I 3643)	75 ml
Concentrated Hydrochloric acid (BDH; Code: 103076 P)	25 ml

Lactic Acid

Univar, AR (Min. 88% w/w). Ajax Chemicals, Auburn, NSW, Australia

Methyl Red reagent The indicator solution was prepared by dissolving 0.4 g Methyl Red (BDH; Code: 20068) in 100 ml of distilled water.

Nitrate reagents Reagent A: Sulfanilic acid (Sigma; Code: S-5263) 8 g in 1 L of 5N acetic acid (Sigma; Code: A 6283)

Reagent B: Alpha-napthylamine (Sigma; Code: N-9005) 5 g in 1 L of 5N acetic acid

Oxidase reagent Tetramethyl-paraphenylenediamine dihydrochloride (Sigma; Code: T 7394) 1% aqueous solution (freshly prepared)

Sodium thiosulphate (10%) (Sigma; Code: 7143) 10 g of sodium thiosulphate was dissolved in 100 ml distilled water, mixed well and kept at room temperature.

Voges-Proskauer reagents

Reagent A: Alpha-naphthol (M&B; Code: N28) 5 g in absolute ethyl alcohol 100 ml
Reagent B: Potassium hydroxide (BDH; Code: 10210) 40 g, in distilled water 100 ml

Water The water used in the preparation of reagents and media was prepared by glass distillation of deionised water.

A.1.2 CULTURE MEDIA AND TEST KIT

Preparation, storage and quality control of the following media and reagents were as described below or according to the manufacturer's directions.

API Listeria (BioMérieux; Code: 10 300)

Bile aesculin agar (Oxoid; Code: CM888)

Brain heart infusion broth (BHIB) (Oxoid; Code: CM375) Brain heart infusion agar (BHIA) was prepared from BHIB by addition of 1.5% bacteriological grade agar (Oxoid, L11) prior to sterilisation, and then autoclaved at 121°C x 15 min.

Carbohydrate fermentation broth
Basal medium:

Bacto Peptone (Oxoid; Code: L37)	10.0 g
Lab Lemco Powder (Oxoid; Code:CM15)	1.0 g
Sodium chloride (Univar, Code: 465)	5.0 g
Distilled Water	900 ml
Phenol Red (360 mg/20 ml 0.1 N NaOH)	1 ml

The pH was adjusted to 7.4 ± 0.2 and sterilized at 121°C x 15 minutes. To the cooled basal fermentation broth, 100 ml of filter sterilized carbohydrate solution was added as indicated : Mannitol 10%, Rhamnose 5%, Xylose 5%. 3 ml aliquots were aseptically dispensed to small bijouxs.

Columbia blood agar (Oxoid; Code: CM331) The basal medium was prepared according to the manufacturer's instructions, sterilised, then cooled to 50°C and 8 ml added to 100 mm diameter petri dishes. While still warm, these were overlayed with horse blood agar as described below.

Overlay 4% Defibrinated Horse Blood (Oxoid, Code: HB050) was added aseptically to melted columbia blood agar base which has been cooled to 46°C, mixed with gentle rotation and 3 or 4 ml poured on top of the base layer (warm). Plates were tilted to spread the top layer evenly. A thin overlay was necessary to demonstrate haemolysis produced by surface colonies.

Fraser broth (FB) (Oxoid; Code: CM895)

Lauryl tryptose broth (LTB) (Oxoid; Code: CM451)

Listeria enrichment broth base (UVM Formulation) (Oxoid; Code: CM863)

500 ml basal medium was prepared, sterilisedby autoclaving and cooled to 50°C. The contents of one vial of Listeria Primary Selective Enrichment Supplement (UVM I) Code SR 142 reconstituted with 2 ml of sterile distilled water was added aseptically. The media was mixed well and distributed into sterile containers.

Listeria selective agar base (Oxford formulation, OXF) (Oxoid; Code: CM856)

500 ml basal medium was sterilised by autoclaving and cooled to 50°C. The contents of one vial of Listeria Selective Supplement (Oxford Formulation) Code SR 140 reconstituted with 5 ml of ethanot/sterile distilled water (1:1) was added aseptically. The media was mixed well and poured into sterile petridishes.

Membrane lauryl sulphate agar (MLSA) The media was prepared by adding 1.5% bacteriological grade agar (Oxoid, Code: L11) to Membrane Lauryl Sulphate broth (Oxoid; Code: MM615) prior to sterilisation.

Motility test medium (Difco, Code: 0105-01-3)

MRVP medium (Oxoid; Code: CM43)

Nitrate broth:

Bacto Beef Extract (Oxoid; Code: 0126-01)	3.0	g
Bacto Peptone	5.0	g
Potasium nitrate (Sigma; Code: P 8394)	1.0	g
Distilled water	1	L

Adjust final pH to 7.0 ± 0.2 at 25°C

Sheep blood agar (SBA, CAMP Test agar) (Oxoid; Code: CM854) The basal medium was prepared and sterilised according to the manufacturer's instructions, then cooled to 50°C and 8 ml poured to 100 mm diameter petri dish. The media was allowed to solidify and, while still warm, overlayed with sheep blood as described below.

Overlay 5% Defibrinated Sheep Blood (Oxoid, Code: SB50) was aseptically added to melted sheep blood agar base which had been cooled to 46°C. Media was mixed with gentle rotation and 3 or 4 ml poured on top of the base layer (warm). Plates were tilted to spread the top layer evenly. A thin overlay was necessary to demonstrate haemolysis produced by surface colonies.

Tryptone Soya Broth (TSB), (Oxoid, Code: CM 129)

Tryptone Soya Agar-Yeast Extract (TSA-YE) was prepared from TSB by the addition of 0.6% yeast extract (Oxoid, Code: L21) and 1.5% bacteriological grade agar (Oxoid, Code: L11) prior to sterilisation.

Tryptone Soya Broth-Yeast Extract (TSB-YE) was prepared from TSB by the addition of 0.6% yeast extract (Oxoid, Code: L21) prior to sterilisation. Media were sterilised by autoclaving at 121°C x 15 min.

Tryptone water (Oxoid; Code: CM87) containing 0.1% bacteriological peptone (Oxoid; Code: L37) and 0.85% NaCl was used for serial dilution and for suspension of food samples for homogenisation. It was sterilised by autoclaving at 121°C x 15 min. Final pH 7.2 ± 0.2 at 25°C.

A.1.3 PCR REAGENTS, REAGENTS, AND PRIMERS

1.5% Agarose gel (GibcoBRL; Code: 15510-027) 1.5 g agarose gel was dissolved in 100 ml of TAE buffer (*see* below), and heated until boiling so that the gel completely dissolved. It was cooled to ca. 60°C before pouring onto a gel mould.

Chloroform: Isoamyl alcohol 24:1 (Sigma; Code: C 0549)

DNA Molecular Weight Markers:

1. pUC19 DNA/*Hpa* II (Bresatec, Adelaide, Australia): The *Hpa* II digest of plasmid pUC19 DNA produces low molecular weight DNA fragments ranging from 26 bp to 501 bp which were used as DNA molecular weight markers for the lower register.

2. SPP-1 (Bresatec, Adelaide, Australia): The double-stranded DNA isolated from bacteriophage SPP-1 which was digested with *Eco* RI was used as a DNA molecular weight marker. It produces 15 DNA fragments in different sizes range from 360 bp to 8,510 bp.

Ethidium Bromide (10 mg/ml) (Sigma; Code: E-1510) The gel staining solution was freshly prepared by adding 15 μl of Ethidium bromide solution to 400 ml of TAE buffer.

6x Gel Loading Buffer:

Bromphenol blue (Sigma; Code: B8026)	0.125 g
Sucrose (Sigma; Code: S 2395)	20.0 g
Distilled water	50 ml

Lysozyme (Sigma, Code: L-7001)

40 mg lysozyme was dissolved in 2 ml saline EDTA, shaken vigorously and dispensed to 1 ml aliquots which were kept at -18°C until required.

Phenol: chloroform:isoamyl alcohol 25:24:1 (Sigma; Code: P-3803)

Primers: The REP-PCR, ERIC-PCR, and BOX-PCR primers were synthesized by Life Technologies Inc., Melbourne, Australia.

Proteinase K

Proteinase K (amRESCO; Code: E634)	10.0 mg
Tris-EDTA	1 ml

Saline-EDTA

Sodium chloride	8.75	g
EDTA, disodium salt (amRESCO; Code: 0105)	37.2	g
Distilled water	1	L

Mixed and adjusted the pH to 8.0 with NaOH.

Sodium dodecyl sulphate (SDS, 10%) (GibcoBRL; Code: 5525UB) 10 g of SDS were dissolved in 100 ml distilled water, mixed well and the pH adjusted to 7.0 with 0.1M NaOH.

70.2% Sodium perchlorate (Sigma; Code: S-1401)

Solutions for Calf thymus DNA calibration

1. 10x TNE Buffer (Standard fluorometer assay solution)

Tris (hydroxymethyl) aminomethane (Sigma, Code: T-8524)	6.06	g
Potasium nitrate	1.0	g
Distilled water	500	ml

The pH was adjusted to 7.4 with conc. HCl. Buffer was filtered before use (0.45 μm) and stored at 4°C for up to 3 months.

2. Calf thymus DNA- 1:10 dilution for low range assay (100μg/ml)

100 μl calf thymus DNA standard

100 μl 10x TNE

800 μl distilled water

The DNA soulution was shaken to mix thoroughly and stored at 4°C for up to 3 months.

3. DNA-specific dye (Hoechst 33258): stock dye solution

H 33258 (Hoefer TKO 310)	1.000 mg/ml

Distilled water was added to make 10.00 ml of stock solution which was stored at 4°C for up to 6 months in an amber bottle.

4. Dye solution A - for low range DNA assay (10-500 ng/ml final conc.)

H 33258 stock solution	10 μl
10x TNE	10.0 ml
Distilled filtered water	90.0 ml

Dye solution was freshly prepared on the day required and kept at room temperature.

Thermostable DNA Polymerase Reaction Buffer I (10x) (*Taq* DNA polymerase, Reaction buffer, and MgCl$_2$ Solution) (Advanced Biotechnologies; Code: AB-0194) These PCR reagents consist of 3 separate vials:

1. 250 units *Taq*: The enzyme is extracted from *Thermus species* and prepared at a concentration of 5 Units/μl. The enzyme has 5' to 3' polymerisation-dependent exonuclease replacement activity but lacks a 3' to 5' exonuclease activity.

2. 1.25 ml of 10x Reaction buffer consisting of 100mM Tris-HCl (pH 8.3 at 25°C), 500mM KCl.

3. 1.25ml of MgCl$_2$

TAE buffer

Tris (hydroxymethyl) aminomethane (Sigma; Code: T 1378)	48.4	g
Glacial acetic acid (Sigma; Code: A6283)	11.42	ml
0.5M EDTA (pH 8)	20	ml
Distilled water to final volume	900	ml

Tris-EDTA

Tris (hydroxymethyl) methylamine (UNILAB; Code: 563)	0.121	g
Disodium EDTA	0.074	g
Distilled water to final volume	100	ml

Reagents were mixed and the pH adjusted to 8.1 with NaOH. Tris-EDTA sloution was kept refrigerated.

Milli-Q Water (Distilled filtered water)

The water used to dissolve bacterial DNA, and prepare some solutions was prepared by a distilled filtered water machine model Milli-Q PLUS (MILLIPORE S.A., France).

A.1.4 SOURCES OF ORGANISMS

The cultures with ACM label were obtained from Australian Collection of Micro-organisms, University of Queensland, St. Lucia, Brisbane, Australia. The cultures with ATCC label were obtained from Dr. C.D. Garland, Aquahealth, University of Tasmania, Hobart, Tasmania.

Escherichia coli ATCC 25922

Listeria innocua ACM 3178

Listeria ivanovii ACM 3179

Listeria monocytogenes ACM 98

Rhodococcus equi ACM 702

Staphylococcus aureus ATCC 25923

Streptococcus faecalis ATCC 19433

L. monocytogenes strain Scott A was obtained from Dr. F. Grau, CSIRO Division of Food Processing, Brisbane, Queensland.

L. monocytogenes strain L5, isolated from commercially prepared cold smoked salmon was also obtained from Dr. C.D. Garland.

A.1.5 EQUIPMENT

Anaerobic Jars:
Polycarbonate anaerobic jars 3.5 L model 60627 (BBL) and model HPO 11A (Oxoid) were used.

Balances
1. Mettler PJ 3600 DeltaRange®.± 0.01 g precision. Mettler Instrumente AG, Zurich, Switzerland.
2. MC1 Analytic AC210P (Sartorius Australia Pty Ltd, PO Box 84 Chadstone, Vic 3148, Aus). Precision ± 0.0001 g.

Conductance Meter
Conduktometer, LF 191 WTW

DNA Fluorometer
The DNA fluorometer model TKO-100 (Hoefer Scientific Instruments, USA). $\lambda_{ex} = 365$ nm, $\lambda_{em} = 460$ nm.

DNA Thermocycler

A FTS-960 Fast Thermal Sequencer (Corbett Research, Australia) was used. The unit cycles 96 well polycarbonate microplates, or 0.2 ml microtubes in strips of eight, and operates from 4°C to 96°C and incorporates an 'Active Thermoelectric Module' (Patent Pending).

Ekman Grab

Similar to that depicted in Figure 10500:7 page 10-102 in APHA (1989).

Electronic Temperature Loggers :

Delphi loggers with a teflon freezer probe (MIRINZ, Hamilton, New Zealand). Quoted accuracy ± 0.25°C over the operating range (-20°C to +40°C).

Filter Housing

Diameter 47 mm (Nalgene) and 90 mm (Schott Glaswerke, Duran-Screw Filters).

Gel Electrophoresis Apparatus

A horizontal gel electrophoresis apparatus (Horizon® 58, GibcoBRL, Life Technologies, USA). Current range : 4-360 mA, Voltage Range : 200 VDC Max.

Incubators

A range of Qualtex incubators were used (Manufactured by Watson Victor Ltd., Aus).

Laminar Flow Cabinet

A laminar flow cabinet model CF43S (Gelman Sciences, Aus) and model DF-44 (Clemco Contamminaton Control, Clemco Ultra-Violet Products Pty. Ltd., 71 Dickson Ave., Artarmon, N.S.W.) were used.

pH Metering

1. General: Microprocessor pH-temperature Meter (portable), pH 196 WTW.

2. pH - measurement of cultures: Orion Model 250A (portable) with calomel sealed flat tip probe (AEP433). Orion Research Inc., Boston, Mass., USA.

Pipettors

A range of fixed and variable volume pipettors were used throughout this study.

1. 'Transferpette', Germany: 100 μl, 1 ml

2. 'Pipetman': 1-20 μl, 1-100 μl, 1-200 μl and 200-1000 μl. Gilson Medical Electronics (France) S.A., B.P. 45-95400 Villiers-le-Bel, France.

3. 'Oxford Macro-set': 5-10 ml, 'Oxford Adjustable': 40-200 μl. Oxford Laboratories, Inc., California. USA.

4. 'Eppendorf': 0.5-10 μl and 10-100 μl.

5. Electronic Digital Pipette 'EDP-Plus Motorized Microliter Pipette' (Rainin Instrument Co, Inc., Mack Road, Woburn, MA 0188-4026 USA).

Dispensed volume of fixed volume pipettors was checked periodically by weighing of water at room temperature, and was typically found to be within ±1% of nominal volume. Variable volume pipettors were calibrated, by weighing of water, before use.

Spectrophotometer

Spectronic 20 (analogue display), Milton Roy Co., USA.

Stomacher

Colworth, Stomacher 400, Model BA6021, Single Phase, A.J. Seward, UAC House, Blackfriars Road, London, SE1 9UG

Temperature Gradient Incubator

Model TN3. Advantec, Toyo Roshi International, California, USA.

Timer

An alarm clock-timer (Model 870A, Jadco, China) was used for all growth rate experiments. At the commencement of inoculation, the timer was set to zero, and the real time recorded in case of timer failure.

Triplet Outlet Filter Manifold

The equipment (Nalgene) was connected with a 10 L liquid reservoir, water trap and 240 volt vacuum pump, Clements.

Chamber Vacuum Packing Machine

BUSCH type 100-132 (Boss 6380 Bad Homburg 6, West Germany), vacuum 0.5 mbar, motor oil type SAE 30, Timer: second (manual) or automatic.

Vortex Mixer

Model MT19 (Chiltern Scientific). Variable speed control from 300 to 2,200 rpm.

Water Activity Meter

Aqualab CX2 (Decagon Devices, Inc. PO Box 835, Pullman, Washington 99163, USA). Quoted acuracy ± 0.003. The instrument was checked on each occasion before use by distilled water and satuated NaCl.

Water Baths

1. A range of Lauda waterbaths (Lauda DR.R. Wobser GMBH & Co. K.G., Lauda-Könighofen, West Germany) was used; Models RC20, RM20 (R denotes refrigerated, the number indicates the bath capacity in litres).

2. Shaking waterbath Model SWB20 (Ratek instruments, 1/3 Wadhurst Drive Boronia, Aus 3155).

A.1.6 CONSUMABLES

Anaerobic Gas Generating Kit : Anaerogen (Oxoid Code: AN 035A).

Centrifuge Tubes

1. Conical Tubes: 15 ml and 50 ml sterile/Gamma irradiated graduated conical tube (Opticul™ Polypropylene) with cap (Falcon, Becton Dickson Labware, 2 Bridgewater Lane, Lincoln Park, New Jersey USA).

2. Microcentrifuge tubes: 0.5 and 1.5 ml microcentrifuge tube with cap graduated (Kartell) made in Italy.

Filtering

1. Cellulose acetate membrane filter pore size 0.45 μm, diameter 47 mm (GN-6, Gelman-Sciences) and diameter 90 mm (Supor®-450, GelmanSciences).

2. Cellulose ester prefilter, diameter 90 mm (AW06 90 25, Millipore).

3. Filter paper, Whatman No. 3.

4. Sterile filter unit pore size 0.45 μm hydrophilic cellulose acetate membrane, diameter 25 mm, acrylic Housing (PRO-X™, Lida Manufacturing Corp.).

Gauze Pads

Conforming cotton gauze bandage width 3 and 10 cm were used.

L-Tubes

L-shaped glass tube, 150 mm diameter, capacity approximately 25 ml. Topped with metal cap.

Petri Dishes

Sterile plastic Petri dishes 150x860 mm (LABSERV, Australia), and 150x560 mm (Disposable Products, South Australia).

Plastic Bags

Stomacher bags 100×160 mm (Disposable Products, Australia), and 172×253 mm factory's plastic bags used for packaging its retail product.

Sterile Well Plates

Linbro® Tissue Culture multi-well plate with cover, 24 flat bottom wells 1.7x1.6 cm approx., Well capacity: 3.5 ml approx. Area per well: 2.0 cm^2 approx. (ICN Biomedicals, Inc. 1263 South Chillicothe Road Aurora, Ohio 44202).

A.2 METHODS

A.2.1 CATALASE TEST

The catalase reagent was dropped onto a slide, then smeared with the bacterial culture. Gas bubbles observed from the smear constitutes a positive catalase test. No gas bubbles constitutes a negative test. A *S. aureus* culture was used as a positive-control test and *Pseudomonas aeruginosa* was used as a negative-control test.

A.2.2 COLONY COUNTING METHODS

Three appropriate dilutions of samples were routinely plated. For growth rate determination experiments, numbers of organisms at each sampling time were predicted on the basis of models developed in broth systems. From this prediction, the sample dilution expected to yield 30-300 colonies on a 0.1 ml spread plate, and the tenfold higher and tenfold lower dilutions were plated. Duplicate spread plates of each dilution were usually prepared.

The colonies on all plates were counted and recorded, except in the case of very high numbers, for which an estimate based on the number of colonies within a subsection of the plate was used. All plates having between 30 and 300 colonies were included in the calculation of the number of organisms present in the sample, using the method of Farmiloe *et al.* (1954).

A.2.3 CORRECTION FUNCTION FOR NON-LINEARITY OF ABSORBANCE (CELL YIELD) DATA

The deviation of the OD response from the cell density is reported to be non-linear when the OD value is above 0.3 (Koch, 1981). The observed absorbance for the "apparent" cell yield at the maximum growth of *L. monocytogenes* in an enriched nutrient used in Chapter 4 may be well above the upper sensitivity limit of the instrument (McMeekin *et al.*, 1993). Therefore, the "apparent" yield was corrected for the non-linearity of the OD-concentration relationship, using the correction function derived by Dalgaard *et al.* (1994). The equation can be written as:

$$ABS = ABS_{obs} \times (1 + (k_1(ABS_{obs})^{k_2}))$$

where ABS is the corrected absorbance, ABS_{obs} is the observed absorbance, k_1 and k_2 are the constant values of 0.51 and 2.49 respectively.

A.2.4 INDOLE TEST

0.2 ml of Kovacs reagent was added to inoculated Tryptone Water after incubation at 35°C for 24-48 hr. The culture was shaken, then allowed to stand for 10 min. A dark red colour in the amyl alcohol surface layer constitutes a positive indole test; no change in the original colour of the reagent constitutes a negative test.

A.2.5 MAINTENANCE OF CULTURES AT -80°C (LONG TERM STORAGE)

All cultures were maintained in triplicate. One was used for routine recovery, while the others were held in reserve.

Plastic beads (3 mm) were washed in tap water with detergent, followed by dilute HCl to neutralise alkalinity. The beads were washed several times in tap water, then in distilled water and dried. Approximate 20 beads were placed in each small bijoux, which was then autoclaved at 121°C x 15 min.

A single colony from each strain of bacteria was grown overnight on appropriate agar plates at the optimum temperature for each bacterial strain. Approximately 1 ml of sterile (autoclaved: 121°C x 15 min) 15% (v/v) glycerol in NB was dispensed onto the plate. Using a wire loop the growth was emulsified to make a thick suspension. The bacterial suspension was aseptically transferred into the prepared vials. The suspension was aspirated several times to ensure the air bubbles inside the bead were displaced. Excess suspension was removed to prevent the beads sticking together when frozen. Vials were placed on their sides (to facilitate removal of beads when frozen) and stored overnight at -20°C before being transferred to -80°C.

The recovery was done by removing a bead and rubbing over the surface of a suitable solid medium and also selective medium (to check the purity and identity), which were then incubated appropriately.

A.2.6 MAINTENANCE OF CULTURES AT 4°C (SHORT TERM STORAGE)

The cultures used routinely as the reference cultures in CAMP test and other reagents tests were maintained aerobically on BHIA slopes at 4°C and periodically subcultured. Purity and identity of the culture was checked at subculture by gram reaction and colony morphology on selective media.

A.2.7 METHYL RED TEST

To 5 ml of MRVP medium culture (after incubation at 35°C for not less than 48 hr) was added a few drops of methyl red solution and the colour on the surface of the medium read immediately. A positive reaction is indicated by a distinct red colour, showing the presence of acid. A negative reaction is indicated by a yellow colour.

A.2.8 NITRATE TEST

A few drops of each nitrate reagent were added to inoculated nitrate broth after incubation at 35°C for 24 hr. A distinct red or pink colour indicates the presence of nitrite reduced from original nitrate. The test was controlled by comparing with an uninoculated tube of the medium which had been kept under the same conditions as the inoculated tubes. The evolution of gas in nitrate meduim containing no sugar or fermentable substance is a definite indication of reduction to free nitrogen.

A.2.9 OXIDASE TEST

A filter paper was soaked with a few drops of the freshly prepared reagent. A bacterial colony was picked and streaked on the soaked filter paper. A distinct purple colour on the streak line constitutes a positive test. No change in colour constitutes a negative test. Note that the reagent oxidizes rapidly which makes the colour change from transparent to purple, leading to possible false ositive results.

A.2.10 PREPARATION OF CHLORINATED WATER SAMPLE BOTTLES

Sample bottles used for collecting chlorinated water and ice were added with sodium thiosulphate (10% w/v) at a rate of 0.4 ml per 500 ml expected sample volume. The bottles were then autoclaved at 121°C x 15 min.

A.2.11 QUANTITATION OF BACTERIAL DNA

The DNA fluorometer, TKO-100, was calibrated with the Calf thymus DNA standard before use.:

1. Two ml of dye solution A (see section A.1.3) was added into the glass cuvette and used as a blank solution (set the instrument to zero).

2. 2 μl of Calf thymus DNA (see section A.1.3) was added and the solution mixed (without introducing bubbles into the solution). The scale was set to 100%.

3. Steps 1 to 2 were repeated at least once to verify that the results were reproducible. The cuvette was rinsed with distilled filtered water and drained between each measurement.

The bacterial DNA concentration was measured by following the above steps but in step 2, 2 μl of bacterial DNA solution was used instead. The DNA concentration was read directly as ng/μl.

A.2.12 RELATIONSHIP BETWEEN ABSORBANCE AND PERCENT TRANSMITTANCE

Transmittance and absorbance are defined:

$$\text{absorbance} = \log_{10}(I_{incident}/I_{transmitted})$$
$$\text{transmittance} = \log_{10}(I_{transmitted}/I_{incident})$$

therefore:
$$\text{absorbance} = \log_{10}(1/\text{transmittance})$$
$$= -\log_{10}(\text{transmittance})$$

by adding and subtracting $\log_{10} 100$

$$= (\log_{10} 100 - \log_{10} 100) - \log_{10}(\text{transmittance})$$

and rearranging and evaluating $\log_{10} 100$

$$= 2 - \{\log_{10} 100 + \log_{10}(\text{transmittance})\}$$
$$= 2 - \log_{10}(100 \times \text{transmittance})$$
$$= 2 - \log_{10}(\text{percent transmittance})$$
$$= 2 - \log_{10}(\%T)$$

A.2.13 VOGES-PROSKAUER TEST

To 5 ml of MRVP medium culture (after incubation at 35°C for 24 hr) was added 0.6 ml of reagent A and 0.2 ml of reagent B. The culture was shaken well, allowed to stand exposed to the air, and observed at intervals of 2, 12, and 24 hr. A positive test was indicated by the development of an eosin pink colour.

B MULTILOCUS ENZYME ELECTROPHORESIS

B.1 MATERIALS AND METHODS

The multilocus enzyme electrophoresis tests in this section were performed at The Food Safety Solutions, Sydney, NSW. All the materials and equipment used were provided by P. Sutherland, The Food Safety Solutions.

B.1.1 SAMPLE PREPARATION

100 ml of BHI was inoculated with *L. monocytogenes* and incubated at 37°C on a shaker for 24 hrs. Cells were harvested by spinning at 2,500 rpm for 15 mins in a Clements 2000 bench centrifuge. The supernatant was discarded and the pellet was resuspended in 2.4 ml of Breaking buffer, pH 6.8, and transferred into a 5 ml screw cap polyethylene tube in readiness for sonication.

Breaking Buffer pH 6.8

Tris (Sigma 7-9)	120	mg
Disodium EDTA (Boehringer:808 270)	37	mg
NADP (Sigma:N0505)	37	mg
Distilled Water	100	ml

Adjusted to pH 6.8. Kept refrigerated (4°C).

Cells were lysed using a sonicator with microtip (Branson Sonifer 450, Branson Sonic Power Company, USA) operating at an output of 2.5 and a 90% duty cycle for a total of 4 mins, made up of periods of 60 seconds. The sample was chilled well on ice between bursts to reduce any possible loss of enzyme activity.

1.5 ml of the resulting lysate was transferred into a 1.5 ml microcentrifuge tube and spun at 9,000 g (10,000 rpm) for 5 min using a Centra M-2 microcentrifuge. This process removed whole cells and cell wall fractions that could interfere with electrophoresis. The clarified cell lysate (supernatant) fraction was kept in a microcentrifuge tube and 60 μl aliquots (at least 3) were also distributed into separate microcentrifuge tubes to avoid repeated thawing and refreezing of the original sample in each electrophoresis run. Lysates were stored in a freezer ($\sim$-20°C) for up to a month (short-term storage) or below -50°C for long-term storage.

B.1.2 STARCH GELS PREPARATION

Starch gels were prepared from commercially available potato starch (Sigma, USA). 30.8 or 57 g of starch was weighed and suspended in the volume of 270 ml or 500 ml (11.4% starch gel solution) of appropriate buffer for small or large gel plate, respectively. The starch suspension was heated until dissolved, and then degassed with suction for approximately 30 sec, until there were no bubbles visible in the solution. The solution was gently swirled until the temperature was reduced to 70°C, and then poured into a gel tray with a continuous action.

The gel was left at room temperature for 5 min and a perspex plate was then placed on top of the gel, taking care not to trap any air bubbles. The covered gel was left at room temperature for at least an hour before placing in a refrigerator. Gels were not stored for longer than 24 hours.

Gel Buffer Preparation

1 . Tris-citrate pH 8 (TC 8): Tris (Sigma 7-9) 83.20 g and Citric acid monohydrate (BDH:10081) 33.09 g were dissolved in 1 L of distilled water, and the pH was adjusted to 8.0 using HCl (conc.). The buffer was stored at 0-5°C and used neat for electrode buffer or diluted 1:29 with distilled water for gels.

2. Tris-maleate pH 8.2 (TM 8.2): Tris (Sigma 7-9) 12.10 g, Maleic acid (Sigma: M9138) 11.60 g, Disodium EDTA 3.72 g, and $MgCl_2.6H_2O$ 2.03 g were dissolved in 1 L of distilled water, and the pH adjusted to 8.2 using NaOH (approx. 7 g). The buffer was stored at 0-5°C, and used neat for electrode buffer or diluted 1:9 with distilled water for gels.

B.1.3 ELECTROPHORESIS

B.1.3.1 Sample application

Sample lysates were removed from the freezer and kept on ice. The sample solution was soaked with a sample insert (4x8 mm of Whatman No.3 filter paper), and allowed to thaw on the bench while preparing gels.

The excess edges were removed from the gel tray and a cut made across the gel about 3 cm from and parallel to the shorter end. Excess liquid was blotted from inserts and lined up along the exposed cut using a template. At least 1 mm was left between samples. The forceps used were rinsed in deionised water and wiped dry between successive uses. Bromophenol Blue was used as a migration marker in each electrophoretic run. In

addition, to ensure good contact between cut surfaces and inserts, a spacer was placed at the end of the gel so that there were no gaps in the cut.

B.1.3.2 Electrophoresis

A continuous buffer system (i.e. the buffer in the gel and in the electrode were the same) was used in the study. In each chamber, approximately 300 ml of suitable buffer was added. Each gel was run in a 4°C refrigerator at an initial current of 30 mA, and a voltage limit of 120 V. After electrophoresis, three or four horizontal slices per small or large gel were cut in preparation for enzyme staining.

B.1.4 ENZYME VISUALISATION

Enzyme assays were performed essentially in accordance with those described by Selander *et al.* (1986). The substrates, coenzymes, reagents and mechanisms of the 12 different enzymes are given below: Alanine dehydrogenase (ALA), Catalase (CAT), Fumarate hydratase (FUM), Glucose-6-phosphate dehydrogenase (G6PD), Glyceral-dehyde-3-phos-phate dehydrogenase (GP), Mannose phosphate isomerase (MPI), Nucleoside phospho-rylase (NP), Peptidase-leucyl-leucyl-glycine (PLG), Phosphogluco-mutase (PGM), Phos-phoglucose isomerase (PGI), 6-Phosphogluconate dehydrogenase (6PGD), and Super-oxide dismutase (SOD).

B.1.4.1 Solutions for stains

1.) 0.2 M Tris-HCl pH 8 buffer: Tris (Sigma 7-9) 24.2 g in 1 L of distilled water. Adjusted to pH 8.2 using HCl (conc.). Stored at 0-5°C.

2.) Phosphate buffer pH 7: $NaH_2PO_4.2H_2O$; 0.62 g and $NaHPO_4$; 0.56 g in 1 L of distilled water. Stored at 0-5°C.

3.) 1% Phenazine methosulfate (PMS): Phenazine methosulfate (Sigma: P9625) 0.2 g in 20 ml of distilled water. Protected from light and kept refrigerated.

4.) 1% Dimethylthiazol tetrazolium (MTT): Dimethylthiazol tetrazolium (Sigma: M2128) 0.2 g in 20 ml of distilled water. (n.b. the solution did not dissolve completely). Protected from light and kept refrigerated.

5.) 2% $MgCl_2$: $MgCl_2.6H_2O$ (Sigma: M0250) 2 g in 100 ml of distilled water.

6.) 2% Agar: Bacteriological agar (Oxoid) 4 g in 200 ml 0.2 M Tris-HCl buffer. Boiled to completely dissolve, then cooled to 60°C and kept in a waterbath at 60°C.

7.) 1% α-Napthyl propionate: α-Napthyl propionate (Sigma: N0376) 0.2 g in 20 ml of distilled water.

B.1.4.2 Stain formulae

For all stains components were added in the order that they appear. All ingredients were dissolved before adding PMS or other catalysts. Agar based stains were poured over gel immediately after agar was added. For liquid stains, a gel slice was transferred to a suitable tray and stain solution poured over the gel. All stains were allowed to develop in the dark at 37°C. Staining solutions will react to light.

NB: Rate = rate of travel of enzyme relative to bromophenol blue marker. Bromophenol blue travels approximately 15 cm, at 140 volts after 6 hours in a 9 mm thick starch gel.

1.) Alanine Dehydrogenase (ALA)

Buffer :	TC 8; pH 8.0		
Rate :	54%		
Staining :	D,L-alanine	35	mg
	NAD	4	mg
	phosphate buffer	12	ml
	MTT	400	μl
	PMS	60	μl
	Agar	12	ml

2.) Catalase (CAT)

Buffer : TM 8.2; pH 8.2
Rate : 70%
Staining : Stage 1. Distilled water   100 ml
        100 vol. $H_2 O_2$ 100 μl
        Poured on gel and incubated at 25°C for 15 minutes.
      Stage 2. Poured off solution and rinsed gel well in tap water. Immersed in fresh 50:50 mixtures of 2% potassium ferricyanide and 2% Iron (III) chloride. Mixed gently. Removed stain as yellow zones appeared on blue background (approx. 30 secs).

3.) Fumarate Hydratase (FUM)

Buffer :	TM 8.2; pH 8.2		
Rate :	44%		
Staining :	Fumaric acid (K salt)	100	mg
	NAD	20	mg
	Tris-HCl pH 8 buffer	50	ml
	MTT	1	ml
	PMS	400	μl
	Malic dehydrogenase	50	units

Staining time : 1-2 hours

4.) 6-Phosphogluconate Dehydrogenase (6PGD)

Buffer :	TC 8; pH 8		
Rate :	75%		
Staining :	6-phosphogluconic acid	10	mg
	NADP	3	mg
	Tris-HCl pH 8 buffer	12	ml
	$MgCl_2$	2	ml
	MTT	300	μl
	PMS	40	μl
	Agar	12	ml

Staining time : 20 mins.

5.) Glucose-6-Phosphate Dehydrogenase (G6PD)

Buffer :	TC 8; pH 8		
Rate :	66%		
Staining :	Glucose-6-phosphate	20	mg
	NADP	3	mg
	Tris-HCl pH 8 buffer	12	ml
	$MgCl_2$	2	drops
	MTT	300	μl
	PMS	60	μl
	Agar	12	ml

Staining time : 20 mins. Stain diffuses overnight.

6.) Glyceraldehyde-3-Phosphate Dehydrogenase (GP)

Buffer :	TC 8; pH 8		
Rate :	70%		
Staining :	Stage 1. make up fresh GP stock solution;		
	Tris-HCl pH 8 buffer	2	ml
	Fructose-1,6-diphosphate	50	mg
	aldolase	5	units

Incubate at 37°C for 30 mins.

	Stage 2. Sodium arsenate	50	mg
	NAD	5	mg
	Tris-HCl pH 8 buffer	12	ml
	MTT	400	μl
	GP stock solution	2	ml (all)
	PMS	80	μl
	Agar	12	ml

Staining time :1-2 hours

7.) Mannose Phosphate Isomerase (MPI)

Buffer :   TC 8; pH 8
Rate :   95%
Staining :   Mannose-6-phosphate   10   mg
                  NAD   1.5 mg
                  Tris-HCl pH 8 buffer   12   ml
                  MTT   350 μl
                  G6PD   5   units
                  PGI   30   units
                  PMS   40 μl
                  Agar   12   ml
Use bottom slice for heavy enzymes.

Staining time : 1-2 hours

8.) Nucleoside Phosphorylase (NP)

Buffer :   TC 8; pH 8
Rate :   95%
Staining :   Inosine   15   mg
                  Phosphate buffer pH 7   12   ml
                  MTT   200 μl
                  Xanthine oxidase   0.5 units
                  PMS   50 μl
                  Agar   12   ml

Staining time : 10 mins. Stain fades and diffuses overnight at room temp.

9.) Peptidase-Leucyl-Leucyl-Glycine (PLG)

Buffer :   TC 8; pH 8
Rate :   64%
Staining :   PLG   10   mg
                  O-dianisadine   5   mg
                  (dissolve both in 4 drops 0.1M HCl)
                  L-amino acid oxidase   5   mg
                  Peroxidase   300 U
                  Phosphate buffer pH 7   12   ml
                  $MgCl_2$ 2   drops
                  Agar   12   ml
Staining time : 1-2 hours. Stain strengthens overnight but background stain increases and resolution decreases. Orange stain.

10.) Phosphoglucomutase (PGM)

Buffer :   TM 8.2; pH 8.2
Rate :   80%

Staining:	Glucose-1-phosphate	100	mg
	NADP	10	mg
	Tris-HCl pH 8 buffer	12	ml
	MTT	400	μl
	MgCl$_2$	2	ml
	PMS	80	μl
	G6PD	6	units
	Agar	12	ml

Staining time : 30 mins-1 hours.

11.) Phosphoglucose Isomerase (PGI)

Buffer:	TC 8; pH 8		
Rate:	70%		
Staining:	Fructose-6-phosphate	10	mg
	NADP	1.5	mg
	Tris-HCl pH 8 buffer	12	ml
	MTT	300	μl
	G6PD	5	units
	PMS	20	μl
	Agar	12	ml

Staining time : 10 mins. Stain fades and diffuses overnight at room temperature.

12.) Superoxide Dismutase (SOD)

Buffer:	TM 8.2; pH 8.2		
Rate:	60%		
Staining:	Tris-HCl pH 8 buffer	12	ml
	MTT	400	μl
	PMS	100	μl
	Agar	12	ml

Usually appears as an incidental stain on TC8 or TM8.2 gels e.g. FUM, as white on blue background. Staining time : 2-3 hours (FUM gel)

B.1.5 ANALYSIS

For each enzyme, the relative mobility was established by scoring the relative migration distance from the cathode, i.e. the enzymes nearest to the anode were given the lowest score. Each different combination of electromorphs was assigned to an Electrophoretic Type (ET). Table B.1 shows the results of assigning 85 ETs from the 113 *L. mono-cytogenes* isolates collected from the North West Bay survey (Chapter 2). Statistical analyses of the data were performed by using a Fortran programming language designed

by Whittam T.S., kindly provided by P. Sutherland, Pacific Analysis Co. Ltd., Sydney, NSW. To express the genetic relationships among strains, a dendrogram was produced from cluster analysis by using the average distance method and matrices of weighted proportion. Genetic diversity (h) for an enzyme locus was calculated by the following formula:

$$h = (1 - \Sigma x_i^2) \frac{n}{n - 1}$$

where x_i is the frequency of the ith allele and n is the number of ETs.

Genetic distance between ETs was expressed as a proportion of loci at which dissimilar alleles occur (Selander *et al.*, 1986). The ET diversity was calculated from the same formula as genetic diversity, with x_i being the frequency of the ith ET and n being the number of isolates.

Table B.1 Results of MEE, the enzymes profile from 113 *L. monocytogenes* isolates were classified into 85 ETs. Number of loci is 12.

ET	Reference isolate[a]	n[b]	PGI	MPI	ALA	G6P	6PG	GP	PLG	SOD	FUM	PGM	CAT	NP
1	W5/1	1	1	6	2	2	2	3	2	1	2	2	3	1
2	W10/1	1	1	4	2	1	2	3	2	2	1	3	4	2
3	W12/1	1	1	6	2	2	3	1	2	1	1	3	1	1
4	S8/1	2	1	7	2	2	2	3	2	1	3	1	3	1
5	S9/1	2	1	5	2	1	2	3	2	1	3	3	2	1
6	W10a/2	1	1	7	2	3	3	1	1	1	3	3	2	1
7	W10b/2	2	1	3	2	1	2	3	2	2	4	3	4	2
8	W12/2	2	1	5	2	1	2	3	2	1	2	3	1	1
9	W8/3	1	1	6	2	3	3	1	1	1	2	1	2	1
10	W10b/3	1	1	5	1	1	2	3	2	1	3	3	2	1
11	W12/3	1	1	7	2	2	3	1	2	1	1	2	1	1
12	W8/4	1	1	6	2	2	2	1	1	1	2	1	3	1
13	W10/4	1	1	4	2	1	1	3	1	2	2	2	4	2
14	W12/4	1	1	7	2	2	3	1	1	1	1	3	1	1
15	W8/5	1	1	7	2	2	3	1	4	1	1	2	1	1
16	W10/5	1	1	5	2	1	1	3	2	1	2	3	1	1
17	W12/5	1	1	7	2	2	2	1	4	1	1	2	1	1
18	S7/5	1	1	5	2	1	1	3	2	1	3	3	2	1
19	S8/5	1	1	6	2	2	1	3	2	1	3	1	2	1
20	W10/6	1	1	3	2	1	1	3	3	2	4	5	5	2
21	W11/6	1	1	5	1	2	2	3	3	1	2	4	2	1
22	W12/6	1	1	7	2	3	3	3	1	1	2	4	1	1
23	W8/7	1	1	6	2	3	2	3	2	1	2	4	4	1
24	W10/7	1	1	3	2	2	2	3	2	2	4	4	5	2
25	W12/7	1	1	7	2	2	3	3	1	1	1	4	5	1
26	S4/7	1	1	6	2	3	2	3	2	1	2	3	3	1
27	S8/7	1	1	6	2	3	2	3	2	1	1	3	3	1
28	W8/8	1	1	6	2	3	2	3	2	1	2	2	3	1
29	W10a/8	1	1	5	1	3	2	3	2	1	1	3	1	1
30	W10b/8	1	1	3	2	2	2	3	2	1	2	2	3	2
31	W12/8	1	1	7	2	2	3	2	1	1	1	3	1	1
32	W8/9	2	1	7	2	3	3	2	1	1	2	2	1	1
33	W9/9	2	1	6	2	3	2	2	2	1	2	2	1	1
34	W12/9	2	1	7	2	3	3	1	1	1	1	3	1	1
35	W5/10	1	1	3	3	1	2	3	1	2	4	1	1	1
36	W12/10	1	1	7	2	2	3	3	1	1	1	2	1	1
37	W1/11	1	1	6	2	2	2	3	1	1	2	2	3	1
38	W3/11	2	1	6	2	3	2	3	1	1	2	2	3	1
39	W5/11	1	1	5	2	1	2	3	1	1	2	3	1	1
40	W6/11	1	1	5	2	3	2	3	1	1	2	4	3	1
41	W10/11	1	1	5	1	1	2	2	1	1	3	4	2	1
42	W12/11	1	1	7	2	3	3	3	1	1	1	1	1	1
43	S3/11	1	1	6	3	3	1	2	1	1	2	1	3	1
44	S8/11	1	1	5	2	1	1	2	1	1	2	3	1	1

(continued overleaf)

Table B.1 (contd.) Results of MEE, the enzymes profile from 113 *L. monocytogenes* isolates were classified into 85 ETs. Number of loci is 12.

ET	Reference isolate[a]	n[b]	PGI	MPI	ALA	G6P	6PG	GP	PLG	SOD	FUM	PGM	CAT	NP
45	O3/11	1	1	6	2	4	3	3	1	1	2	3	3	1
46	O5/11	1	1	6	2	1	2	2	1	1	2	2	1	1
47	M/11	1	1	5	2	3	3	3	1	1	1	2	1	1
48	W8/12	1	1	6	2	3	3	3	2	1	2	2	2	1
49	W12/12	1	1	7	2	3	2	3	1	1	1	1	1	1
50	W8/13	2	1	7	2	3	1	3	3	1	2	1	4	1
51	W12/13	1	1	7	2	3	2	1	2	1	1	2	1	1
52	S7/13	1	1	5	2	2	1	3	4	1	2	2	2	1
53	W12/14	4	1	7	2	3	2	1	1	1	1	2	1	1
54	W8/15	1	1	7	2	3	1	3	2	1	2	1	4	1
55	W11/15	1	1	5	1	2	1	3	4	1	3	1	3	1
56	W12/15	1	1	7	2	3	2	1	1	1	2	1	1	1
57	S5/15	1	1	5	2	1	1	3	3	1	3	2	3	1
58	S7/15	1	1	5	2	2	1	2	3	1	2	2	2	1
59	S8/15	1	1	6	2	3	1	2	2	1	2	1	4	1
60	O5/15	1	1	5	1	2	1	2	2	1	3	2	3	1
61	W10/16	1	1	2	2	2	1	2	2	2	4	2	4	2
62	W11/16	1	1	3	3	2	1	2	2	2	3	3	5	2
63	W11/19	1	1	5	1	2	1	3	4	1	3	1	2	1
64	W12/19	1	1	5	1	2	1	3	2	1	2	1	2	1
65	W8/20	1	1	7	2	2	1	1	1	1	1	2	1	1
66	W11/20	1	1	3	2	1	1	3	3	2	4	2	4	2
67	W12/20	2	1	7	2	2	2	1	1	1	1	2	1	1
68	W8/21	14	1	6	2	2	1	3	2	1	3	1	3	1
69	W11a/21	1	1	4	3	1	1	2	2	2	4	6	2	2
70	W11b/21	1	1	5	1	1	1	3	4	1	3	1	2	1
71	W10/22	1	1	5	1	1	1	3	2	1	3	2	2	1
72	W12/23	1	1	6	2	3	3	2	0	1	2	2	1	1
73	W3/24	1	1	7	2	2	3	2	0	1	1	2	1	1
74	W8/24	2	1	6	2	2	3	3	2	1	2	1	3	1
75	W10a/24	1	1	5	2	1	2	3	3	1	3	2	2	1
76	W10b/24	1	1	3	2	1	2	3	2	2	0	3	4	1
77	W11/24	1	1	5	1	1	2	3	4	1	3	1	2	1
78	W9/25	1	2	4	2	1	1	3	2	2	4	4	5	1
79	W11/25	1	1	2	2	1	1	3	2	2	0	3	4	1
80	W12/25	2	1	6	2	2	3	2	1	1	1	1	1	1
81	S5/25	1	1	1	2	1	2	3	2	2	0	6	1	1
82	S6/25	1	1	1	3	1	2	1	2	2	2	5	4	1
83	S9/25	1	2	3	2	1	2	3	2	2	4	4	5	1
84	W10/26	1	1	5	2	2	2	3	2	1	2	1	3	1
85	W11/26	1	1	2	2	1	2	3	2	2	0	3	2	1

[a] Sample type, Site/ Sampling round. W, water sample. S, sediment sample. O, oysters sample. M, mussel sample. [b] Number of isolates.

C RESULTS OF THE OCCURRENCE OF *LISTERIA* SPP. IN NORTH WEST BAY

Table C.1 Physicochemical parameters of, and occurrence of *Listeria* spp., faecal coliforms and *E. coli* in water samples, and occurrence of *Listeria* spp in sediments from samples at Tinderbox (Site 1).

Sample Round	Sampling Date	Water							Sediment	
		pH	Temp. (°C)	Salinity (‰)	FC /100 ml	*E. coli* /100 ml	*L. mono-cytogenes* /L	Other *Listeria* species/L	*L. mono-cytogenes* /25 g	Other *Listeria* species/25 g
1	20/5/94	7.83	10.8	29	<1	<1	A	*L. welshimeri*	A	A
2	3/6/94	7.75	10.8	26.4	3	1	A	*L. seeligeri*		
3	17/6/94	7.96	10.9	23.5	<1	<1	A	A	A	A
4	1/7/94	7.75	10.5	26.1	1	1	A	A		
5	14/7/94	7.84	9.8	25.4	<1	<1	A	A	A	A
6	29/7/94	7.71	9.6	23.9	<1	<1	A	A		
7	12/8/94	7.82	8.7	23.8	2	2	A	A	A	A
8	26/8/94	7.92	9.7	26.4	2	2	A	A		
9	9/9/94	7.99	10.8	26.4	2.7x10	2.7x10^2	A	A	A	*L. seeligeri*
10	22/9/94	7.96	9.8	25.6	8	8	A	A		
11	7/10/94	8.03	10.9	25.4	2.0x10^2	2.0x10^2	P; ET 37	A	A	*L. seeligeri*
12	20/10/94	8.2	12.9	27.9	24	24	A	A		
13	4/11/94	7.89	14.0	28.2	<1	<1	A	A	A	A
14	18/11/94	7.95	13.6	27.3	1	1	A	A		
15	1/12/94	7.84	13.6	27.8	4	4	A	A	A	*L. seeligeri*
16	15/12/94	7.93	16.7	28.4	<1	<1	A	A		
17	5/1/95	7.66	17.2	28.7	11	11	A	A	A	A
18	13/1/95	7.96	18.2	26.4	1	1	A	A		
19	27/1/95	8.03	17.5	27.8	<1	<1	A	A	A	A
20	10/2/95	7.15	16.9	20.1	1	1	A	A		
21	24/2/95	8.04	17.4	23.9	2	<1	A	A	A	A
22	10/3/95	8.22	18.1	23.4	<1	<1	A	A		
23	24/3/95	8.02	15.6	23.5	13	13	A	A	A	A
24	7/4/95	7.64	13.8	22.5	1.3x10^2	1.3x10^2	P; ET 74	*L. seeligeri*		
25	21/4/95	7.83	12.9	22.8	2	2	A	A	A	*L. seeligeri*
26	5/5/95	7.98	12.6	25.2	4	2	A	A		
Mean±S.D.		7.88±0.2	13.2±3.1	25.6±2.3	1.7x10±45	1.7x10±45			-	-
Median		7.93	12.9	25.9	2	1				
Min-Max		7.15-8.22	8.7-18.2	20.1-29.0	<1-2.0x10^2	<1-2.0x10^2			-	-
Total *Listeria*							7.7%	11.5%	0%	30.8%

FC = Faecal Coliforms; A = Absent; P = Present, ET = Electrophoretic Type

Table C.2 Physicochemical parameters of, and occurrence of *Listeria* spp., faecal coliforms and *E. coli* in water samples, and occurrence of *Listeria* spp. in sediments from samples at Salmon farm (Site 2).

Sample Round	Sampling Date	Water							Sediment	
		pH	Temp (°C)	Salinity (‰)	FC /100 ml	*E. coli* /100 ml	*L. mono-cytogenes* /L	Other *Listeria* species/L	*L. mono-cytogenes* /25 g	Other *Listeria* species/25 g
1	20/5/94	7.92	11.2	29.0	<1	<1	A	A	A	A
2	3/6/94	7.94	10.8	26.4	<1	<1	A	A		
3	17/6/94	7.95	10.9	25.8	<1	<1	A	A	A	A
4	1/7/94	7.82	10.2	27.2	<1	<1	A	A		A
5	14/7/94	7.87	10	27.8	1	<1	A	A	A	A
6	29/7/94	7.68	9.4	27.3	1	1	A	A		
7	12/8/94	7.86	8.9	21.4	1	1	A	A	A	A
8	26/8/94	8.04	9.8	27.7	<1	<1	A	A		
9	9/9/94	8.05	10.1	27.6	<1	<1	A	A	A	A
10	22/9/94	8.05	9.6	26.8	3.1x10	3.1x10	A	A		
11	7/10/94	8.10	10.8	27.0	6.9x10	5.2x10	A	*L. innocua*	A	A
12	20/10/94	8.12	13.4	27.4	<1	<1	A	A		
13	4/11/94	7.98	14.1	28.3	<1	<1	A	A	A	A
14	18/11/94	8.04	13.7	27.0	<1	<1	A	A		
15	1/12/94	7.95	14.2	27.5	1	1	A	A	A	*L. seeligeri*
16	15/12/94	7.95	16.6	28.3	<1	<1	A	A		
17	5/1/95	7.84	17.4	28.1	<1	<1	A	A	A	A
18	13/1/95	8.01	18.9	27.8	<1	<1	A	A		
19	27/1/95	7.83	17.7	27.7	1	1	A	A	A	*L. seeligeri*
20	10/2/95	7.51	16.4	20.5	<1	<1	A	A		
21	24/2/95	8.02	17.8	24.0	<1	<1	A	A	A	A
22	10/3/95	8.07	18.4	23.4	<1	<1	A	A		
23	24/3/95	8.11	15.8	23.5	<1	<1	A	A	A	A
24	7/4/95	7.80	14.7	22.5	1.7x10	1.7x10	A	*L. seeligeri*		
25	21/4/95	7.87	13.2	22.8	<1	<1	A	A	A	*L. seeligeri*
26	5/5/95	7.99	12.8	24.3	<1	<1	A	A		
Mean±S.D		7.94±0.1	13.3±3.2	26.0±2.4	4.7±15	4.0±12	-	-	-	-
Median		7.95	13.3	27.1	<1	<1	-	-	-	-
Min.-Max		7.51-8.12	8.9-18.9	20.5-29.0	<1-6.9x10	<1-5.2x10	-	-	-	-
Total *Listeria*							0%	7.7%	0%	23.1%

FC = Faecal Coliforms; A = Absent

Table C.3 Physicochemical parameters of, and occurrence of *Listeria* spp., faecal coliforms and *E. coli* in water samples, and occurrence of *Listeria* spp. in sediments and oysters from samples at Stinkpot Bay (Site 3).

Sample Round	Sampling Date	Water							Sediment		Oysters	
		pH	Temp. (°C)	Salinity (‰)	FC / 100 ml	*E. coli* / 100 ml	*L. monocytogenes* /L	Other *Listeria* species/L	*L. monocytogenes* /25 g	Other *Listeria* species/25 g	*L. monocytogenes* /25 g	Other *Listeria* species/25 g
1	20/5/94	7.83	7.8	28.1	1.9x10	1.9x10	A	*L. seeligeri*	A	*L. welshimeri*	P; ET 4	*L. innocua*
2	3/6/94	7.92	9.7	26.2	1.6x10	1.6x10	A	A				
3	17/6/94	7.9	8.8	25.8	6.0x10	4.5x10	A	*L. seeligeri*	A	A	A	*L. seeligeri*
4	1/7/94	7.85	9.2	25.7	<1	<1	A	A				
5	14/7/94	7.91	7.6	27.7	1	1	A	A	A	*L. seeligeri, L. welshimeri*	A	A
6	29/7/94	7.81	8.4	26.9	3	3	A	A				
7	12/8/94	7.9	8.3	27.3	2	2	A	*L. ivanovii*	A	*L. innocua*	A	A
8	26/8/94	8.09	9.4	27.7	2.6x10	2.6x10	A	A				
9	9/9/94	8.16	11.9	27.6	1.5x10	1.5x10	A	*L. seeligeri*	A		A	A
10	22/9/94	8.16	11.1	27.2	6.9x10	6.9x10	A	A				
11	7/10/94	7.62	9.5	21.1	1.9x10^3	1.9x10^3	P; ET 38	*L. innocua*	P; ET 43	A	P; ET 45	A
12	20/10/94	7.93	14.3	27.2	9	9	A	*L. innocua*				
13	4/11/94	7.98	16.1	27.9	5	5	A	A	A	A	A	*L. ivanovii*
14	18/11/94	8 07	13.4	27.3	6.1x10	6.1x10	A	A				
15	1/12/94	7.96	15.2	27.5	1.9x10	1.9x10	A	A	A	*L. innocua*	A	A
16	15/12/94	7.93	20.7	28.6	4	4	A	A				
17	5/1/95	7.92	18.8	28.7	3.0x10	3.0x10	A	A	A	A	A	A
18	13/1/95	8.04	22.7	23.4	1.2x10^2	1.2x10^2	A	A				
19	27/1/95	8.16	19.2	26.3	3.0x10	3.0x10	A	A	A	A	A	*L. innocua*
20	10/2/95	7.71	18.6	19.8	1.6x10	1.6x10	A	A				
21	24/2/95	8.09	19.8	23.7	7	7	A	A	A	A	A	A
22	10/3/95	8.09	23.6	23.4	1.6x10	1.6x10	A	A				
23	24/3/95	8.14	15.3	23.4	2.5x10	2.5x10	A	A	A	A	A	A
24	7/4/95	7.84	12.9	20	3.6x10^3	3.6x10^3	P; ET 73	*L. innocua, L. seeligeri*				
25	21/4/95	7.97	13.3	22.3	1.1x10^2	1.1x10^2	A	A	A	*L. seeligeri*	A	A
26	5/5/95	7.95	12.7	24.7	5.4x10^2	5 4x10^2	A	A				
Mean±S.D.		7.96±0.1	13.8±4.9	25.6±2.6	2.6x10^2±779	2.6x10^2±779						
Median		7 94	13.1	26.6	19.0	19.0						
Min.-Max.		7.62-8.16	7.6-23.6	19.8-28.7	<1-3.6x10^3	<1-3.6x10^3						
Total *Listeria*							7.7%	26.9%	7.7%	38.5%	15.4%	30.8%

FC = Faecal Coliforms; A = Absent; P = Present; ET = Electrophoretic Type

Table C.4 Physicochemical parameters of, and occurrence of *Listeria* spp., faecal coliforms and *E. coli* in water samples, and occurrence of *Listeria* spp. in sediments from samples at 'Sanctuary' (Site 4).

Sample Round	Sampling Date	Water							Sediment	
		pH	Temp. (°C)	Salinity (‰)	FC /100 ml	*E. coli* /100 ml	*L. mono-cytogenes* /L	Other *Listeria* species/L	*L. mono-cytogenes* /25 g	Other *Listeria* species/25 g
1	20/5/94	7.66	7.7	28.2	2.4×10	2.4×10	A	A	A	A
2	3/6/94	7.78	8.7	26.4	1.2×10	1.2×10	A	A		
3	17/6/94	7.91	8.4	26.2	8	8	A	A	A	A
4	1/7/94	7.89	9.6	26	5.8×10	5.8×10	A	A		
5	14/7/94	7.91	5.3	28.5	1	1	A	A	A	A
6	29/7/94	7.63	6.7	27	1.7×10	1.7×10	A	A		
7	12/8/94	7.92	7.4	27.7	1.3×10	1.3×10	A	A	P; ET 26	A
8	26/8/94	7.96	9.2	28.2	7.2×10	7.2×10	A	A		
9	9/9/94	8.08	11.2	27.3	9	9	A	*L. seeligeri*	A	A
10	22/9/94	8.03	10.4	20.8	1.5×10^2	1.5×10^2	P, ET 32	*L. seeligeri*		
11	7/10/94	7.96	10.7	27.2	1.3×10^3	1.2×10^3	P, ET 38	A	A	*L. seeligeri*
12	20/10/94	7.79	14.2	24.9	3.0×10^2	3.0×10^2	A	*L. seeligeri*		
13	4/11/94	8.17	16.2	27.5	3.3×10	3.3×10	A	A	A	A
14	18/11/94	7.94	13.1	27.5	5.5×10^2	5.5×10^2	A	A		
15	1/12/94	7.74	18.2	26.8	6.4×10	6.4×10	A	A	A	*L. seeligeri*
16	15/12/94	7.71	23	30.1	1.2×10^2	1.2×10^2	A	A		
17	5/1/95	7.93	19	29	<1	<1	A	A	A	A
18	13/1/95	7.64	27.9	23.7	1.1×10^2	1.1×10^2	A	A		
19	27/1/95	7.71	20.1	25.6	1.4×10^2	1.4×10^2	A	A	A	A
20	10/2/95	7.67	23.4	18.88	3.6×10	3.6×10	A	A		
21	24/2/95	8.09	21.8	24	1.1×10^2	1.1×10^2	A	*L. seeligeri*	P; ET 68	*L. seeligeri*
22	10/3/95	8.09	24	23.3	5.8×10	5.8×10	A	A		
23	24/3/95	8.07	18.4	22.1	1.7×10^2	1.7×10^2	A	A	A	A
24	7/4/95	7.76	13.1	11.38	3.3×10^2	3.3×10^2	A	*L. seeligeri*		
25	21/4/95	7.97	14.3	20.8	3.0×10	3.0×10	A	A	A	A
26	5/5/95	8.1	14.6	16.8	2.0×10	2.0×10	A	A		
Mean±S.D.		7.89±0.2	14.5±6.2	24.8±4.3	$1.4 \times 10^2 \pm 265$	$1.4 \times 10^2 \pm 250$				
Median		7.92	13.7	26.3	5.8×10	5.8×10				
Min.-Max.		7.63-8.17	5.3-27.9	11.4-30.1	$<1-1.3 \times 10^3$	$<1-1.2 \times 10^3$				
Total *Listeria*						7.7%	19.2%		15.4%	23.1%

FC = Faecal Coliforms, A = Absent; P = Present; ET = Electrophoretic Type

Table C.5 Physicochemical parameters of, and occurrence of *Listeria* spp., faecal coliforms and *E. coli* in water samples, and occurrence of *Listeria* spp in sediments and oysters from samples at Dru Point (Site 5).

Sample Round	Sampling Date	Water							Sediment		Oysters	
		pH	Temp. (°C)	Salinity (‰)	FC /100 ml	*E. coli* /100 ml	*L. mono-cytogenes* /L	Other *Listeria* species/L	*L. mono-cytogenes* /25 g	Other *Listeria* species/25 g	*L. mono-cytogenes* /25 g	Other *Listeria* species/25 g
1	20/5/94	7.54	9.4	26.0	1.3×10^3	1	P; ET 1	A	A	*L. seeligeri*	A	*L. innocua*
2	3/6/94	7.77	10.2	26.0	2.9×10^2	2.9×10^2	A	*L. seeligeri*				
3	17/6/94	7.85	9.8	26.1	1.2×10^2	9.9×10	A	A	A	*L. seeligeri*	A	A
4	1/7/94	7.74	8.7	26.6	4.0×10	3.3×10	A	A				
5	14/7/94	7.89	8.4	27.6	4.6×10	4.6×10	A	A	A	A	A	*L. innocua*
6	29/7/94	7.84	8.6	28.3	1.1×10^2	1.1×10^2	A	A				
7	12/8/94	7.79	8.1	25.1	5.1×10^2	5.1×10^2	A	*L. innocua*	A	*L. seeligeri*	A	*L. seeligeri*
8	26/8/94	8.12	9.2	28.2	3.1×10^2	3.1×10^2	A	*L. seeligeri*				
9	9/9/94	8.14	11.0	27.1	2.9×10	2.9×10	A	A	A	*L. seeligeri*	A	*L. seeligeri*
10	22/9/94	7.98	10.4	24.2	4.7×10^2	4.7×10^2	P; ET 35	A				
11	7/10/94	7.90	10.5	26.7	5.2×10^2	5.2×10^2	P; ET 39	*L. innocua*	A	*L. innocua*	P; ET 46	A
12	20/10/94	7.98	14.7	27.0	2.5×10	2.1×10	A	*L. seeligeri*				
13	4/11/94	7.90	16.3	22.6	3.0×10^2	3.0×10^2	A	*L. seeligeri*	A	A	A	A
14	18/11/94	8.00	14.9	24.5	1.5×10^2	1.5×10^2	A	A				
15	1/12/94	7.74	17.2	23.1	2.2×10^2	2.2×10^2	A	A	P; ET 57	A	P; ET 60	A
16	15/12/94	7.75	20.8	27.3	3.1×10	3.1×10	A	A				
17	5/1/95	7.82	19.0	28.7	2.2×10	2.2×10	A	A	A	A	A	A
18	13/1/95	7.70	24.2	26.6	1.3×10^2	1.3×10^2	A	A				
19	27/1/95	7.58	19.6	25.8	1.1×10^2	1.1×10^2	A	A	A	A	A	A
20	10/2/95	7.61	19.6	25.4	1.9×10^2	1.9×10^2	A	A				
21	24/2/95	7.76	19.9	22.8	2.7×10^2	2.7×10^2	A	A	P; ET 68	*L. seeligeri*	A	A
22	10/3/95	7.83	19.3	22.9	1.6×10^2	1.6×10^2	A	A				
23	24/3/95	7.97	15.3	23.0	3.0×10^2	1.5×10^2	A	A	A	A	A	*L. seeligeri*
24	7/4/95	7.41	12.0	3.14	1.7×10^4	1.7×10^4	A	*L. innocua*				
25	21/4/95	7.93	13.3	19.0	2.3×10^2	2.3×10^2	A	A	P; ET 81	*L. seeligeri*	A	*L. seeligeri, L. innocua*
26	5/5/95	7.87	13.0	21.4	1.5×10^2	1.5×10^2	A	A				
Mean±S.D.		7.82±0.03	13.97±22	24.4±24	$1.0 \times 10^3 \pm 1.2 \times 10^7$	$1.0 \times 10^4 \pm 1.2 \times 10^7$						
Median		7.84	13.2	25.9	2.1×10^2	1.6×10^2						
Min.-Max.		7.41-8.14	8.1-24.2	3.1-28.7	$2.2 - 1.7 \times 10^4$	$1 - 1.7 \times 10^4$						
Total *Listeria*							11.5%	26.9%	23.1%	53.8%	15.4%	30.8%

FC = Faecal Coliforms; A = Absent; P = Present; ET = Electrophoretic Type

Table C.6 Physicochemical parameters of, and occurrence of *Listeria* spp., faecal coliforms and *E. coli* in water samples, and occurrence of *Listeria* spp. in sediments from samples at NWB Jetty (Site 6a) and occurrence of *Listria* spp. in mussel samples at Beach Road Jetty (site 6b).

Sample Round	Sampling Date	Water							Sediment		Mussels	
		pH	Temp. (°C)	Salinity (‰)	FC / 100 ml	E. coli / 100 ml	L. mono-cytogenes /L	Other Listeria species/L	L. mono-cytogenes /25 g	Other Listeria species/25 g	L. mono-cytogenes /25 g	Other Listeria species/25 g
1	20/5/94	7.94	11.1	29	4.7x10	4.7x10	A	A	A	A	A	A
2	3/6/94	7.96	10.7	27	8	4	A	A				
3	17/6/94	7.91	10.7	26.6	7	7	A	A	A	A	A	A
4	1/7/94	7.89	10.7	27.2	3	<1	A	A				
5	14/7/94	7.93	9.8	28	1.8x10	1.5x10	A	A	A	A	A	A
6	29/7/94	7.91	9.5	28.8	1.1x10	1.1x10	A	A				
7	12/8/94	7.91	9.4	28.4	<1	<1	A	A	A	L. seeligeri	A	L. seeligeri
8	26/8/94	8.01	9.8	28.7	<1	<1	A	A				
9	9/9/94	8.27	11.3	28.1	<1	<1	A	A	A	A	A	A
10	22/9/94	8.14	11.4	27.1	1.1x10	1.1x10	A	A				
11	7/10/94	8.11	10.8	27.1	3.9x10	3.2x10	P; ET 40	A	A	A	P; ET 47	A
12	20/10/94	8	12.9	27	2	2	A	A				
13	4/11/94	8.15	15.6	28.3	9	9	A	A	A	A	A	A
14	18/11/94	8.13	14.4	27.2	4	4	A	A				
15	1/12/94	8.17	15.8	27.7	<1	<1	A	A	A	A	A	A
16	15/12/94	8.15	18.6	28.4	2	2	A	A				
17	5/1/95	7.79	18.6	28.6	2	2	A	A	A	L. ivanovii	A	L. ivanovii
18	13/1/95	8.26	22.9	26.6	3.6x10	3.6x10	A	A				
19	27/1/95	8.21	19.2	28	9	7	A	A	A	A	A	A
20	10/2/95	8.02	19.5	24.8	3	3	A	A				
21	24/2/95	8.36	20.5	23.8	3.2x10	3.2x10	A	A	A	A	P; ET 68	A
22	10/3/95	8.37	22.4	23.2	4.4x10	4.1x10	A	A				
23	24/3/95	8.18	17.1	23.3	1.2x10	1.2x10	P; ET 68	A	A	A	A	A
24	7/4/95	7.58	13	22.2	1.3x10²	1.3x10²	A	L. innocua				
25	21/4/95	8.26	13.9	22.5	5	5	A	A	P; ET 82	A	A	A
26	5/5/95	8.18	12.9	25.2	4	4	A	A				
Mean±S.D.		8.07±0.2	14.3±4.2	26.6±2.1	1.7x10±27	1.6x10±27						
Median		8.12	12.95	27.2	7.5	6.0						
Min.-Max.		7.58-8.37	9.4-22.9	22.2-29	<1-1.3x10²	<1-1.3x10²						
Total Listeria							7.7%	3.8%	7.7%	15.4%	15.4%	15.4%

FC = Faecal Coliforms, A = Absent, P = Present; ET = Electrophoretic Type

Table C.7 Physicochemical parameters of, and occurrence of *Listeria* spp., faecal coliforms and *E. coli* in water samples, and occurrence of *Listeria* spp in sediments from samples at NWB Marina (Site 7).

Sample Round	Sampling Date	Water							Sediment	
		pH	Temp. (°C)	Salinity (‰)	FC /100 ml	*E. coli* /100 ml	*L. mono-cytogenes* /L	Other *Listeria* species/L	*L. mono-cytogenes* /25 g	Other *Listeria* species/25 g
1	20/5/94	7.92	11	29.4	<1	<1	A	A	A	A
2	3/6/94	7.95	10.9	26.7	<1	<1	A	A		
3	17/6/94	7.92	10.7	28.0	2	2	A	A	A	A
4	1/7/94	7.86	9.9	28.6	2	2	A	A		
5	14/7/94	7.94	10.2	28.7	1.1x10	9	A	A	P; ET 18	A
6	29/7/94	7.86	9.0	28.3	3	3	A	A		
7	12/8/94	7.89	9.5	28.3	5	5	A	A	A	A
8	26/8/94	8.01	9.9	28.8	4	4	A	A		
9	9/9/94	8.13	10.6	28.1	4.2x10	3.9x10	A	A	P; ET 34	A
10	22/9/94	8.15	11.1	27.5	5	5	A	A		
11	7/10/94	7.96	11.1	27.8	1.3x10^2	1.2x10^2	A	*L. innocua*	A	*L. innocua*
12	20/10/94	7.89	13.1	25.8	<1	<1	A	A		
13	4/11/94	8.05	15.6	28.0	<1	<1	A	A	P; ET 52	A
14	18/11/94	8.16	13.7	27.4	1	1	A	A		
15	1/12/94	7.85	16.1	27.8	6.4x10	6.4x10	A	A	P; ET 58	A
16	15/12/94	7.99	19.9	28.6	9	9	A	A		
17	5/1/95	7.98	19.6	28.2	1	1	A	A	A	A
18	13/1/95	7.99	24.1	26.8	2.4x10	2.4x10	A	A		
19	27/1/95	8.03	19.6	27.1	5.5x10	5.1x10	A	A	A	A
20	10/2/95	7.78	18.8	24.7	<1	<1	A	*L. innocua*		
21	24/2/95	8.34	23.1	24.0	11	11	A	A	A	A
22	10/3/95	8.04	19.8	23.4	1	1	A	A		
23	24/3/95	8.08	16.2	23.6	6	6	A	A	A	A
24	7/4/95	7.65	13.2	19.5	1.5x10^2	1.5x10^2	A	*L. innocua*		
25	21/4/95	8.08	14.5	23.1	1.1x10	1.1x10	A	A	A	*L. innocua*
26	5/5/95	8.06	13.1	25.5	6	6	A	A		
Mean±S.D.		7.98±0.1	14.4±4.47	26.7±2.4	2.1x10±39	4.0±12				
Median		7.99	13.2	27.7	5	5				
Min.-Max.		7.65-8.34	9.0-24.1	19.5-29.4	<1-1.5x10^2	<1-1.5x10^2				
Total *Listeria*							0%	11.5%	31%	15.4%

FC = Faecal Coliforms; A = Absent; P = Present; ET = Electrophoretic Type

Table C.8 Physicochemical parameters of, and occurrence of *Listeria* spp., faecal coliforms and *E. coli* in water samples, and occurrence of *Listeria* spp. in sediments from samples at Coffee Creek (Site 8).

Sample Round	Sampling Date	Water							Sediment	
		pH	Temp. (°C)	Salinity (‰)	FC /100 ml	*E. coli* /100 ml	*L. mono-cytogenes* /L	Other *Listeria* species/L	*L. mono-cytogenes* /25 g	Other *Listeria* species/25 g
1	20/5/94	7.52	7.3	0.30	2.1×10^2	1.1×10^2	A	*L. innocua*	P; ET 4	A
2	3/6/94	7.0	8.2	0.60	5.0×10	5.0×10	A	*L. innocua*		
3	17/6/94	7.43	8.3	18.40	8.0×10	8.0×10	P, ET 9	A	P; ET 8	A
4	1/7/94	7.22	6.2	0.70	1.5×10^2	1.5×10^2	P, ET 12	A		
5	14/7/94	7.50	6.2	19.10	4.7×10	4.7×10	P, ET 15	A	P; ET 19	A
6	29/7/94	7.48	6.5	17.80	9.0×10	9.0×10	A	*L. seeligeri*		A
7	12/8/94	7.38	7.0	11.30	1.2×10^2	1.2×10^2	P, ET 23	A	P; ET 27	A
8	26/8/94	7.48	8.8	0.30	1.6×10^2	1.6×10^2	P, ET 28	A		
9	9/9/94	7.33	8.6	8.50	1.3×10^2	1.3×10^2	P, ET 32	A	P; ET 33	A
10	22/9/94	8.20	7.6	1.20	1.5×10^3	1.5×10^3	A	*L. innocua*		
11	7/10/94	7.81	10.2	0.10	7.0×10^3	4.7×10^3	A	*L. innocua*	P; ET 44	A
12	20/10/94	7.58	11.0	0.30	2.2×10^3	1.8×10^3	P, ET 48	A		
13	4/11/94	7.14	13.0	13.99	1.7×10^3	1.7×10^3	P, ET 50	*L. seeligeri*	A	*L. seeligeri*
14	18/11/94	7.12	13.2	9.00	8.2×10^2	6.0×10^2	P, ET 50	*L. seeligeri*		
15	1/12/94	7.10	15.2	12.20	2.3×10^3	2.3×10^3	P, ET 54	A	P; ET 59	A
16	15/12/94	7.00	16.6	16.80	5.3×10^2	5.3×10^2	A	*L. seeligeri*		
17	5/1/95	7.29	17.9	27.40	7.8×10	7.8×10	A	*L. seeligeri*	A	A
18	13/1/95	7.15	20.4	24.20	9.0×10^2	9.0×10^2	A	*L. seeligeri*		
19	27/1/95	7.21	18.0	16.18	2.0×10^3	2.0×10^3	A	*L. seeligeri, L. welshimeri*	A	*L. seeligeri*
20	10/2/95	7.28	16.1	3.50	2.0×10^2	2.0×10^2	P, ET 65	*L. seeligeri*		
21	24/2/95	7.45	14.3	19.40	7.4×10^2	7.4×10^2	P, ET 68	*L. seeligeri*	P; ET 68	*L. seeligeri*
22	10/3/95	7.38	18.4	18.20	3.2×10^3	3.2×10^3	P, ET 68	A		
23	24/3/95	7.47	14.2	20.30	7.0×10^2	7.0×10^2	P, ET 68	*L. seeligeri*	P; ET 68	A
24	7/4/95	7.41	10.7	0.24	8.0×10^3	8.0×10^3	P, ET 74	*L. seeligeri, L. innocua*		
25	21/4/95	7.58	10.7	0.94	1.5×10^3	1.5×10^3	P, ET 68	*L. seeligeri*	A	*L. seeligeri*
26	5/5/95	7.64	10.4	2.70	8.2×10^4	7.4×10^4	A	*L. innocua*		
Mean±S.D.		7.39±0.3	11.7±4.3	10.14±8.9	$4.5 \times 10^3 \pm 1.6 \times 10^4$	$4.0 \times 10^3 \pm 1.4 \times 10^4$				
Median		7.39	10.7	10.13	7.5×10^2	6.5×10^2				
Min.-Max.		7.00-8.20	6.2-20.4	0.10-27.40	4.7×10-8.2×10^4	4.7×10-7.4×10^4				
Total *Listeria*							62%	65.4%	69%	30.8%

FC = Faecal Coliforms; A = Absent; P = Present; ET = Electrophoretic Type

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Table C.9 Physicochemical parameters of, and occurrence of *Listeria* spp., faecal coliforms and *E. coli* in water samples, and occurrence of *Listeria* spp. in sediments from samples at NWB River (Site 9).

Sample Round	Sampling Date	Water							Sediment	
		pH	Temp. (°C)	Salinity (‰)	FC /100 ml	*E. coli* /100 ml	*L. mono-cytogenes* /L	Other *Listeria* species/L	*L. mono-cytogenes* /25 g	Other *Listeria* species/25 g
1	20/5/94	7.66	5.9	0.11	1.2×10^3	1.2×10^3	A	*L. innocua*	P; ET 5	*L. seeligeri*
2	3/6/94	7.61	7.2	0.00	1.2×10^2	1.2×10^2	P, ET 5	A		
3	17/6/94	7.93	6.6	0.10	7.0×10	4.7×10	A	*L. innocua*	A	*L. seeligeri*
4	1/7/94	7.62	5.3	0.13	2.0×10	2.0×10	A	*L. seeligeri*		
5	14/7/94	7.93	5.0	0.10	9.0×10	9.0×10	A	*L. seeligeri*	A	A
6	29/7/94	8.01	5.7	0.10	6.0×10	6.0×10	A	*L. ivanovii*		
7	12/8/94	7.64	6.2	0.00	5.9×10	5.9×10	A	*L. innocua*	A	A
8	26/8/94	8.5	7.6	0.03	5.4×10	5.4×10	A	*L. innocua*		
9	9/9/94	8.64	7.2	0.07	7.5×10	7.5×10	P, ET 33	A	A	A
10	22/9/94	8.66	6.8	0.00	3.7×10^2	3.7×10^2	A	*L. innocua*		
11	7/10/94	8.57	8.2	0.00	9.0×10^3	6.0×10^3	A	*L. innocua*	A	*L. seeligeri*
12	20/10/94	8.46	12.1	0.00	1.2×10^2	1.2×10^2	A	*L. seeligeri*		
13	4/11/94	8.26	14.5	0.12	1.7×10^2	1.7×10^2	A	A	A	*L. seeligeri*
14	18/11/94	8.35	13.0	0.09	1.5×10^2	1.5×10^2	A	*L. seeligeri*		
15	1/12/94	8.55	13.5	0.12	2.6×10^2	2.6×10^2	A	*L. seeligeri*	A	A
16	15/12/94	8.13	18.3	0.14	9.0×10^2	9.0×10^2	A	*L. seeligeri*		
17	5/1/95	8.5	18.6	0.20	7.0×10^2	7.0×10^2	A	*L. seeligeri*	A	*L. seeligeri*
18	13/1/95	7.92	22.1	0.22	5.0×10^2	5.0×10^2	A	*L. seeligeri*		
19	27/1/95	8.23	17.4	0.12	9.0×10^2	9.0×10^2	A	*L. seeligeri, L. welshimeri*	A	*L. seeligeri*
20	10/2/95	8.81	17.1	0.09	3.6×10^2	3.6×10^2	A	*L. seeligeri*		
21	24/2/95	9.06	16.9	0.12	3.3×10^2	3.3×10^2	A	*L. seeligeri*	P; ET 68	A
22	10/3/95	8.11	17.3	0.17	4.9×10^2	4.9×10^2	A	A		
23	24/3/95	8.34	14.1	0.16	6.0×10^2	6.0×10^2	A	*L. innocua*	A	*L. seeligeri*
24	7/4/95	7.54	10.3	0.10	3.4×10^4	3.4×10^4	A	*L. innocua*		
25	21/4/95	8.45	9.7	0.07	2.0×10^2	2.0×10^2	P, ET 78	*L. innocua*	P; ET 83	A
26	5/5/95	8.53	9.0	0 07	3.3×10^2	3.3×10^2	A	*L. seeligeri*		
Mean±S.D.		8.23±0.4	11.4±5.1	0.09±0.06	2.0×10^3±6.8×10^3	1.9×10^3±6.7×10^3				
Median		8.3	10.0	0.1	1.2×10^2	1.2×10^2				
Min.-Max.		7.54-9.06	5.0-22.1	0.00-0.22	2.0×10-3.4×10^4	2.0×10-3.4×10^4				
Total *Listeria*							12%	84.6%	23%	53.8%

FC = Faecal Coliforms, A = Absent; P = Present; ET = Electrophoretic Type

Table C.10 Physicochemical parameters of, and occurrence of *Listeria* spp., faecal coliforms and *E. coli* in effluent samples from Sewage Treatment Pond, Dru Point (Site 10).

Sample Round	Sampling Date	pH	Temp. (°C)	Salinity (‰)	FC /100 ml	*E coli* /100 ml	*L. mono-cytogenes* / L	Other *Listeria* species/ L
1	20/5/94	7.97	7.6	0.30	1.2×10^4	1.2×10^4	P; ET 2	A
2	3/6/94	7.7	8.3	0.20	5.5×10^3	5.3×10^3	P; ET 6, 7	A
3	17/6/94	7.93	7.4	0.20	5.1×10^3	1.3×10^3	P; ET 7,10	A
4	1/7/94	7.72	6.0	0.30	2.6×10^4	2.6×10^4	P; ET 13	*L. innocua*
5	14/7/94	8.2	5.8	0.30	1.7×10^3	1.2×10^3	P; ET 16	A
6	29/7/94	7.92	6.4	0.30	5.0×10^3	5.0×10^3	P; ET 20	*L. innocua*
7	12/8/94	7.69	7.1	0.20	2.7×10^4	2.7×10^4	P; ET 24	A
8	26/8/94	8.78	9.4	0.20	1.3×10^4	1.3×10^4	P; ET 29,30	A
9	9/9/94	9.39	12.4	0.24	2.0×10^3	2.0×10^3	A	A
10	22/9/94	9.08	10.9	0.02	4.8×10^4	4.8×10^4	A	*L. innocua*
11	7/10/94	8.51	12.9	0.20	2.1×10^4	1.6×10^4	P; ET 41	*L. innocua*
12	20/10/94	7.41	15.5	0.20	2.9×10^2	2.1×10^2	A	A
13	4/11/94	8.1	20.0	0.24	2.9×10^2	2.9×10^2	A	A
14	18/11/94	8.31	17.0	0.26	1.0×10^3	5.0×10^2	A	*L. seeligeri*
15	1/12/94	9.55	17.2	0.34	5.0×10^3	4.4×10^3	A	A
16	15/12/94	9.43	21.5	0.27	1.8×10^3	1.7×10^3	P; ET 61	A
17	5/1/95	9.24	21.7	0.32	1.4×10^2	1.4×10^2	A	A
18	13/1/95	9.78	24.4	0.32	3.5×10^3	2.0×10^3	A	A
19	27/1/95	8.77	19.8	0.34	3.2×10^3	2.4×10^3	A	*L. seeligeri*
20	10/2/95	9.06	20.1	0.32	4.8×10^3	2.6×10^3	A	A
21	24/2/95	9.06	16.9	0.31	3.3×10^3	3.3×10^3	P; ET 68	A
22	10/3/95	8.9	20.2	0.28	5.0×10^2	5.0×10^2	P; ET 71	A
23	24/3/95	8.48	15.4	0.27	2.0×10^3	1.4×10^3	A	*L. seeligeri*
24	7/4/95	7.79	13.0	0.34	2.1×10^3	2.1×10^3	P; ET 75,76	A
25	21/4/95	8.54	12.9	0.22	2.0×10^3	2.0×10^3	A	A
26	5/5/95	8.05	11.6	0.40	2.6×10^3	2.6×10^3	P; ET 84	A
Mean ± S.D.		8.51 ± 0.5	13.9 ± 31.9	0.27 ± 0.01	$7.6 \times 10^3 \pm 1 \times 10^9$	$7.0 \times 10^3 \pm 1 \times 10^9$		
Median		8.50	13.0	0.28	2.7×10^3	2.1×10^3		
Min.-Max.		7.41-9.78	5.8-24.4	0.02-0.4	1.4×10^2 -4.8×10^3	1.4×10^2 -4.8×10^4		
Total *Listeria*							53.8%	26.9%

FC = Faecal Coliforms; A = Absent; P = Present; ET = Electrophoretic Type

<u>Table C 11</u> Physicochemical parameters of, and occurrence of *Listeria* spp., faecal coliforms and *E. coli* in effluent samples from Fish Processing Factory 1 (Site 11)

Sample Round	Sampling Date	pH	Temp. (°C)	Salinity (‰)	FC/100 ml	E. coli /100 ml	L mono-cytogenes / L	Other Listeria species/ L
1	20/5/94	5.45	9.1	7.1	4.0×10^2	4.0×10^2	A	*L. innocua*
2	3/6/94	5.71	7.1	6.8	6.0×10^2	6.0×10^2	A	*L. innocua*
3	17/6/94	6.22	12.3	13.1	1.0×10^4	6.7×10^3	A	*L. innocua*
4	1/7/94	5.33	10.6	11.8	5.0×10	5.0×10	A	*L. innocua*
5	14/7/94	6.11	10.3	10.2	2.7×10^3	2.7×10^3	A	*L. innocua*
6	29/7/94	6.23	9.6	10.2	8.0×10	6.0×10	P; ET 21	A
7	12/8/94	5.58	9.1	12.0	9.0×10	9.0×10	A	A
8	26/8/94	5.77	11.3	9.1	5.0×10	5.0×10	A	A
9	9/9/94	5.52	13.3	9.4	4.0×10^4	4.0×10^4	A	A
10	22/9/94	5.41	11.1	8.4	3.0×10^2	3.0×10^2	A	A
11	7/10/94	6.54	14.7	10.2	1.9×10^2	1.9×10^3	A	A
12	20/10/94	6.44	14.3	13.3	5.0×10	5.0×10	A	A
13	4/11/94	5.84	17.8	9.1	1.5×10^3	1.1×10^3	A	A
14	18/11/94	6.03	16.6	6.5	7.7×10^2	4.7×10^2	A	A
15	1/12/94	6.43	18.2	11.6	6.5×10^4	5.0×10^2	P; ET 55	*L. innocua*
16	15/12/94	5.89	22.4	10.5	3.6×10^3	3.0×10^3	P; ET 62	A
17	5/1/95	6.1	22	9.0	4.8×10^4	3.4×10^4	A	A
18	13/1/95	6.33	24.6	5.8	1.5×10^3	5.0×10^2	A	A
19	27/1/95	6.25	19.4	4.9	3.0×10^3	7.7×10^2	P; ET 63	A
20	10/2/95	6.61	19.6	3.1	3.5×10^3	7.7×10^2	P; ET 66	A
21	24/2/95	6.77	21.3	5.9	8.5×10^3	7.5×10^3	P; ET 69,70	A
22	10/3/95	6.48	20.9	10.8	9.7×10^2	7.0×10^2	A	*L. innocua*
23	24/3/95	5.75	18.2	9.7	1.9×10^4	1.0×10^4	A	*L. innocua*
24	7/4/95	6.03	15.7	9.3	6.0×10^3	4.0E+3	P; ET 77	*L. innocua*
25	21/4/95	6.46	15.4	8.4	1.3×10^4	2.0×10^4	P; ET 79	*L. innocua*
26	5/5/95	6.62	14.4	9.5	2.0×10^2	2.0×10^2	P; ET 85	A
Mean ± S.D.		6.07±0.4	15.4±4.8	9.06±2.5	3.1×10^3 ±4.7×10^3	1.6×10^3 ±2.6×10^3	-	
Median		6.11	15.1	9.35	6.8×10^2	4.8×10^2	-	
Min.-Max.		5.33-6.77	7.1-24.6	3.08-13.3	5.0×10 -1.9×10^4	5.0×10 -1.0×10^4	-	
Total *Listeria*							53.8%	26.9%

FC = Faecal Coliforms; A = Absent; P = Present; ET = Electrophoretic Type

Table C.12 Physicochemical parameters of, and occurrence of *Listeria* spp., faecal coliforms and *E. coli* in effluent samples from Fish Processing Factory 2 (Site 12)

Sample Round	Sampling Date	pH	Temp. (°C)	Salinity (‰)	FC /100 ml	E. coli /100 ml	L mono-cytogenes / L	Other Listeria species/ L
1	20/5/94	6.49	10.1	9.2	1.7×10^4	1.7×10^4	P; ET 3	A
2	3/6/94	6.61	11.3	9.2	3.7×10^4	3.7×10^4	P; ET 8	A
3	17/6/94	6.31	11	9.1	1.9×10^4	1.9×10^4	P; ET 11	A
4	1/7/94	6.36	9.6	9.7	5.0×10^3	5.0×10^3	P; ET 14	A
5	14/7/94	7.1	11	10.8	2.9×10^2	2.9×10^2	P; ET 17	A
6	29/7/94	7.07	9.7	10.2	1.0×10^3	1.0×10^3	P; ET 22	A
7	12/8/94	7.18	10.1	8.3	5.8×10^3	5.8×10^3	P; ET 25	A
8	26/8/94	6.81	11.5	7.9	2.0×10^3	2.0×10^3	P; ET 31	A
9	9/9/94	6.58	12.7	0.8	6.5×10^4	5.0×10^3	P; ET 34	A
10	22/9/94	6.13	10.8	7.1	1.3×10^3	1.3×10^3	P; ET 36	A
11	7/10/94	6.56	13.9	8.4	1.0×10^6	1.0×10^6	P; ET 42	L. innocua
12	20/10/94	6.58	14.6	10.2	5.0×10	5.0×10	P; ET 49	A
13	4/11/94	6.74	16.4	8.43	4.0×10^3	5.0×10^3	P; ET 51	L. innocua
14	18/11/94	6.63	15.3	8.62	1.5×10^2	5.0×10	P; ET 53	A
15	1/12/94	6.53	16.6	9.1	2.4×10^3	1.9×10^3	P; ET 56	A
16	15/12/94	6.39	20.7	10.01	6.5×10^2	6.5×10^2	P; ET 53	A
17	5/1/95	6.13	21.4	6.61	2.1×10^3	2.1×10^3	P; ET 53	A
18	13/1/95	6.57	22	7.33	2.6×10^3	2.6×10^3	P; ET 53	A
19	27/1/95	6.56	19.1	7.52	5.3×10^4	5.3×10^4	P; ET 64	A
20	10/2/95	6.22	19.1	7.51	4.3×10^3	4.3×10^3	P; ET 67	A
21	24/2/95	6.52	20.2	8.3	1.6×10^4	9.0×10^2	P; ET 68	A
22	10/3/95	6.6	19.9	8.5	2.5×10^2	2.0×10^2	P; ET 67	A
23	24/3/95	4.86	18	8.9	1.1×10^4	2.0×10^3	P; ET 72	A
24	7/4/95	6.73	16.3	9.8	1.2×10^4	4.0×10^3	P; ET 68	A
25	21/4/95	6.75	15.7	9	5.6×10^2	5.6×10^2	P; ET 80	A
26	5/5/95	6.15	14.1	8.2	5.0×10^3	5.0×10^2	P; ET 80	A
Mean±S.D.		6.51±0.4	15.0±4.1	8.41±1.9	$4.9 \times 104 \pm 1.9 \times 10^5$	$4.5 \times 104 \pm 2 \times 10^5$	-	-
Median		6.57	15.0	8.56	4.1×10^3	2.1×10^3	-	-
Min.-Max.		4.86-7.18	9.6-22	0.8-10.8	$5.0 \times 10 - 1 \times 10^6$	$5.0 \times 10 - 1 \times 10^6$	-	-
Total *Listeria*							100%	7.7%

FC = Faecal Coliforms; A = Absent; P = Present; ET = Electrophoretic Type

D RECORDED RAINFALL

Table D.1 Rainfall recorded (mm) at Margate station (Hobart, Tasmania) used in North West Bay study (Chapter 2).

Sampling Round	Sampling Date	Rainfall Recorded at Margate Station (mm)			
		24 h	48 h	72 h	1 week
1	20-May-94	0.0	21.0	21.8	26.5
2	3-Jun-94	0.0	0.0	2.4	6.6
3	17-Jun-94	1.0	2.4	7.0	9.8
4	1-Jul-94	0.0	0.2	0.2	2.8
5	14-Jul-94	0.0	0.2	0.2	0.6
6	29-Jul-94	0.0	0.2	0.2	0.8
7	12-Aug-94	0.0	0.0	0.2	27.6
8	26-Aug-94	0.0	0.2	2.2	18.2
9	9-Sep-94	0.0	0.8	4.6	15.4
10	22-Sep-94	0.2	4.2	10.2	37.2
11	7-Oct-94	14.4	16.6	20.2	53.6
12	20-Oct-94	0.0	0.0	0.0	0.0
13	4-Nov-94	1.0	1.6	4.0	7.1
14	18-Nov-94	0.0	0.0	0.6	13.2
15	1-Dec-94	1.4	7.0	7.0	8.5
16	15-Dec-94	0.0	0.0	0.0	0.0
17	5-Jan-95	1.0	1.0	1.0	2.8
18	13-Jan-95	0.0	0.0	0.0	10.2
19	27-Jan-95	1.4	2.6	6.8	19.8
20	10-Feb-95	0.4	0.4	0.6	13.4
21	24-Feb-95	0.0	1.2	1.2	1.8
22	10-Mar-95	0.0	4.4	4.5	4.5
23	24-Mar-95	8.6	8.6	8.7	10.5
24	7-Apr-95	47.0	47.2	53.2	61.6
25	21-Apr-95	1.0	1.0	3.4	10.3
26	5-May-95	0.0	0.0	0.0	1.0

E LOGISTIC ANALYSIS FOR NORTH WEST BAY

E.1 THE LOGISTIC PROCEDURE

The presence or absence data of *Listeria* spp. or *L. monocytogenes* were grouped by the type of samples; river water, effluent and inshore marine water, and analysed using the SAS[1] LOGISTIC procedure (Release 6.10 SAS Institute Inc. Cary USA, 1995) by having *Listeria* spp. and *L. monocytogenes* as the response variables, and the environmental factors (temperature, pH, salinity and rainfall) and the level of faecal coliforms and *E. coli* as the independent variables. Examples of the results of the analysis for inshore marine water with *L. monocytogenes* as the response variables using 1, 2, and 3 independent variable(s) respectively are shown below. An increase in the Chi-Square for covariates statistic (-2 LOG L) of more than 3.84 for a single added independent variable is considered to be significant (α = 0.05). For 2 independent variables, the critical value is 5.99; for 3 independent variables, it is 7.81, etc. The parameter estimates for the 3 independent variable cases shown here were fitted to Eqn. 2.1 and presented as Eqn.2.8 (see Chapter 2). The agreement between the predicted probabilities given by the fitted model and the observed responses used to generate the model is determined from the area 'c' under the receiver operating characteristic (ROC) curve as discussed in Chapter 2, section 2.2.4. Summary of the logistic analysis of 52 river water samples, 78 effluent samples, and 182 inshore water samples when *Listeria* spp. or *L. monocytogenes* was the dependent variable are given in Tables E.1-2, E.3-4, and E.5-6 respectively.

Examples of the results of the analysis for inshore marine water with L. monocytogenes as the response variables using SAS[1] LOGISTIC procedure.

The LOGISTIC Procedure
Response Profile

Ordered Value	L_MONO	Count
1	1	11
2	0	171

Example 1: With 1 independent variable (1 DF)

Criterion	Intercept Only	Intercept and Covariates	Chi-Square for Covariates
AIC	85.056	59.155	.
SC	88.260	65.563	.
-2 LOG L	83.056	55.155	27.901 with 1 DF (p=0.0001)
Score	.	.	29.237 with 1 DF (p=0.0001)

Analysis of Maximum Likelihood Estimates

Variable	DF	Parameter Estimate	Standard Error	Wald Chi-Square	Pr > Chi-Square	Standardized Estimate	Odds Ratio
INTERCPT	1	-6.3319	1.1613	29.7277	0.0001	.	0.002
LOGFC	1	0.8510	0.2093	16.5340	0.0001	1.008003	2.342

Association of Predicted Probabilities and Observed Responses

Concordant	=	89.6%	Somers' D	=	0.798
Discordant	=	9.8%	Gamma	=	0.803
Tied	=	0.6%	Tau-a	=	0.091
(1881 pairs)			c	=	0.899

Example 2: With 2 independent variables (2 DF)

Criterion	Intercept Only	Intercept and Covariates	Chi-Square for Covariates
AIC	85.056	56.014	.
SC	88.260	65.626	.
-2 LOG L	83.056	50.014	33.042 with 2 DF (p=0.0001)
Score	.	.	45.862 with 2 DF (p=0.0001)

Analysis of Maximum Likelihood Estimates

Variable	DF	Parameter Estimate	Standard Error	Wald Chi-Square	Pr > Chi-Square	Standardized Estimate	Odds Ratio
INTERCPT	1	-6.2250	1.1858	27.5570	0.0001	.	0.002
LOGFC	1	0.6775	0.2298	8.6961	0.0032	0.802493	1.969
LOG24	1	0.6126	0.2680	5.2232	0.0223	0.323597	1.845

Association of Predicted Probabilities and Observed Responses

Concordant	=	94.0%	Somers' D	=	0.882
Discordant	=	5.8%	Gamma	=	0.883
Tied	=	0.1%	Tau-a	=	0.101
(1881 pairs)			c	=	0.941

Example 3: With 3 independent variable (3 DF)

Criterion	Intercept Only	Intercept and Covariates	Chi-Square for Covariates
AIC	85.056	54.231	.
SC	88.260	67.047	.
-2 LOG L	83.056	46.231	36.825 with 3 DF (p=0.0001)
Score	.	.	50.722 with 3 DF (p=0.0001)

Analysis of Maximum Likelihood Estimates

Variable	DF	Parameter Estimate	Standard Error	Wald Chi-Square	Pr > Chi-Square	Standardized Estimate	Odds Ratio
INTERCPT	1	3.5306	5.4575	0.4185	0.5177	.	34.143
LOGT	1	-3.6274	2.1041	2.9721	0.0847	-0.550149	0.027
LOGFC	1	0.6303	0.2306	7.4686	0.0063	0.746507	1.878
LOG24	1	0.6899	0.2892	5.6892	0.0171	0.364426	1.993

Association of Predicted Probabilities and Observed Responses

Concordant	=	94.7%	Somers' D	=	0.897
Discordant	=	5.0%	Gamma	=	0.900
Tied	=	0.3%	Tau-a	=	0.102
(1881 pairs)			c	=	0.948

<u>Table E.1</u> A summary of the logistic analysis of 52 river water samples when *Listeria* was the dependent variable. Of these 50 were positive and 2 were negative for *Listeria* spp.

Independent Variable	Chi-Square statistic	Independent Variables	Chi-Square statistic
1 Predictor	*1 df*	*2 Predictors*	*2 df*
Temperature (T)	2.18 (p=0.14)	T and salinity	4.68 (p=0.097)
Salinity (S)	1.65 (p=0.199)	T and pH	3.24 (p=0.198)
pH	1.17 (p=0.28)	T and Rf 24	2.26 (p=0.323)
Rainfall 24 hr (Rf 24)	0.096 (p=0.76)	T and Rf 48	2.83 (p=0.244)
Rainfall 48 hr (Rf 48)	0.22 (p=0.64)	T and Rf 72	3.27 (p=0.195)
Rainfall 72 hr (Rf 72)	0.23 (p=0.63)	T and FC	3.34 (p=0.188)
Rainfall 7 days (Rf 7d)	0.098 (p=0.75)	T and *E. coli*	3.31 (p=0.191)
Faecal coliforms (FC)	0.12 (p=0.73)	S and FC	1.69 (p=0.431)
E. coli (E)	0.09 (p=0.76)	S and *E. coli*	1.67 (p=0.434)

Table E.2 A summary of the logistic analysis of 52 river water samples when *L. mono-cytogenes* was the dependent variable. Of these 19 were positive and 33 were negative for *L. monocytogenes*. The independent variable which had high statistical significance and was deemed practical is shown in bold face. The parameter estimates for this independent variable were fitted to Eqn. 2.1 and resulted as Eqn. 2.2 (see Chapter 2).

Independent Variable (s)	Chi-Square statistic	Independent Variables	Chi-Square statistic
1 Predictor	*1 df*	Rf 48 and FC	0.31 (p=0.86)
Temperature (T)	0.26 (p=0.61)	Rf 48 and E	0.34 (p=0.84)
Salinity (S)	8.18 (p<0.01)	Rf 72 and FC	0.03 (p=0.98)
pH	**10.87 (p<0.01)**	Rf 72 and E	0.02 (p=0.99)
Rainfall 24 hr (Rf 24)	0.00 (p=0.99)	*3 Predictors*	*3 df*
Rainfall 48 hr (Rf 48)	0.24 (p=0.63)	T, S and pH	12.88 (p<0.01)
Rainfall 72 hr (Rf 72)	0.24 (p=0.88)	T, S and Rf 24	10.61 (p=0.014)
Rainfall 7 days (Rf 7d)	0.09 (p=0.77)	T, S and Rf 48	10.29 (p=0.016)
Faecal coliforms (FC)	0.00 (p=0.98)	T, S and Rf 72	10.74 (p=0.013)
E. coli (E)	0.002 (p=0.97)	T, S and FC	10.48 (p=0.015)
2 Predictors	*2 df*	T, S and E	10.53 (p=0.015)
T and S	10.29 (p<0.01)	T, Rf 24 and FC	0.296 (p=0.961)
T and pH	11.40 (p<0.01)	S, Rf 24 and FC	8.40 (p=0.038)
T and Rf 24	0.26 (p=0.88)	S, Rf 24 and E	8.37 (p=0.039)
T and Rf 48	0.49 (p=0.79)	*4 Predictors*	*4 df*
T and Rf 72	0.27 (p=0.87)	T, S, pH and Rf 24	13.08 (p=0.011)
T and FC	0.29 (p=0.86)	T, S, pH and Rf 48	12.89 (p=0.012)
T and E	0.33 (p=0.85)	T, S, pH and Rf 72	13.27 (p=0.010)
S and FC	8.20 (p=0.017)	T, S, pH and FC	12.95 (p=0.012)
S and E	8.19 (p=0.017)	T, S, pH and E	12.99 (p=0.011)
S and pH	11.60 (p<0.01)	T, S, Rf 24 and FC	10.64 (p=0.03)
S and Rf 24	8.26 (p=0.017)	T, S, Rf 24 and E	10.67 (p=0.03)
S and Rf 48	8.21 (p=0.017)	T, S, Rf 72 and FC	10.76 (p=0.03)
S and Rf 72	8.62 (p=0.013)	T, S, Rf 72 and E	10.79 (p=0.03)
pH and Rf 24	10.87 (p<0.01)	S, pH, Rf 24 and FC	11.82 (p=0.019)
pH and Rf 48	10.97 (p<0.01)	S, pH, Rf 24 and E	11.77 (p=0.019)
pH and Rf 72	11.00 (p<0.01)	*5 Predictors*	*5 df*
pH and FC	10.96 (p<0.01)	T, S, pH, FC and Rf 24	13.09 (p=0.022)
pH and E	10.93 (p<0.01)	T, S, pH, FC and Rf 72	13.27 (p=0.02)
Rf 24 and FC	0.00 (p=0.99)	T, S, pH, E and Rf 24	13.10 (p=0.023)
Rf 24 and E	0.00 (p=0.99)	T, S , pH, E and Rf 72	13.27 (p=0.02)

Table E.3 A summary of the logistic analysis of 78 effluent samples when *Listeria* spp. was the dependent variable. Of these 60 were positive and 18 were negative for *Listeria* spp. The independent variable which had high statistical significance and was deemed practical is shown in bold face. The parameter estimates for this independent variable were fitted to Eqn. 2.1 and resulted as Eqn. 2.3 (see Chapter 2).

Independent Variable (s)	Chi-Square statistic	Independent Variables	Chi-Square statistic
1 Predictor	*1 df*	*3 Predictors*	*3 df*
Temperature (T)	3.74 (p=0.053)	T, S and pH	6.36 (p=0.095)
Salinity (S)	0.78 (p=0.38)	T, S and Rf 24	5.68 (p=0.13)
pH	0.06 (p=0.81)	T, S and Rf 48	6.83 (p=0.08)
Rainfall 24 hr (Rf24)	0.35 (p=0.55)	T, S and Rf 72	5.53 (p=0.14)
Rainfall 48 hr (Rf48)	1.61 (p=0.20)	T, S and FC	15.63 (p<0.01)
Rainfall 72 hr (Rf72)	0.77 (p=0.38)	T, S and *E*	13.12 (p<0.01)
Rainfall 7 days (RF7d)	0.38 (p=0.54)	T, pH and Rf 48	5.65 (p=0.13)
Faecal coliforms (FC)	7.93 (p<0.01)	T, FC and Rf 24	12.46 (p<0.01)
E. coli (E)	6.31 (p=0.012)	T, FC and Rf 72	12.59 (p<0.01)
2 Predictors	*2 df*	T, *E* and Rf 24	9.94 (p=0.019)
T and S	4.97 (p=0.08)	T, *E* and Rf 72	9.92 (p=0.019)
T and pH	3.77 (p=0.15)	S, pH and Fc	11.77 (p<0.01)
T and Rf 24	4.49 (p=0.11)	S, pH and *E*	10.01 (p=0.019)
T and Rf 48	5.64 (p=0.06)	S, Rf 24 and FC	11.13 (p=0.011)
T and Rf 72	4.29 (p=0.11)	S, Rf 48 and FC	11.25 (p=0.011)
T and FC	**12.46 (p<0.01)**	S, Rf 72 and FC	11.10 (p=0.011)
T and *E*	9.91 (p<0.01)	S, Rf 24 and *E*	9.51 (p=0.02)
S and pH	1.36 (p=0.51)	*4 Predictors*	*4 df*
S and Rf 24	1.11 (p=0.57)	T, S, pH and Rf 24	7.22 (p=0.12)
S and Rf 48	2.38 (p=0.30)	T, S, pH and Rf 48	8.69 (p=0.07)
S and Rf 72	1.58 (p=0.45)	T, S, pH and Rf 72	7.198 (p=0.13)
S and FC	11.07 (p<0.01)	T, S, pH and FC	18.04 (p<0.01)
S and *E*	9.50 (p<0.01)	T, S, pH and *E*	14.73 (p<0.01)
pH and Rf 24	0.39 (p=0.82)	T, S, Rf 24 and FC	15.63 (p<0.01)
pH and Rf 48	1.64 (p=0.44)	T, S, Rf 24 and *E*	13.14 (p=0.01)
pH and Rf 72	0.80 (p=0.67)	T, S, Rf 72 and FC	15.78 (p<0.01)
pH and FC	8.77 (p=0.012)	T, S, Rf 72 and *E*	13.18 (p=0.01)
pH and *E*	7.23 (p=0.027)	S, pH, Rf 24 and FC	11.81 (p=0.019)
Rf 24 and FC	7.97 (p=0.019)	S, pH, Rf 24 and *E*	10.02 (p=0.04)
Rf 24 and *E*	6.32 (p=0.04)	*5 predictors*	*5 df*
Rf 48 and FC	8.15 (p=0.017)	T, S, pH, FC and Rf 24	18.05 (p<0.01)
Rf 48 and *E*	6.65 (p=0.04)	T, S, pH, FC and Rf 72	18.06 (p<0.01)
Rf 72 and FC	7.94 (p=0.019)	T, S, pH, *E* and Rf 24	14.82 (p<0.01)
Rf 72 and *E*	6.32 (p=0.04)	T, S, pH, *E* and Rf 72	14.73 (p=0.012)

Table E.4 A summary of the logistic analysis of 78 effluent samples when *L. mono-cytogenes* was the dependent variable. Of these 49 were positive and 29 were negative for *L. monocytogenes*. The independent variable which had high statistical significance and was deemed practical is shown in bold face. The parameter estimates for this independent variable were fitted to Eqn. 2.1 and presented as Eqn. 2.4 (see Chapter 2).

Independent Variable (s)	Chi-Square statistic	Independent Variables	Chi-Square statistic
1 Predictor	*1 df*	*3 Predictors*	*3 df*
Temperature (T)	1.36 (p=0.24)	T, S and pH	6.35 (p=0.096)
Salinity (S)	0.56 (p=0.45)	T, S and Rf 24	2.57 (p=0.46)
pH	0.21 (p=0.65)	T, S and Rf 48	2.36 (p=0.50)
Rainfall 24 hr (Rf24)	0.17 (p=0.68)	T, S and Rf 72	2.22 (p=0.53)
Rainfall 48 hr (Rf48)	0.12 (p=0.73)	T, S and FC	14.18 (p<0.01)
Rainfall 72 hr (Rf72)	0.007 (p=0.94)	T, S and *E*	10.90 (p=0.012)
Rainfall 7 days (RF7d)	0.84 (p=0.36)	T, pH and Rf 24	1.99 (p=0.57)
Faecal coliforms (FC)	9.70 (p<0.01)	T, FC and Rf 24	11.25 (p=0.01)
E. coli *(E)*	7.09 (p<0.01)	T, FC and Rf 72	13.13 (p<0.01)
2 Predictors	*2 df*	T, *E* and Rf 24	8.06 (p=0.04)
T and S	2.21 (p=0.33)	T, *E* and Rf 72	9.33 (p=0.025)
T and pH	1.58 (p=0.46)	**S, pH and FC**	**15.47 (p<0.01)**
T and Rf 24	1.73 (p=0.42)	S, pH and *E*	12.13 (p<0.01)
T and Rf 48	1.53 (p=0.47)	S, Rf 24 and FC	12.60 (p<0.01)
T and Rf 72	1.38 (p=0.50)	S, Rf 48 and FC	12.81 (p<0.01)
T and FC	11.13 (p<0.01)	S, Rf 72 and FC	14.26 (p<0.01)
T and *E*	8.05 (p=0.018)	S, Rf 24 and *E*	9.76 (p=0.02)
S and pH	3.48 (p=0.18)	*4 Predictors*	*4 df*
S and Rf 24	0.71 (p=0.70)	T, S, pH and Rf 24	7.00 (p=0.14)
S and Rf 48	0.67 (p=0.72)	T, S, pH and Rf 48	6.69 (p=0.15)
S and Rf 72	0.57 (p=0.75)	T, S, pH and Rf 72	6.36 (p=0.17)
S and FC	12.26 (p<0.01)	T, S, pH and FC	18.91 (p<0.01)
S and *E*	9.62 (p<0.01)	T, S, pH and *E*	14.51 (p<0.01)
pH and Rf 24	0.40 (p=0.82)	T, S, Rf 24 and FC	14.33 (p<0.01)
pH and Rf 48	0.35 (p=0.84)	T, S, Rf 24 and *E*	10.94 (p=0.03)
pH and Rf 72	0.21 (p=0.90)	T, S, Rf 72 and FC	16.55 (p<0.01)
pH and FC	9.73 (p<0.01)	T, S, Rf 72 and *E*	12.54 (p=0.014)
pH and *E*	7.14 (p=0.028)	S, pH, Rf 24 and FC	15.66 (p<0.01)
Rf 24 and FC	9.96 (p<0.01)	S, pH, Rf 24 and *E*	12.18 (p=0.016)
Rf 24 and *E*	7.16 (p=0.028)	*5 predictors*	*5 df*
Rf 48 and FC	10.11 (p<0.01)	T, S, pH, FC and Rf 24	18.93 (p<0.01)
Rf 48 and *E*	7.31 (p=0.026)	T, S, pH, FC and Rf 72	20.36 (p<0.01)
Rf 72 and FC	11.43 (p<0.01)	T, S, pH, *E* and Rf 24	14.51 (p=0.013)
Rf 72 and *E*	8.29 (p=0.016)	T, S, pH, *E* and Rf 72	15.43 (p<0.01)

Table E.5 A summary of the logistic analysis of 182 inshore water samples when *Listeria* spp. was the dependent variable. Of these 34 were positive and 148 were negative for *Listeria* spp. The independent variables which had high statistical significances and were deemed practical are shown in bold face. The parameter estimates for these independent variables were fitted to Eqn. 2.1 and resulted as Eqns. 2.5 and 2.6 (see Chapter 2).

Independent Variable (s)	Chi-Square statistic	Independent Variables	Chi-Square statistic
1 Predictor	*1 df*	T, S and Rf 72	44.86 (p<0.01)
Temperature (T)	6.34 (p=0.012)	T, S and FC	47.56 (p<0.01)
Salinity (S)	9.25 (p<0.01)	T, S and E	43.03 (p<0.01)
pH	4.71 (p=0.03)	T, pH and Rf 24	45.03 (p<0.01)
Rainfall 24 hr (Rf 24)	33.38 (p<0.01)	T, pH and Rf 72	42.11 (p<0.01)
Rainfall 48 hr (Rf 48)	32.58 (p<0.01)	T, pH and FC	45.43 (p<0.01)
Rainfall 72 hr (Rf 72)	35.47 (p<0.01)	T, pH and E	41.13 (p<0.01)
Rainfall 7 days (Rf 7d)	31.43 (p<0.01)	**T, FC and Rf 24**	**61.09** (p<0.01)
Faecal coliforms (FC)	36.78 (p<0.01)	T, FC and Rf 72	59.82 (p<0.01)
E. coli (E)	31.05 (p<0.01)	T, E and Rf 24	56.88 (p<0.01)
2 Predictors	*2 df*	T, E and Rf 72	57.19 (p<0.01)
T and S	19.53 (p<0.01)	S, pH and Rf 24	35.13 (p<0.01)
T and pH	10.22 (p<0.01)	S, pH and Rf 48	35.52 (p<0.01)
T and Rf 24	**44.14** (p<0.01)	S, pH and Rf 72	39.32 (p<0.01)
T and Rf 48	39.75 (p<0.01)	S, pH and Fc	37.71 (p<0.01)
T and Rf 72	40.12 (p<0.01)	S, pH and E	32.72 (p<0.01)
T and FC	45.27 (p<0.01)	S, Rf 24 and FC	50.78 (p<0.01)
T and E	40.36 (p<0.01)	S, Rf 48 and FC	51.75 (p<0.01)
S and pH	10.89 (p<0.01)	S, Rf 72 and FC	53.93 (p<0.01)
S and Rf 24	33.61 (p<0.01)	S, Rf 24 and E	45.78 (p<0.01)
S and Rf 48	34.42 (p<0.01)	S, Rf 48 and E	48.68 (p<0.01)
S and Rf 72	37.66 (p<0.01)	S, Rf 72 and E	50.76 (p<0.01)
S and FC	37.50 (p<0.01)	*4 Predictors*	*4 df*
S and E	32.02 (p<0.01)	T, S, pH and Rf 24	45.56 (p<0.01)
pH and Rf 24	35.12 (p<0.01)	T, S, pH and Rf 48	44.40 (p<0.01)
pH and Rf 48	34.34 (p<0.01)	T, S, pH and Rf 72	45.46 (p<0.01)
pH and Rf 72	38.08 (p<0.01)	T, S, pH and FC	47.59 (p<0.01)
pH and FC	37.10 (p<0.01)	T, S, pH and E	43.36 (p<0.01)
pH and E	32.05 (p<0.01)	T, S, Rf 24 and FC	61.10 (p<0.01)
Rf 24 and FC	50.55 (p<0.01)	T, S, Rf 24 and E	56.93 (p<0.01)
Rf 24 and E	45.66 (p<0.01)	T, S, Rf 72 and FC	60.48 (p<0.01)
Rf 48 and FC	51.75 (p<0.01)	T, S, Rf 72 and E	58.00 (p<0.01)
Rf 48 and E	48.68 (p<0.01)	S, pH, Rf 24 and FC	50.94 (p<0.01)
Rf 72 and FC	53.92 (p<0.01)	S, pH, Rf 24 and E	46.36 (p<0.01)
Rf 72 and E	50.73 (p<0.01)	*5 predictors*	*5 df*
3 Predictors	*3 df*	T, S, pH, FC and Rf 24	61.11 (p<0.01)
T, S and pH	20.04 (p<0.01)	T, S, pH, FC and Rf 72	60.52 (p<0.01)
T, S and Rf 24	45.03 (p<0.01)	T, S, pH, E and Rf 24	57.20 (p<0.01)
T, S and Rf 48	44.29 p<0.01)	T, S, pH, E and Rf 72	58.36 (p<0.01)

<u>Table E.6</u> A summary of the logistic analysis of 182 inshore water samples when *L. monocytogenes* was the dependent variable. Of these 11 were positive and 171 were negative for *L. monocytogenes*. The independent variables which had high statistical significances and were deemed practical are shown in bold face. The parameter estimates for these independent variables were fitted to Eqn. 2.1 and resulted as Eqns. 2.7 and 2.8 (see Chapter 2).

Independent Variable (s)	Chi-Square statistic	Independent Variables	Chi-Square statistic
1 Predictor	*1 df*	T, S and Rf 48	30.75 (p<0.01)
Temperature (T)	3.17 (p=0.08)	T, S and Rf 72	31.90 (p<0.01)
Salinity (S)	0.80 (p=0.37)	T, S and FC	33.86 (p<0.01)
pH	0.61 (p=0.44)	T, S and *E. coli*	24.68 (p<0.01)
Rainfall 24 hr (Rf 24)	21.18 (p<0.01)	T, pH and Rf 24	29.14 (p<0.01)
Rainfall 48 hr (Rf 48)	27.08 (p<0.01)	T, pH and Rf 72	33.33 (p<0.01)
Rainfall 72 hr (Rf 72)	28.67 (p<0.01)	T, pH and FC	34.23 (p<0.01)
Rainfall 7 days (Rf 7d)	34.18 (p<0.01)	T, pH and *E. coli*	24.00 (p<0.01)
Faecal coliforms (FC)	27.90 (p<0.01)	**T , F C and Rf 2 4**	**3 6 . 8 3** (p<0.01)
E. coli (E)	19.34 (p<0.01)	T, FC and Rf 72	40.47 (p<0.01)
2 Predictors	*2 df*	T, *E. coli* and Rf 24	31.11 (p<0.01)
T and salinity	4.33 (p=0.11)	T, *E. coli* and Rf 72	36.20 (p<0.01)
T and pH	3.50 (p=0.17)	S, pH and Rf 48	29.00 (p<0.01)
T and Rf 2 4	**27.37** (p<0.01)	S, pH and Rf 72	31.12 (p<0.01)
T and Rf 48	30.11 (p<0.01)	S, pH and FC	32.98 (p<0.01)
T and Rf 72	30.93 (p<0.01)	S, pH and *E. coli*	21.35 (p<0.01)
T and Rf 7d	34.39 (p<0.01)	S, Rf 24 and FC	41.43 (p<0.01)
T and FC	30.95 (p<0.01)	S, Rf 48 and FC	46.29 (p<0.01)
T and *E. coli*	23.17 (p<0.01)	S, Rf 72 and FC	47.29 (p<0.01)
S and pH	1.03 (p=0.59)	S, Rf 24 and *E. coli*	32.12 (p<0.01)
S and Rf 24	23.37 (p<0.01)	S, Rf 48 and *E. coli*	39.49 (p<0.01)
S and Rf 48	28.31 (p<0.01)	S, Rf 72 and *E. coli*	40.36 (p<0.01)
S and Rf 72	30.30 (p<0.01)	*4 Predictors*	*4 df*
S and FC	31.35 (p<0.01)	T, S, pH and Rf 24	29.83 (p<0.01)
S and *E. coli*	21.18 (p<0.01)	T, S, pH and Rf 48	32.85 (p<0.01)
pH and Rf 24	21.63 (p<0.01)	T, S, pH and Rf 72	33.41 (p<0.01)
pH and Rf 48	28.50 (p<0.01)	T, S, pH and FC	35.48 (p<0.01)
pH and FC	30.88 (p<0.01)	T, S, pH and *E. coli*	24.84 (p<0.01)
pH and *E. coli*	20.08 (p<0.01)	T, S, Rf 24 and FC	44.32 (p<0.01)
Rf 24 and FC	33.04 (p<0.01)	T, S, Rf 24 and *E. coli*	35.92 (p<0.01)
Rf 24 and *E. coli*	26.51 (p<0.01)	S, pH, Rf 72 and FC	53.20 (p<0.01)
Rf 48 and FC	37.68 (p<0.01)	S, pH, Rf 72 and *E. coli*	41.86 (p<0.01)
Rf 48 and *E. coli*	32.82 (p<0.01)	*5 predictors*	*5 df*
Rf 72 and FC	38.46 (p<0.01)	T, S, pH, FC and Rf 24	46.20 (p<0.01)
Rf 72 and *E. coli*	33.60 (p<0.01)	T, S, pH, FC and Rf 72	53.45 (p<0.01)
3 Predictors	*3 df*	T, S, pH, FC and Rf 7d	49.09 (p<0.01)
T, S and pH	4.35 (p=0.23)	T, S, pH, *E. coli* and Rf 24	36.56 (p<0.01)
T, S and Rf 24	29.34 (p<0.01)	T, S , pH, *E. coli* and Rf 72	43.17 (p<0.01)

F CALIBRATION AND VALIDATION OF ECOMETRIC TECHNIQUE

The ecometric technique was introduced as a rapid, semi-quantitative screening method for large numbers of samples (Mossel *et al.*, 1980; 1981). The method involves a continuous streaking, and thereby dilution, of bacterial culture on to media, a similar concept to spiral plating, in a rigorously standardised way (Mossel *et al.*, 1983). The technique was used as a criteria for growth or no growth in the probability model study when there was no visible turbidity in the wells. In order to obtain a consistent result, the materials and method used were standardised (Mossel *et al.*, 1983) as follow:

1. To obtain a constant depth of the agar layer, 15 ml of TSA-YE at ca. $50 \pm 1°C$ was dispensed to each plate.

2. The water activity at the test surfaces was standardised by drying the plates upside down with lids closed for 18 ± 1 hr at $37°C$ incubator in stacks not less than 2 cm apart (4 plates/ stack).

3. A template of inoculation pattern was used. The continuous streaking was started from the first line at the perimeter followed the five consecutive parallel lines of the four sectors and finally one streak through the centre (Fig. F.1).
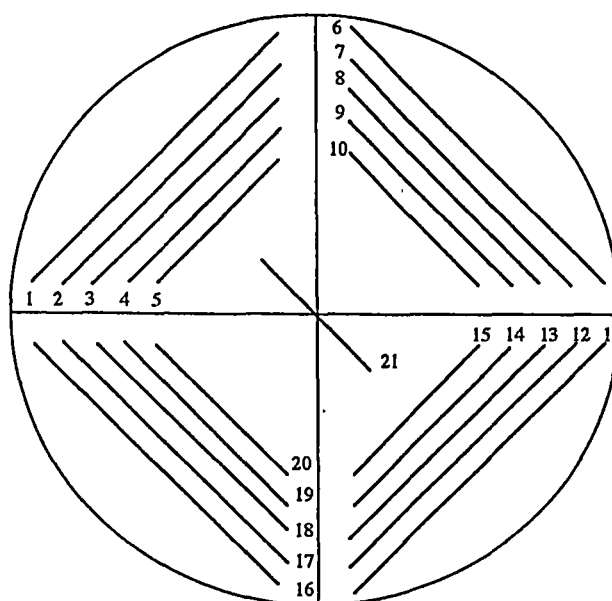
Figure F.1 The template for Ecometric streaking. Line numbers show sequence of streaking. (Adapted from Mossel *et al.*, 1983)

4. The same size of volumetric loop, 1/300 ml, was used. The test culture was mixed by pipetting up and down, then the loop but not the stem was immersed in the test culture. The inoculation was processed with the loop held at a shallow angle flat against the surface of the agar followed the pattern guided by the template placed under the plate.

The assessment of each ecometrically streaked plate was simplified to counting the numbers of lines on which colonies were observed. The technique was compared to the viable count, i.e. pour plates method, on different concentrations of L. monocytogenes Scott A. The bacterial dilutions were kept cold (10°C) during the process of dilution, plating and streaking to minimise the growth of the organism. Duplicates of the proper bacterial dilutions were prepared using pour plate method in TSA-YE. Four replicate plates for each dilution including the original culture were streaked using the standardised Ecometric technique previously described. The relationship between the bacterial plate count and line numbers with growth is shown in Fig. F.2. There is a gradual change in line numbers when the amount of bacteria is less than ca. 1×10^7 cfu/ml, following by a dramatic change with the higher amount of bacteria. Since the inoculum of L. monocytogenes prepared in the probability study was approximately 1×10^7 cfu/ml, the growth or no growth of L. monocytogenes in each well was considered to be adequately detected by this method.
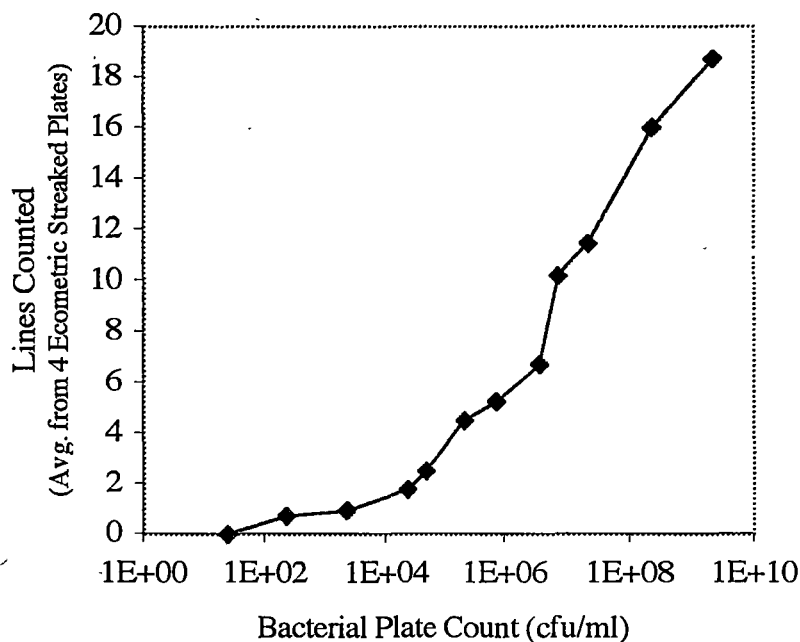
Figure F.2 Calibration of the assessment of Ecometric technique (numbers of lines counted) to a bacterial plate count (cfu/ml).

G DATA SETS USED FOR MODELS GENERATION

G.1 KINETIC MODEL

The data sets upon which the kinetic models for *L. monocytogenes* Scott A (Eqn. 4.17a) and L5 (Eqn. 4.18a) are presented in Tables G.1-G.2 and G.3-G.4 respectively. The modles 4.17b and 4.18b for Scott A and L5 were generated from the data presented in Tables G.1 and G.3 respectively. The variables space covered by the data sets for the kinetic models are shown diagrammatically in Fig. G.1.

Table G.1 *L. monocytogenes* Scott A data set for kinetic models (Eqns. 4.17a,b).

TLA	[UD]	pH	T	a_w	GT		TLA	[UD]	pH	T	a_w	GT
0	0	7.27	3.1	0.995	57.88		20	0.3	5.70	21.7	0.967	3.60
0	0	7.27	5.6	0.995	29.80		20	0.2	5.88	21.7	0.967	3.39
0	0	7.27	7.6	0.995	16.59		20	0.1	6.13	21.8	0.967	3.09
0	0	7.27	9.0	0.995	11.13		20	0.1	6.40	21.8	0.967	2.83
0	0	7.27	10.6	0.995	7.02		20	0.0	6.69	21.8	0.967	2.80
0	0	7.27	11.5	0.995	5.27		20	0.0	7.29	21.8	0.967	2.51
0	0	7.27	12.5	0.995	4.24		20	0.0	7.39	21.9	0.967	2.60
0	0	7.27	13.8	0.995	3.58		20	0.0	7.80	21.9	0.967	2.52
0	0	7.27	14.8	0.995	3.10		50	2.6	5.12	21.7	0.968	68.67
0	0	7.27	15.6	0.995	2.68		50	2.1	5.21	21.7	0.968	13.01
0	0	7.27	16.6	0.995	2.27		50	1.5	5.38	21.7	0.968	5.80
0	0	7.27	17.4	0.995	2.07		50	1.0	5.56	21.7	0.968	4.44
0	0	7.27	18.4	0.995	1.76		50	0.6	5.75	21.8	0.968	3.66
0	0	7.27	19.2	0.995	1.66		50	0.4	5.98	21.8	0.968	3.36
0	0	7.27	20.0	0.995	1.52		50	0.3	6.12	21.8	0.968	3.16
0	0	7.27	21.0	0.995	1.37		50	0.2	6.31	21.9	0.968	2.95
0	0	7.27	21.8	0.995	1.26		50	0.1	6.69	21.9	0.968	2.87
0	0	7.27	22.5	0.995	1.19		50	0.0	7.08	21.9	0.968	2.79
0	0	7.27	23.3	0.995	1.10		50	0.0	7.36	22.0	0.968	2.79
0	0	7.27	24.1	0.995	1.09		50	0.0	7.75	22.0	0.968	2.57
0	0	7.27	25.2	0.995	0.97		100	3.4	5.31	22.4	0.965	51.30
0	0	7.27	26.0	0.995	0.90		100	2.7	5.41	22.3	0.965	12.50
0	0	7.27	27.1	0.995	0.85		100	1.9	5.57	22.3	0.965	6.43
0	0	7.27	28.2	0.995	0.79		100	1.3	5.75	22.2	0.965	4.31
0	0	7.27	29.2	0.995	0.75		100	0.7	6.04	22.2	0.965	3.32
0	0	7.27	30.2	0.995	0.72		100	0.4	6.21	22.1	0.965	3.22
0	0	7.27	31.7	0.995	0.67		100	0.2	6.51	22.1	0.965	2.94
0	0	7.27	33.1	0.995	0.63		100	0.1	6.78	22.1	0.965	2.64
0	0	7.27	34.4	0.995	0.59		100	0.1	7.14	22.0	0.965	2.68
0	0	7.27	35.8	0.995	0.56		100	0.0	7.42	21.9	0.965	2.69
20	1.4	4.99	21.6	0.967	20.95		100	0.0	7.67	21.9	0.965	2.24
20	0.9	5.17	21.6	0.967	11.49		200	3.7	5.59	22.5	0.962	45.47
20	0.4	5.52	21.6	0.967	4.00		200	2.8	5.70	22.5	0.962	13.73

Table G.1 (contd.) *L. monocytogenes* Scott A data set for kinetic models.

TLA	[UD]	pH	T	a_w	GT
200	2.4	5.78	22.4	0.962	8.42
200	1 7	5.93	22.4	0.962	5.07
200	1.3	6.05	22.3	0.962	3.83
200	1.0	6.16	22.3	0.962	3.28
200	0.7	6.30	22.2	0.962	3.33
200	0.4	6.52	22.2	0.962	2.37
200	0.3	6.69	22.2	0.962	2.46
200	0.2	6.78	22.1	0.962	2.68
200	0.2	6.97	22.1	0.962	2.59
200	-0.1	7.28	22.1	0.962	2.64
200	0.0	7.65	22.0	0.962	2.39
0	0	5.53	20.0	0.994	2.57
0	0	5.42	20.0	0.985	2.99
0	0	5.49	20.0	0.980	3.04
0	0	5.50	20.0	0.974	3.38
0	0	5.48	20.0	0.965	4.71
0	0	5.45	19.8	0.958	6.58
0	0	5.36	19.9	0.952	8.06
0	0	5.37	19.9	0.945	11.99
0	0	5.35	19.9	0.939	17.88
0	0	5.47	19.9	0.929	31.35
0	0	5.82	20.2	0.994	2.13
0	0	5.75	20.2	0.985	2.51
0	0	5.81	20.1	0.980	2.69
0	0	5.79	20.1	0.974	2.74
0	0	5.78	20.0	0.965	3.71
0	0	5.78	20.0	0.958	4.66
0	0	5.73	20.1	0.952	5.52
0	0	5.68	20.1	0.945	7.28
0	0	5.76	20.0	0.939	9.18
0	0	5.77	20.0	0.929	18.80
0	0	6.15	20.4	0.994	1.85
0	0	6.10	20.4	0.985	2.01
0	0	6.26	20.4	0.980	2.23
0	0	6.22	20.4	0.974	2.37

TLA	[UD]	pH	T	a_w	GT
0	0	6.15	20.4	0.965	3.18
0	0	6.09	20.3	0.958	3.97
0	0	6.08	20.3	0.952	4.89
0	0	6.09	20.3	0.945	6.01
0	0	6.10	20.3	0.939	8.06
0	0	6.07	20.2	0.929	14.29
50	1.1	5.51	20.0	0.991	3.83
50	1.1	5.49	20.0	0.984	5.16
50	1.3	5.44	20.0	0.978	5.09
50	1.4	5.41	19.9	0.973	7.18
50	1.6	5.35	19.9	0.963	10.48
50	1.6	5.34	19.9	0.956	13.18
50	1.6	5.33	19.9	0.950	32.74
50	1.5	5.37	19.9	0.943	48.98
50	0.6	5.79	20.1	0.991	2.61
50	0.6	5.81	20.1	0.984	2.63
50	0.7	5.73	20.1	0.978	2.96
50	0.7	5.71	20.0	0.973	3.13
50	0.6	5.75	20.0	0.963	4.09
50	0.6	5.78	20.0	0.956	5.00
50	0.7	5.70	20.1	0.950	6.35
50	0.5	5.82	20.0	0.943	7.01
50	0.7	5.73	20.0	0.937	11.65
50	0.6	5.75	20.0	0.930	25.33
50	0.2	6.19	20.4	0.991	1.88
50	0.3	6.12	20.4	0.984	2.08
50	0.2	6.18	20.4	0.978	2.29
50	0.3	6.10	20.4	0.973	2.50
50	0.3	6.11	20.3	0.963	3.29
50	0.3	6.13	20.3	0.956	4.01
50	0.3	6.07	20.2	0.950	4.92
50	0.2	6.20	20.2	0.943	6.00
50	0.2	6.18	20.2	0.937	8.66
50	0.3	6.14	20.2	0.930	11.28

Table G.2 The additional *L. monocytogenes* Scott A data set for kinetic model (Eqn. 4.17a).

TLA	[UD]	pH	T	a_w	GT
0	0	4.23	20.7	0.995	73.85
0	0	4.31	20.7	0.995	38.87
0	0	4.48	20.7	0.995	15.95
0	0	4.59	20.7	0.995	9.35
0	0	4.82	20.8	0.995	4.80
0	0	4.98	20.8	0.995	3.04
0	0	5.24	20.9	0.995	2.09

TLA	[UD]	pH	T	a_w	GT
0	0	5.47	20.9	0.995	1.70
0	0	5.68	20.9	0.995	1.52
0	0	5.99	21.0	0.995	1.41
0	0	6.21	21.0	0.995	1.46
0	0	6.53	21.0	0.995	1.36
0	0	6.79	21.1	0.995	1.33

Table G.3 *L. monocytogenes* L5 data set for kinetic models (Eqns. 4.18a,b).

TLA	[UD]	pH	T	a_w	GT
0	0	7.29	3.06	0.995	47.00
0	0	7.29	4.96	0.995	28.34
0	0	7.29	7.40	0.995	15.53
0	0	7.29	8.84	0.995	9.71
0	0	7.29	9.86	0.995	6.92
0	0	7.29	11.14	0.995	5.04
0	0	7.29	12.36	0.995	3.92
0	0	7.29	13.68	0.995	3.43
0	0	7.29	14.53	0.995	3.08
0	0	7.29	15.70	0.995	2.66
0	0	7.29	16.50	0.995	2.30
0	0	7.29	17.48	0.995	1.93
0	0	7.29	18.40	0.995	1.72
0	0	7.29	19.35	0.995	1.53
0	0	7.29	20.10	0.995	1.45
0	0	7.29	20.98	0.995	1.37
0	0	7.29	21.88	0.995	1.23
0	0	7.29	22.70	0.995	1.15
0	0	7.29	23.60	0.995	1.07
0	0	7.29	24.50	0.995	1.05
0	0	7.29	25.43	0.995	0.96
0	0	7.29	26.44	0.995	0.88
0	0	7.29	27.38	0.995	0.87
0	0	7.29	28.40	0.995	0.82
0	0	7.29	29.58	0.995	0.77
0	0	7.29	30.74	0.995	0.75
0	0	7.29	31.96	0.995	0.70
0	0	7.29	33.24	0.995	0.66
0	0	7.29	34.90	0.995	0.62
0	0	7.29	36.24	0.995	0.61
20	1.4	4.98	19.90	0.969	23.61
20	1.2	5.04	20.04	0.969	16.10
20	0.8	5.26	20.12	0.969	5.71
20	0.6	5.36	20.28	0.969	5.12
20	0.3	5.65	20.44	0.969	3.17
20	0.1	6.09	20.64	0.969	2.91
20	0.1	6.24	20.68	0.969	2.70
20	0.1	6.40	20.74	0.969	2.56
20	0.0	6.78	20.86	0.969	2.51
20	0.0	7.09	20.96	0.969	2.32
20	0.0	7.41	21.04	0.969	2.56
20	0.0	7.67	21.16	0.969	2.22
50	3.6	4.97	19.34	0.969	99.83
50	2.9	5.07	19.50	0.969	29.10
50	2.8	5.09	19.68	0.969	24.08
50	1.9	5.27	19.86	0.969	7.26
50	1.7	5.31	19.98	0.969	6.57
50	0.9	5.59	20.12	0.969	4.32
50	0.4	5.99	20.22	0.969	3.02
50	0.2	6.18	20.38	0.969	2.83

TLA	[UD]	pH	T	a_w	GT
50	0.2	6.34	20.46	0.969	2.75
50	0.1	6.73	20.58	0.969	2.49
50	0.0	7.01	20.70	0.969	2.42
50	0.0	7.33	20.80	0.969	2.36
50	0.0	7.58	20.90	0.969	2.61
100	4.5	5.19	21.70	0.966	66.63
100	3.5	5.3	21.68	0.966	24.92
100	3.3	5.33	21.66	0.966	15.19
100	1.9	5.58	21.60	0.966	6.29
100	1.2	5.77	21.60	0.966	3.91
100	0.7	6.00	21.54	0.966	3.13
100	0.5	6.17	21.50	0.966	3.06
100	0.3	6.36	21.48	0.966	2.85
100	0.1	6.7	21.38	0.966	2.55
100	0.1	7.03	21.36	0.966	2.40
100	0.0	7.37	21.30	0.966	2.40
100	0.0	7.56	21.22	0.966	2.29
200	3.8	5.57	21.56	0.964	25.59
200	3.5	5.61	21.50	0.964	17.36
200	2.4	5.77	21.46	0.964	8.54
200	1.6	5.96	21.40	0.964	4.21
200	1.3	6.03	21.34	0.964	4.06
200	0.9	6.21	21.26	0.964	3.37
200	0.7	6.31	21.20	0.964	2.68
200	0.3	6.63	21.16	0.964	2.62
200	0.2	6.96	21.06	0.964	2.66
200	0.1	7.22	21.00	0.964	2.65
450	4.3	5.88	21.28	0.962	56.77
450	3.5	5.97	21.30	0.962	33.21
450	3.0	6.04	21.33	0.962	19.46
450	2.3	6.15	21.38	0.962	10.39
450	1.8	6.25	21.43	0.962	6.32
450	1.4	6.38	21.48	0.962	4.79
450	0.8	6.60	21.53	0.962	3.41
0	0	5.42	20.24	0.995	2.41
0	0	5.32	20.24	0.987	2.61
0	0	5.39	20.22	0.982	2.81
0	0	5.41	20.22	0.976	3.09
0	0	5.39	20.22	0.966	4.23
0	0	5.36	20.20	0.961	5.75
0	0	5.39	20.16	0.954	6.34
0	0	5.35	20.16	0.948	8.69
0	0	5.38	20.14	0.941	12.98
0	0	5.34	20.12	0.936	26.44
0	0	5.72	20.50	0.995	1.83
0	0	5.64	20.48	0.987	2.23
0	0	5.71	20.46	0.982	2.26
0	0	5.68	20.44	0.976	2.45
0	0	5.67	20.42	0.966	3.14
0	0	5.70	20.40	0.961	3.94

Table G.3 (contd.) *L. monocytogenes* L5 data set for kinetic models.

TLA	[UD]	pH	T	a_w	GT
0	0	5.64	20.32	0.954	4.65
0	0	5.59	20.32	0.948	6.00
0	0	5.67	20.30	0.941	7.62
0	0	5.65	20.30	0.936	11.63
0	0	6.05	20.82	0.995	1.63
0	0	6.00	20.80	0.987	1.90
0	0	6.15	20.76	0.982	1.98
0	0	6.11	20.74	0.976	2.01
0	0	6.05	20.70	0.966	2.55
0	0	5.98	20.68	0.961	3.18
0	0	5.98	20.62	0.954	3.62
0	0	5.99	20.60	0.948	4.69
0	0	5.99	20.60	0.941	6.36
0	0	6.02	20.52	0.936	8.69
50	1.3	5.42	20.28	0.995	3.21
50	1.4	5.41	20.26	0.987	3.95
50	1.5	5.37	20.24	0.982	4.48
50	1.6	5.33	20.22	0.976	5.34
50	1.6	5.33	20.22	0.966	8.76
50	1.6	5.35	20.18	0.961	10.07
50	1.6	5.35	20.16	0.954	11.86

TLA	[UD]	pH	T	a_w	GT
50	1.7	5.31	20.14	0.948	21.05
50	0.7	5.71	20.64	0.995	2.18
50	0.7	5.72	20.62	0.987	2.26
50	0.8	5.64	20.60	0.982	2.63
50	0.9	5.62	20.54	0.976	2.89
50	0.8	5.66	20.52	0.966	3.94
50	0.7	5.69	20.48	0.961	4.45
50	0.9	5.62	20.48	0.954	6.07
50	0.6	5.79	20.38	0.948	5.07
50	0.8	5.66	20.38	0.941	14.02
50	0.8	5.67	20.36	0.936	14.37
50	0.3	6.12	20.76	0.995	1.66
50	0.3	6.04	20.76	0.987	1.96
50	0.3	6.11	20.74	0.982	1.93
50	0.3	6.02	20.74	0.976	2.15
50	0.3	6.03	20.74	0.966	2.76
50	0.3	6.03	20.68	0.961	3.16
50	0.4	5.99	20.64	0.954	3.94
50	0.3	6.13	20.64	0.948	4.24
50	0.3	6.10	20.56	0.941	6.03
50	0.3	6.06	20.56	0.936	8.39

Table G. 4 The additional *L. monocytogenes* L5 data set for kinetic model (Eqn. 4.18a).

TLA	[UD]	pH	T	a_w	GT
0	0	4.25	20.70	0.995	64.06
0	0	4.32	20.73	0.995	40.14
0	0	4.50	20.75	0.995	16.62
0	0	4.61	20.80	0.995	10.00
0	0	4.84	20.80	0.995	4.19
0	0	4.99	20.88	0.995	2.75
0	0	5.26	20.90	0.995	2.00

TLA	[UD]	pH	T	a_w	GT
0	0	5.48	20.90	0.995	1.83
0	0	5.69	20.95	0.995	1.61
0	0	6.00	20.98	0.995	1.48
0	0	6.21	21.00	0.995	1.42
0	0	6.54	21.05	0.995	1.34
0	0	6.80	21.08	0.995	1.27

G.2 PROBABILITY MODEL

The probability models for *L. monocytogenes* Scott A (Eqn. 5.1) and L5 (Eqn. 5.2) are based on data presented in Tables G.5 and G.6 respectively. The variables space covered by the data sets for the probability models are shown diagrammatically in Fig. G.2.

Table G.5 *L. monocytogenes* Scott A data set for probability model (Eqn. 5.1) including the 135, and 13 growth data from Tables G.1 and G.2 respectively, and 17 no growth data from those kinetic experiments.

TLA	[UD]	pH	T	a_w	NG	G
0	0	3.89	4	0.994	4	0
0	0	4.18	4	0.994	4	0
0	0	4.36	4	0.994	4	0
0	0	4.50	4	0.994	4	0
0	0	4.61	4	0.994	4	0
0	0	4.71	4	0.994	4	0
0	0	4.92	4	0.994	4	0
0	0	5.12	4	0.994	4	0
0	0	5.59	4	0.994	0	4
0	0	6.08	4	0.994	0	4
0	0	3.89	10	0.994	4	0
0	0	4.18	10	0.994	4	0
0	0	4.36	10	0.994	4	0
0	0	4.50	10	0.994	0	4
0	0	4.61	10	0.994	0	4
0	0	4.71	10	0.994	0	4
0	0	4.92	10	0.994	0	4
0	0.	5.12	10	0.994	0	4
0	0	5.59	10	0.994	0	4
0	0	6.08	10	0.994	0	4
0	0	3.89	20	0.994	4	0
0	0	4.18	20	0.994	4	0
0	0	4.36	20	0.994	0	4
0	0	4.50	20	0.994	0	4
0	0	4.61	20	0.994	0	4
0	0	4.71	20	0.994	0	4
0	0	4.92	20	0.994	0	4
0	0	5.12	20	0.994	0	4
0	0	5.59	20	0.994	0	4
0	0	6.08	20	0.994	0	4
0	0	3.89	30	0.994	4	0
0	0	4.18	30	0.994	4	0
0	0	4.36	30	0.994	4	0
0	0	4.50	30	0.994	0	4
0	0	4.61	30	0.994	0	4
0	0	4.71	30	0.994	0	4
0	0	4.92	30	0.994	0	4
0	0	5.12	30	0.994	0	4
0	0	5.59	30	0.994	0	4
0	0	6.08	30	0.994	0	4
10	5.74	3.73	4	0.994	4	0
10	4.88	3.88	4	0.994	4	0
10	3.49	4.13	4	0.994	4	0

TLA	[UD]	pH	T	a_w	NG	G
10	2.53	4.33	4	0.994	4	0
10	1.54	4.60	4	0.994	2	0
10	1.12	4.76	4	0.994	4	0
10	0.84	4.90	4	0.994	1	0
10	0.23	5.48	4	0.994	0	4
10	0.07	5.99	4	0.994	0	4
10	5.74	3.73	10	0.994	4	0
10	4.88	3.88	10	0.994	4	0
10	3.49	4.13	10	0.994	4	0
10	2.53	4.33	10	0.994	4	0
10	2.16	4.42	10	0.994	1	0
10	1.54	4.60	10	0.994	0	4
10	1.12	4.76	10	0.994	0	4
10	0.84	4.90	10	0.994	0	4
10	0.23	5.48	10	0.994	0	4
10	0.07	5.99	10	0.994	0	4
10	5.74	3.73	20	0.994	4	0
10	4.88	3.88	20	0.994	4	0
10	3.49	4.13	20	0.994	4 -	0
10	2.53	4.33	20	0.994	0	4
10	2.16	4.42	20	0.994	0	4
10	1.54	4.60	20	0.994	0	4
10	1.12	4.76	20	0.994	0	4
10	0.84	4.90	20	0.994	0	4
10	0.23	5.48	20	0.994	0	4
10	0.07	5.99	20	0.994	0	4
10	5.74	3.73	30	0.994	4	0
10	4.88	3.88	30	0.994	4	0
10	3.49	4.13	30	0.994	4	0
10	2.53	4.33	30	0.994	4	0
10	2.16	4.42	30	0.994	4	0
10	1.54	4.60	30	0.994	0	4
10	1.12	4.76	30	0.994	0	4
10	0.84	4.90	30	0.994	0	4
10	0.23	5.48	30	0.994	0	4
10	0.07	5.99	30	0.994	0	4
20	7.63	4.07	4	0.993	4	0
20	5.69	4.26	4	0.993	4	0
20	4.98	4.34	4	0.993	4	0
20	4.09	4.45	4	0.993	4	0
20	3.39	4.55	4	0.993	4	0
20	2.43	4.72	4	0.993	4	0
20	1.35	5.00	4	0.993	4	0

Table G.5 (contd.) *L. monocytogenes* Scott A data set for probability model (Eqn. 5.1).

TLA	[UD]	pH	T	a_w	NG	G
20	0.72	5.29	4	0.993	4	0
20	0.43	5.52	4	0.993	0	4
20	0.19	5.88	4	0.993	0	4
20	7.63	4.07	10	0.993	4	0
20	5.69	4.26	10	0.993	4	0
20	4.98	4.34	10	0.993	4	0
20	4.09	4.45	10	0.993	4	0
20	3.39	4.55	10	0.993	4	0
20	2.43	4.72	10	0.993	0	4
20	1.35	5.00	10	0.993	0	4
20	0.72	5.29	10	0.993	0	4
20	0.43	5.52	10	0.993	0	4
20	0.19	5.88	10	0.993	0	4
20	7.63	4.07	20	0.993	3	0
20	5.69	4.26	20	0.993	3	0
20	4.09	4.45	20	0.993	1	0
20	3.39	4.55	20	0.993	0	4
20	2.43	4.72	20	0.993	0	4
20	1.35	5.00	20	0.993	0	4
20	0.72	5.29	20	0.993	0	4
20	0.43	5.52	20	0.993	0	4
20	0.19	5.88	20	0.993	0	4
20	7.63	4.07	30	0.993	4	0
20	5.69	4.26	30	0.993	4	0
20	4.98	4.34	30	0.993	4	0
20	4.09	4.45	30	0.993	4	0
20	3.39	4.55	30	0.993	4	0
20	2.43	4.72	30	0.993	0	4
20	1.35	5.00	30	0.993	0	4
20	0.72	5.29	30	0.993	0	4
20	0.43	5.52	30	0.993	0	4
20	0.19	5.88	30	0.993	0	4
30	10.96	4.10	4	0.992	4	0
30	6.25	4.44	4	0.992	4	0
30	4.36	4.63	4	0.992	4	0
30	2.78	4.85	4	0.992	4	0
30	2.21	4.96	4	0.992	4	0
30	1.86	5.04	4	0.992	4	0
30	1.20	5.24	4	0.992	4	0
30	0.86	5.39	4	0.992	4	0
30	0.63	5.53	4	0.992	4	0
30	0.26	5.92	4	0.992	0	4
30	10.96	4.10	10	0.992	4	0
30	6.25	4.44	10	0.992	4	0
30	4.36	4.63	10	0.992	4	0
30	2.78	4.85	10	0.992	4	0
30	2.21	4.96	10	0.992	4	0
30	1.86	5.04	10	0.992	0	4
30	1.20	5.24	10	0.992	0	4
30	0.86	5.39	10	0.992	0	4
30	0.63	5.53	10	0.992	0	4
30	0.26	5.92	10	0.992	0	4
30	10.96	4.10	20	0.992	4	0
30	6.25	4.44	20	0.992	4	0

TLA	[UD]	pH	T	a_w	NG	G
30	4.36	4.63	20	0.992	4	0
30	2.78	4.85	20	0.992	0	4
30	2.21	4.96	20	0.992	0	4
30	1.86	5.04	20	0.992	0	4
30	1.20	5.24	20	0.992	0	4
30	0.86	5.39	20	0.992	0	4
30	0.63	5.53	20	0.992	0	4
30	0.26	5.92	20	0.992	0	4
30	10.96	4.10	30	0.992	4	0
30	6.25	4.44	30	0.992	4	0
30	4.36	4.63	30	0.992	4	0
30	2.78	4.85	30	0.992	4	0
30	2.21	4.96	30	0.992	0	4
30	1.86	5.04	30	0.992	0	4
30	1.20	5.24	30	0.992	0	4
30	0.86	5.39	30	0.992	0	4
30	0.63	5.53	30	0.992	0	4
30	0.26	5.92	30	0.992	0	4
50	16.18	4.18	4	0.993	4	0
50	10.22	4.45	4	0.993	4	0
50	7.40	4.62	4	0.993	4	0
50	4.74	4.84	4	0.993	4	0
50	4.01	4.92	4	0.993	4	0
50	3.03	5.05	4	0.993	4	0
50	1.83	5.28	4	0.993	4	0
50	1.12	5.50	4	0.993	4	0
50	0.38	5.98	4	0.993	0	4
50	0.12	6.48	4	0.993	0	4
50	16.18	4.18	10	0.993	4	0
50	10.22	4.45	10	0.993	4	0
50	7.40	4.62	10	0.993	4	0
50	4.74	4.84	10	0.993	4	0
50	4.01	4.92	10	0.993	4	0
50	3.03	5.05	10	0.993	0	4
50	1.83	5.28	10	0.993	0	4
50	1.12	5.50	10	0.993	0	4
50	0.38	5.98	10	0.993	0	4
50	0.12	6.48	10	0.993	0	4
50	16.18	4.18	20	0.993	4	0
50	10.22	4.45	20	0.993	4	0
50	7.40	4.62	20	0.993	4	0
50	4.74	4.84	20	0.993	0	4
50	4.01	4.92	20	0.993	0	4
50	3.03	5.05	20	0.993	0	4
50	1.83	5.28	20	0.993	0	4
50	1.12	5.50	20	0.993	0	4
50	0.38	5.98	20	0.993	0	4
50	0.12	6.48	20	0.993	0	4
50	16.18	4.18	30	0.993	4	0
50	10.22	4.45	30	0.993	4	0
50	7.40	4.62	30	0.993	4	0
50	4.74	4.84	30	0.993	4	0
50	4.01	4.92	30	0.993	4	0
50	3.03	5.05	30	0.993	0	4

Table G.5 (contd.) *L. monocytogenes* Scott A data set for probability model (Eqn. 5.1).

TLA	[UD]	pH	T	a_w	NG	G		TLA	[UD]	pH	T	a_w	NG	G
50	1.83	5.28	30	0.993	0	4		0	0	5.33	30	0.940	0	4
50	1 12	5.50	30	0.993	0	4		0	0	5.04	30	0.929	4	0
50	0 38	5.98	30	0.993	0	4		0	0	5.13	30	0.929	4	0
50	0.12	6.48	30	0.993	0	4		0	0	5.21	30	0.929	0	4
0	0	4.40	20	0.965	4	0		0	0	5.31	30	0.929	0	4
0	0	4.54	20	0.965	0	4		0	0	5.41	30	0.929	0	4
0	0	4.62	20	0.965	0	4		0	0	5.53	30	0.929	0	4
0	0	4.73	20	0.965	0	4		0	0	5.61	30	0.929	0	4
0	0	4.85	20	0.965	0	4		20	2.4	4.72	20	0.955	2	0
0	0	4.94	20	0.965	0	4		20	2.1	4.78	20	0.955	2	0
0	0	5.05	20	0.965	0	4		20	1.5	4.95	20	0.955	0	4
0	0	5.18	20	0.965	0	4		20	1.2	5.04	20	0.955	0	4
0	0	4.61	20	0.954	3	0		20	1.0	5.14	20	0.955	0	4
0	0	4.83	20	0.954	0	4		20	0.8	5.25	20	0.955	0	4
0	0	4.93	20	0.954	0	4		20	0.6	5.38	20	0.955	0	4
0	0	4.99	20	0.954	0	4		20	2.6	4.69	20	0.941	3	0
0	0	5.07	20	0.954	0	4		20	2.0	4.82	20	0.941	4	0
0	0	5.19	20	0.954	0	4		20	1.6	4.92	20	0.941	0	4
0	0	5.38	20	0.954	0	4		20	1.4	5.00	20	0.941	0	4
0	0	4.74	20	0.940	4	0		20	1.1	5.10	20	0.941	0	4
0	0	4.83	20	0.940	1	3		20	0.8	5.23	20	0.941	0	4
0	0	4.91	20	0.940	0	4		20	0.6	5.39	20	0.941	0	4
0	0	5.01	20	0.940	0	4		20	1.8	4.87	20	0.927	4	0
0	0	5.14	20	0.940	0	4		20	1.2	5.04	20	0.927	4	0
0	0	5.23	20	0.940	0	4		20	1.0	5.12	20	0.927	4	0
0	0	5.33	20	0.940	0	4		20	0.9	5.20	20	0.927	0	4
0	0	5.04	20	0.929	0	4		20	0.7	5.32	20	0.927	0	4
0	0	5.13	20	0.929	0	4		20	0.4	5.54	20	0.927	0	4
0	0	5.21	20	0.929	0	4		20	0.3	5.64	20	0.927	0	4
0	0	5.31	20	0.929	0	4		20	2.4	4.72	30	0.955	2	0
0	0	5.41	20	0.929	0	4		20	1.5	4.95	30	0.955	0	4
0	0	5.53	20	0.929	0	4		20	1.2	5.04	30	0.955	0	4
0	0	5.61	20	0.929	0	4		20	1.0	5.14	30	0.955	0	4
0	0	4.40	30	0.965	4	0		20	0.8	5.25	30	0.955	0	4
0	0	4.54	30	0.965	0	4		20	0.6	5.38	30	0.955	0	4
0	0	4.62	30	0.965	0	4		20	2.6	4.69	30	0.941	4	0
0	0	4.73	30	0.965	0	4		20	2.0	4.82	30	0.941	4	0
0	0	4.85	30	0.965	0	4		20	1.6	4.92	30	0.941	0	4
0	0	4.94	30	0.965	0	4		20	1.4	5.00	30	0.941	0	4
0	0	5.05	30	0.965	0	4		20	1.1	5.10	30	0.941	0	4
0	0	5.18	30	0.965	0	4		20	0.8	5.23	30	0.941	0	4
0	0	4.61	30	0.954	4	0		20	0.6	5.39	30	0.941	0	4
0	0	4.83	30	0.954	0	4		20	1.8	4.87	30	0.927	4	0
0	0	4.93	30	0.954	0	4		20	1.2	5.04	30	0.927	4	0
0	0	4.99	30	0.954	0	4		20	1.0	5.12	30	0.927	4	0
0	0	5.07	30	0.954	0	4		20	0.9	5.20	30	0.927	4	0
0	0	5.19	30	0.954	0	4		20	0.7	5.32	30	0.927	1	3
0	0	5.38	30	0.954	0	4		20	0.4	5.54	30	0.927	0	4
0	0	4.74	30	0.940	4	0		20	0.3	5.64	30	0.927	0	4
0	0	4.83	30	0.940	4	0		50	4.8	4.83	20	0.955	4	0
0	0	4.91	30	0.940	0	4		50	4.1	4.91	20	0.955	0	4
0	0	5.01	30	0.940	0	4		50	3.3	5.01	20	0.955	0	4
0	0	5.14	30	0.940	0	4		50	2.6	5.12	20	0.955	0	4
0	0	5.23	30	0.940	0	4		50	1.9	5.26	20	0.955	0	4

Table G.5 (contd.) *L. monocytogenes* Scott A data set for probability model (Eqn. 5.1).

TLA	[UD]	pH	T	a_w	NG	G
50	1.6	5.33	20	0.955	0	4
50	1.1	5.51	20	0.955	0	4
50	3.7	4.96	20	0.939	4	0
50	3.0	5.06	20	0.939	0	4
50	2.5	5.14	20	0.939	0	4
50	2.0	5.25	20	0.939	0	4
50	1.6	5.35	20	0.939	0	4
50	1.3	5.44	20	0.939	0	4
50	0.9	5.58	20	0.939	0	4
50	2.5	5.13	20	0.927	2	0
50	1.9	5.27	20	0.927	2	0
50	1.5	5.37	20	0.927	4	0
50	1.2	5.47	20	0.927	0	4
50	0.9	5.58	20	0.927	0	4
50	0.7	5.69	20	0.927	0	4
50	0.5	5.84	20	0.927	0	4
50	4.8	4.83	30	0.955	4	0
50	4.1	4.91	30	0.955	0	4
50	3.3	5.01	30	0.955	0	4
50	2.6	5.12	30	0.955	0	4
50	1.9	5.26	30	0.955	0	4
50	1.6	5.33	30	0.955	0	4
50	1.1	5.51	30	0.955	0	4
50	3.7	4.96	30	0.939	4	0
50	3.0	5.06	30	0.939	4	0
50	2.5	5.14	30	0.939	1	3
50	2.0	5.25	30	0.939	0	4
50	1.6	5.35	30	0.939	0	4
50	1.3	5.44	30	0.939	0	4
50	0.9	5.58	30	0.939	0	4
50	2.5	5.13	30	0.927	4	0
50	1.9	5.27	30	0.927	4	0
50	1.5	5.37	30	0.927	4	0
50	1.2	5.47	30	0.927	4	0
50	0.9	5.58	30	0.927	1	3
50	0.7	5.69	30	0.927	0	4
50	0.5	5.84	30	0.927	0	4
0	0	4.39	6	0.992	4	0
0	0	4.51	-6	0.992	4	0
0	0	4.61	6	0.992	4	0
0	0	4.72	6	0.992	4	0
0	0	4.84	6	0.992	4	0
0	0	4.98	6	0.992	0	4
0	0	5.09	6	0.992	0	4
0	0	5.28	6	0.992	0	4
0	0	5.46	6	0.992	0	4
0	0	4.28	8	0.992	4	0
0	0	4.39	8	0.992	4	0
0	0	4.51	8	0.992	4	0
0	0	4.61	8	0.992	4	0
0	0	4.72	8	0.992	0	4
0	0	4.84	8	0.992	0	4
0	0	4.03	20.7	0.995	1	0
0	0	4.14	20.7	0.995	1	0
20	4.32	4.42	21.5	0.967	1	0
20	3.02	4.61	21.5	0.967	1	0
20	2.43	4.72	21.5	0.967	1	0
20	1.74	4.88	21.6	0.967	1	0
50	5.4	4.78	21.6	0.968	1	0
50	4.3	4.89	21.6	0.968	1	0
50	3.2	5.03	21.7	0.968	1	0
100	8.7	4.88	22.6	0.965	1	0
100	7.1	4.98	22.5	0.965	1	0
100	5.2	5.12	22.5	0.965	1	0
100	4.6	5.18	22.4	0.965	1	0
200	6.1	5.36	22.6	0.962	1	0
200	4.7	5.48	22.5	0.962	1	0
50	1.4	5.41	19.9	0.937	1	0
50	1.4	5.39	19.9	0.930	1	0

Table G.6 *L. monocytogenes* L5 data set for probability model (Eqn. 5.2) including the 142 and 13 growth data from Tables G.3 and G.4 respectively, and 20 no growth data from those kinetic experiments.

TLA	[UD]	pH	T	a_w	NG	G		TLA	[UD]	pH	T	a_w	NG	G
0	0	3.93	4	0.994	4	0		10	3.1	4.21	10	0.994	4	0
0	0	4.17	-4	0.994	4	0		10	2.8	4.28	10	0.994	4	0
0	0	4.23	4	0.994	4	0		10	2.2	4.42	10	0.994	4	0
0	0	4.35	4	0.994	4	0		10	1.9	4.50	10	0.994	4	0
0	0	4.40	4	0.994	4	0		10	1.3	4.68	10	0.994	0	4
0	0	4.54	4	0.994	4	0		10	0.9	4.86	10	0.994	0	4
0	0	4.65	4	0.994	4	0		10	0.7	4.98	10	0.994	0	4
0	0	4.75	4	0.994	4	0		10	0.2	5.56	10	0.994	0	4
0	0	4.95	4	0.994	0	4		10	4.3	3.99	20	0.994	4	0
0	0	5.63	4	0.994	0	4		10	4.0	4.04	20	0.994	4	0
0	0	3.93	10	0.994	4	0		10	3.1	4.21	20	0.994	4	0
0	0	4.17	10	0.994	4	0		10	2.8	4.28	20	0.994	4	0
0	0	4.23	10	0.994	4	0		10	2.2	4.42	20	0.994	4	0
0	0	4.35	10	0.994	4	0		10	1.9	4.50	20	0.994	0	4
0	0	4.40	10	0.994	4	0		10	1.3	4.68	20	0.994	0	4
0	0	4.54	10	0.994	4	0		10	0.9	4.86	20	0.994	0	4
0	0	4.65	10	0.994	0	4		10	0.7	4.98	20	0.994	0	4
0	0	4.75	10	0.994	0	4		10	0.2	5.56	20	0.994	0	4
0	0	4.95	10	0.994	0	4		10	4.3	3.99	30	0.994	4	0
0	0	5.63	10	0.994	0	4		10	4.0	4.04	30	0.994	4	0
0	0	3.93	20	0.994	4	0		10	3.1	4.21	30	0.994	4	0
0	0	4.17	20	0.994	4	0		10	2.8	4.28	30	0.994	4	0
0	0	4.23	20	0.994	4	0		10	2.2	4.42	30	0.994	4	0
0	0	4.35	20	0.994	0	4		10	1.9	4.50	30	0.994	4	0
0	0	4.40	20	0.994	0	4		10	1.3	4.68	30	0.994	0	4
0	0	4.54	20	0.994	0	4		10	0.9	4.86	30	0.994	0	4
0	0	4.65	20	0.994	0	4		10	0.7	4.98	30	0.994	0	4
0	0	4.75	20	0.994	0	4		10	0.2	5.56	30	0.994	0	4
0	0	4.95	20	0.994	0	4		20	8.9	3.96	4	0.993	4	0
0	0	5.63	20	0.994	0	4		20	7.2	4.11	4	0.993	4	0
0	0	3.93	30	0.994	4	0		20	6.8	4.15	4	0.993	4	0
0	0	4.17	30	0.994	4	0		20	5.1	4.32	4	0.993	4	0
0	0	4.23	30	0.994	4	0		20	4.6	4.38	4	0.993	4	0
0	0	4.35	30	0.994	4	0		20	3.5	4.53	4	0.993	4	0
0	0	4.40	30	0.994	4	0		20	3.0	4.61	4	0.993	4	0
0	0	4.54	30	0.994	0	4		20	2.1	4.79	4	0.993	4	0
0	0	4.65	30	0.994	0	4		20	1.2	5.07	4	0.993	0	4
0	0	4.75	30	0.994	0	4		20	0.4	5.57	4	0.993	0	4
0	0	4.95	30	0.994	0	4		20	8.9	3.96	10	0.993	4	0
0	0	5.63	30	0.994	0	4		20	7.2	4.11	10	0.993	4	0
10	4.3	3.99	4	0.994	4	0		20	6.8	4.15	10	0.993	4	0
10	4.0	4.04	4	0.994	4	0		20	5.1	4.32	10	0.993	4	0
10	3.1	4.21	4	0.994	4	0		20	4.6	4.38	10	0.993	4	0
10	2.8	4.28	4	0.994	4	0		20	3.5	4.53	10	0.993	4	0
10	2.2	4.42	4	0.994	4	0		20	3.0	4.61	10	0.993	4	0
10	1.9	4.50	4	0.994	4	0		20	2.1	4.79	10	0.993	0	4
10	1.3	4.68	4	0.994	4	0		20	1.2	5.07	10	0.993	0	4
10	0.9	4.86	4	0.994	4	0		20	0.4	5.57	10	0.993	0	4
10	0.7	4.98	4	0.994	4	0		20	8.9	3.96	20	0.993	4	0
10	0.2	5.56	4	0.994	0	4		20	7.2	4.11	20	0.993	4	0
10	4.3	3.99	10	0.994	4	0		20	6.8	4.15	20	0.993	4	0
10	4.0	4.04	10	0.994	4	0		20	5.1	4.32	20	0.993	4	0

Table G.6 (contd.) *L. monocytogenes* L5 data set for probability model (Eqn. 5.2).

TLA	[UD]	pH	T	a_w	NG	G
20	4.6	4.38	20	0.993	4	0
20	3.5	4.53	20	0.993	4	0
20	3.0	4.61	20	0.993	0	4
20	2.1	4.79	20	0.993	0	4
20	1.2	5.07	20	0.993	0	4
20	0.4	5.57	20	0.993	0	4
20	8.9	3.96	30	0.993	4	0
20	7.2	4.11	30	0.993	4	0
20	6.8	4.15	30	0.993	4	0
20	5.1	4.32	30	0.993	4	0
20	4.6	4.38	30	0.993	4	0
20	3.5	4.53	30	0.993	4	0
20	3.0	4.61	30	0.993	4	0
20	2.1	4.79	30	0.993	0	4
20	1.2	5.07	30	0.993	0	4
20	0.4	5.57	30	0.993	0	4
30	10.2	4.15	4	0.992	4	0
30	7.6	4.33	4	0.992	4	0
30	5.8	4.48	4	0.992	4	0
30	5.1	4.55	4	0.992	4	0
30	4.2	4.65	4	0.992	4	0
30	3.5	4.74	4	0.992	4	0
30	2.7	4.87	4	0.992	4	0
30	2.1	4.99	4	0.992	4	0
30	1.8	5.05	4	0.992	4	0
30	0.6	5.56	4	0.992	0	4
30	10.2	4.15	10	0.992	4	0
30	7.6	4.33	10	0.992	4	0
30	5.8	4.48	10	0.992	4	0
30	5.1	4.55	10	0.992	4	0
30	4.2	4.65	10	0.992	4	0
30	3.5	4.74	10	0.992	4	0
30	2.7	4.87	10	0.992	4	0
30	2.1	4.99	10	0.992	0	4
30	1.8	5.05	10	0.992	0	4
30	0.6	5.56	10	0.992	0	4
30	10.2	4.15	20	0.992	4	0
30	7.6	4.33	20	0.992	4	0
30	5.8	4.48	20	0.992	4	0
30	5.1	4.55	20	0.992	4	0
30	4.2	4.65	20	0.992	4	0
30	3.5	4.74	20	0.992	0	4
30	2.7	4.87	20	0.992	0	4
30	2.1	4.99	20	0.992	0	4
30	1.8	5.05	20	0.992	0	4
30	0.6	5.56	20	0.992	0	4
30	10.2	4.15	30	0.992	4	0
30	7.6	4.33	30	0.992	4	0
30	5.8	4.48	30	0.992	4	0
30	5.1	4.55	30	0.992	4	0
30	4.2	4.65	30	0.992	4	0
30	3.5	4.74	30	0.992	4	0

TLA	[UD]	pH	T	a_w	NG	G
30	2.7	4.85	30	0.992	0	4
30	2.1	4.99	30	0.992	0	4
30	1.8	5.05	30	0.992	0	4
30	0.6	5.56	30	0.992	0	4
50	15.2	4.22	4	0.993	4	0
50	12.2	4.35	4	0.993	4	0
50	9.5	4.49	4	0.993	4	0
50	8.6	4.54	4	0.993	4	0
50	6.8	4.66	4	0.993	4	0
50	5.5	4.77	4	0.993	4	0
50	4.5	4.86	4	0.993	4	0
50	3.8	4.95	4	0.993	4	0
50	2.9	5.07	4	0.993	4	0
50	1.2	5.48	4	0.993	0	4
50	15.2	4.22	10	0.993	4	0
50	12.2	4.35	10	0.993	4	0
50	9.5	4.49	10	0.993	4	0
50	8.6	4.54	10	0.993	4	0
50	6.8	4.66	10	0.993	4	0
50	5.5	4.77	10	0.993	4	0
50	4.5	4.86	10	0.993	4	0
50	3.8	4.95	10	0.993	4	0
50	2.9	5.07	10	0.993	0	4
50	1.2	5.48	10	0.993	0	4
50	15.2	4.22	20	0.993	4	0
50	12.2	4.35	20	0.993	4	0
50	9.5	4.49	20	0.993	4	0
50	8.6	4.54	20	0.993	4	0
50	6.8	4.66	20	0.993	4	0
50	5.5	4.77	20	0.993	4	0
50	4.5	4.86	20	0.993	0	4
50	3.8	4.95	20	0.993	0	4
50	2.9	5.07	20	0.993	0	4
50	1.2	5.48	20	0.993	0	4
50	15.2	4.22	30	0.993	4	0
50	12.2	4.35	30	0.993	4	0
50	9.5	4.49	30	0.993	4	0
50	8.6	4.54	30	0.993	4	0
50	6.8	4.66	30	0.993	4	0
50	5.5	4.77	30	0.993	4	0
50	4.5	4.85	30	0.993	4	0
50	3.8	4.95	30	0.993	0	4
50	2.9	5.07	30	0.993	0	4
50	1.2	5.48	30	0.993	0	4
0	0	4.51	6	0.991	4	0
0	0	4.63	6	0.991	4	0
0	0	4.74	6	0.991	4	0
0	0	4.84	6	0.991	0	4
0	0	4.96	6	0.991	0	4
0	0	5.05	6	0.991	0	4
0	0	5.18	6	0.991	0	4
0	0	5.27	6	0.991	0	4

Table G.6 (contd.) *L. monocytogenes* L5 data set for probability model (Eqn. 5.2).

TLA	[UD]	pH	T	a_w	NG	G
0	0	5.47	6	0.991	0	4
0	0	4.28	8	0.991	4	0
0	0	4.51	8	0.991	4	0
0	0	4.63	8	0.991	0	4
0	0	4.74	8	0.991	0	4
0	0	4.84	8	0.991	0	4
0	0	5.05	8	0.991	0	4
0	0	4.28	30	0.991	4	0
0	0	4.40	30	0.991	4	0
0	0	4.51	30	0.991	0	4
0	0	4.63	30	0.991	0	4
0	0	4.74	30	0.991	0	4
0	0	4.84	30	0.991	0	4
0	0	4.30	20	0.954	2	0
0	0	4.41	20	0.954	3	0
0	0	4.50	20	0.954	2	0
0	0	4.59	20	0.954	4	0
0	0	4.69	20	0.954	0	4
0	0	4.80	20	0.954	0	4
0	0	4.90	20	0.954	0	4
0	0	5.00	20	0.954	0	4
0	0	5.10	20	0.954	0	4
0	0	5.33	20	0.954	0	4
0	0	4.72	20	0.942	4	0
0	0	4.81	20	0.942	1	3
0	0	4.89	20	0.942	0	4
0	0	5.00	20	0.942	0	4
0	0	5.13	20	0.942	0	4
0	0	5.22	20	0.942	0	4
0	0	5.31	20	0.942	0	4
0	0	5.04	20	0.929	4	0
0	0	5.13	20	0.929	0	4
0	0	5.22	20	0.929	0	4
0	0	5.33	20	0.929	0	4
0	0	5.42	20	0.929	0	4
0	0	5.54	20	0.929	0	4
0	0	4.30	30	0.954	4	0
0	0	4.41	30	0.954	4	0
0	0	4.50	30	0.954	4	0
0	0	4.59	30	0.954	4	0
0	0	4.69	30	0.954	0	4
0	0	4.80	30	0.954	0	4
0	0	4.90	30	0.954	0	4
0	0	5.00	30	0.954	0	4
0	0	5.10	30	0.954	0	4
0	0	5.33	30	0.954	0	4
0	0	4.72	30	0.942	4	0
0	0	4.81	30	0.942	4	0
0	0	4.89	30	0.942	0	4
0	0	5.00	30	0.942	0	4
0	0	5.13	30	0.942	0	4
0	0	5.22	30	0.942	0	4

TLA	[UD]	pH	T	a_w	NG	G
0	0	5.31	30	0.942	0	4
0	0	5.04	30	0.929	4	0
0	0	5.13	30	0.929	4	0
0	0	5.22	30	0.929	4	0
0	0	5.33	30	0.929	0	4
0	0	5.42	30	0.929	0	4
0	0	5.54	30	0.929	0	4
20	2.4	4.72	20	0.955	2	0
20	2.1	4.78	20	0.955	2	0
20	1.5	4.95	20	0.955	0	4
20	1.3	5.03	20	0.955	0	4
20	1.0	5.13	20	0.955	0	4
20	0.8	5.23	20	0.955	0	4
20	0.6	5.39	20	0.955	0	4
20	2.6	4.69	20	0.942	4	0
20	2.2	4.76	20	0.942	4	0
20	1.6	4.91	20	0.942	0	4
20	1.4	5.00	20	0.942	0	4
20	1.1	5.09	20	0.942	0	4
20	0.8	5.23	20	0.942	0	4
20	0.6	5.39	20	0.942	0	4
20	1.7	4.88	20	0.928	4	0
20	1.3	5.03	20	0.928	3	0
20	1.0	5.13	20	0.928	3	0
20	0.9	5.20	20	0.928	2	2
20	0.7	5.33	20	0.928	0	4
20	0.4	5.54	20	0.928	0	4
20	0.3	5.65	20	0.928	0	4
50	4.6	4.85	20	0.955	4	0
50	3.8	4.95	20	0.955	0	4
50	3.2	5.03	20	0.955	0	4
50	2.4	5.15	20	0.955	0	4
50	2.0	5.24	20	0.955	0	4
50	1.7	5.31	20	0.955	0	4
50	1.1	5.51	20	0.955	0	4
50	3.7	4.96	20	0.941	3	0
50	3.0	5.06	20	0.941	4	0
50	2.4	5.15	20	0.941	1	3
50	2.0	5.25	20	0.941	0	4
50	1.6	5.35	20	0.941	0	4
50	1.3	5.44	20	0.941	0	4
50	0.9	5.58	20	0.941	0	4
50	2.5	5.13	20	0.928	2	0
50	1.8	5.28	20	0.928	2	0
50	1.5	5.37	20	0.928	4	0
50	1.2	5.47	20	0.928	0	4
50	1.0	5.57	20	0.928	0	4
50	0.7	5.70	20	0.928	0	4
50	0.5	5.85	20	0.928	0	4
20	2.4	4.72	30	0.955	2	0
20	2.1	4.78	30	0.955	2	0
20	1.5	4.95	30	0.955	0	4

Table G.6 (contd.) *L. monocytogenes* L5 data set for probability model (Eqn. 5.2).

TLA	[UD]	pH	T	a_w	NG	G
20	1.3	5.03	30	0.955	0	4
20	1.0	5.13	30	0.955	0	4
20	0.8	5.23	30	0.955	0	4
20	0.6	5.39	30	0.955	0	4
20	2.6	4.69	30	0.942	4	0
20	2.2	4.76	30	0.942	4	0
20	1.6	4.91	30	0.942	1	3
20	1.4	5.00	30	0.942	0	4
20	1.1	5.09	30	0.942	0	4
20	0.8	5.23	30	0.942	0	4
20	0.6	5.39	30	0.942	0	4
20	1.7	4.88	30	0.928	4	0
20	1.3	5.03	30	0.928	4	0
20	1.0	5.13	30	0.928	4	0
20	0.9	5.20	30	0.928	4	0
20	0.7	5.33	30	0.928	4	0
20	0.4	5.54	30	0.928	0	4
20	0.3	5.65	30	0.928	0	4
50	4.6	4.85	30	0.955	4	0
50	3.8	4.95	30	0.955	3	1
50	3.2	5.03	30	0.955	0	4
50	2.4	5.15	30	0.955	0	4
50	2.0	5.24	30	0.955	0	4
50	1.7	5.31	30	0.955	0	4
50	1.1	5.51	30	0.955	0	4
50	3.7	4.96	30	0.941	4	0
50	3.0	5.06	30	0.941	4	0
50	2.4	5.15	30	0.941	3	1
50	2.0	5.25	30	0.941	1	3
50	1.6	5.35	30	0.941	0	4
50	1.3	5.44	30	0.941	0	4
50	0.9	5.58	30	0.941	0	4
50	2.5	5.13	30	0.928	4	0
50	1.8	5.28	30	0.928	4	0
50	1.5	5.37	30	0.928	4	0
50	1.2	5.47	30	0.928	4	0
50	1.0	5.57	30	0.928	4	0

| TLA	[UD]	pH	T	a_w	NG	G
50	0.7	5.70	30	0.928	4	0
50	0.5	5.85	30	0.928	0	4
20	3.0	4.61	19.3	0.969	1	0
20	2.6	4.68	19.5	0.969	1	0
20	2.6	4.81	19.5	0.969	1	0
50	5.5	4.77	19.1	0.969	1	0
50	4.7	4.84	19.1	0.969	1	0
100	8.0	4.92	21.7	0.966	1	0
100	6.6	5.01	21.7	0.966	1	0
100	5.1	5.13	21.7	0.966	1˙	0
200	9.3	5.17	21.7	0.964	1	0
200	7.2	5.29	21.6	0.964	1	0
200	6.1	5.36	21.6	0.964	1	0
200	4.9	5.46	21.6	0.964	1	0
50	1.6	5.33	20.0	0.941	1	0
50	1.7	5.31	20.0	0.936	1	0
0	0	4.04	20.7	0.995	1	0
0	0	4.14	20.7	0.995	1	0
450	10.5	5.48	21.1	0.962	1	0
450	7.7	5.62	21.2	0.962	1	0
450	6.4	5.70	21.2	0.962	1	0
450	5.7	5.75	21.2	0.962	1	0
200	1.5	5.98	20.0	0.962	0	1
250	1.9	5.98	20.0	0.960	0	1
300	2.3	5.97	20.0	0.959	0	1
350	2.6	5.98	20.0	0.958	0	1
400	3.2	5.96	20.0	0.958	0	1
0	0	6.02	5	0.967	0	1
50	0.4	6.01	5	0.965	0	1
100	0.6	6.06	5	0.962	0	1
150	1.0	6.02	5	0.962	0	1
200	1.4	6.01	5	0.962	0	1
250	1.9	5.98	5	0.960	0	1
300	2.3	5.98	5	0.959	0	1
350	2.7	5.97	5	0.958	1	0
400	3.2	5.95	5	0.958	1	0
500	4.3	5.92	5	0.955	1	0
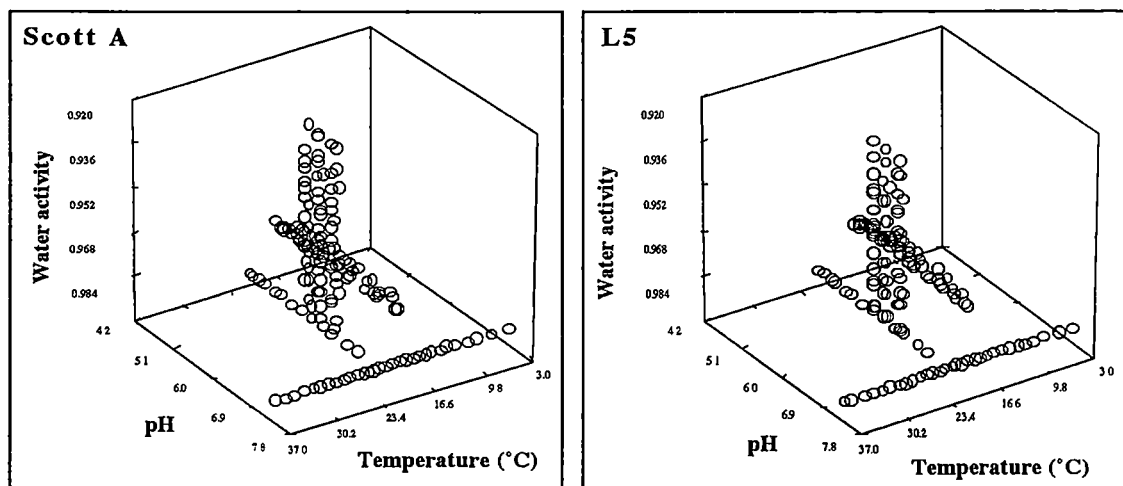
Figure G.1 Diagrammatic representation of variables combinations tested in the kinetic models generation for *L. monocytogenes* Scott A (Eqn. 4.17a) and L5 (Eqn. 4.18a). Note that lactic acid was tested at the levels of 0, 20, 50, 100, and 200 mM for both strains with an additional of 450 mM for strain L5. For data refer to Tables G.1-2 and G.3-4 respectively.
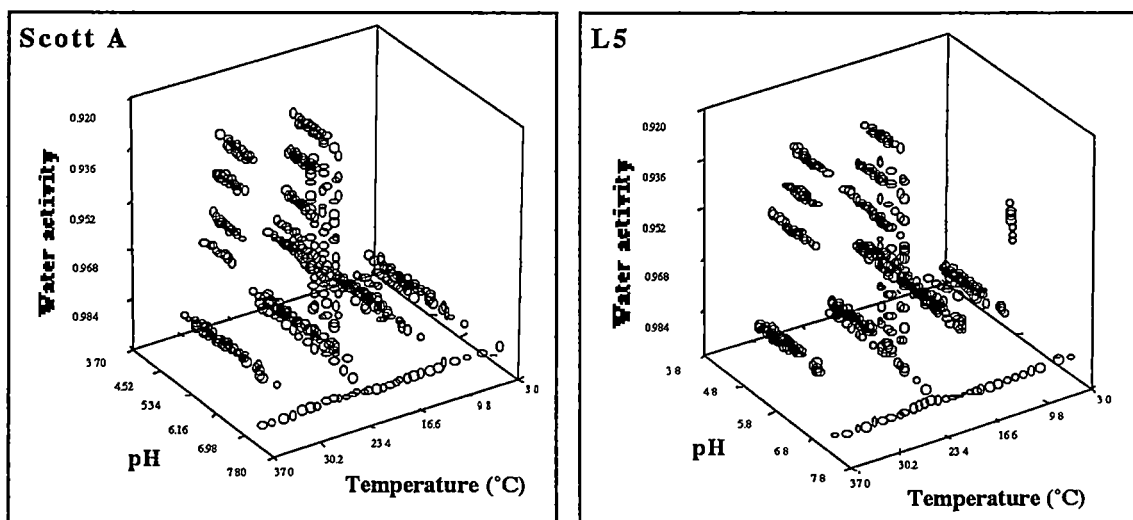

Figure G.2 Diagrammatic representation of variables combinations tested in the growth/no growth interface models generation for *L. monocytogenes* Scott A (Eqn. 5.1) and L5 (Eqn. 5.2). Note that lactic acid was tested at the levels of 0, 10, 20, 30, and 50 mM. For data refer to Tables G.5 and G.6 respectively.