Physiology and Modelling of *Escherichia coli* Growth Inhibition due to pH, Organic Acids Temperature and Water Activity.

by

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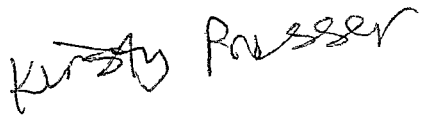

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Abstract

The inhibition of bacterial growth in foods is necessary to prevent food spoilage and food poisoning due to the presence of pathogenic bacteria. Organic acids are inhibitory to bacteria and are present in many foods. In some low pH foods they are present in sufficient concentration to prevent the growth of bacterial pathogens while in others their concentrations merely inhibit their growth rate. Organic acids have yet to be satisfactorily modelled in a way that is simple to understand, covers a range of concentrations and yields meaningful estimates of the concentrations of acids that limit growth. This study aims to describe simply and specifically the inhibition of bacterial growth caused by organic acids in such a way that helps to elucidate the biochemical and metabolic mechanisms of inhibition at a cellular level.

To achieve this aim in Chapter 2 a series of models is developed which describe organic acid inhibition of growth rate of various *Escherichia coli* strains. These models are fitted to datasets that contain data for pH alone, or pH and lactic acid, or finally for pH and acetic acid. The pH responses of both pathogenic and non pathogenic strains were determined and modelled. The square root type pH models were based on the following hypotheses : that growth rate is proportional to the concentration of hydrogen ions, that pH inhibition is separate to organic acid inhibition and that growth rate is also proportional to the concentration of undissociated organic acid and to the concentration of dissociated organic acid. Therefore organic acid inhibition was modelled using terms adapted from the Henderson-Hasselbalch equation which gives the concentration of each form of the acid. The original model type was adapted to describe the individual datasets better. For the lactic acid model it was found that the addition of a term for inhibition due to high pH significantly improved the fit of the model. New terms were developed that better described the inhibition by dissociated acetic acid and for pH inhibition of different pathogenic and nonpathogenic strains. The consequences of these changes and whether these models still support the hypotheses is discussed. However these models fulfil the aim of providing a good mathematical description of growth rate inhibition as shown by the data under the conditions tested.

In Chapter 3 the modelling of organic acid growth inhibition of *Escherichia coli* is extended to describe the boundary between those conditions under which growth is possible and those conditions under which growth is not possible. The simplification of the required information to a binary result (growth/no growth) for each observation allows collection of a larger number of datapoints and hence a much wider range of environmental conditions to be examined. The models are fitted to datasets that contain data for a wide range of pH, water activity, temperature and lactic acid conditions. These models are based on the hypotheses described for Chapter 2. The new hypothesis was that the growth rate model's mathematical equation can be adapted to calculate the probability of growth. The success of the adaptation of the model, and the principles developed for the generation of the type of data necessary to create better models are discussed. These models fulfil the aim of providing a good mathematical description of growth rate inhibition under the severely limiting conditions at the growth/no growth boundary.

In Chapter 4 a method to elucidate the underlying biochemistry and mechanisms of organic acid inhibition is described. The mechanism of inhibitory action of organic acids has been described as the lowering of the internal pH of bacteria due to the permeation of undissociated organic acid ions. Alternatively specific effects of organic acids on cell metabolism, cell membrane transport and other cell functions could be responsible for the inhibitory effect of organic acids on bacteria. Intracellular pH measurement using the fluorescent probe 5 (and 6-) -carboxyfluorescein succinimidyl ester was trialled. This technique could determine the relationship of intracellular pH to variation in pH and organic acid conditions for *E.coli*. The previous technique developed for Gram positive organisms was found to be ineffective for labelling the cells, so new adaptations of techniques for the transitory permeabilisation of *E.coli* to allow labelling were studied. Under particular sets of conditions the cells were found to take up label but then they did not respond to the additions of the assay protocol. This implies the bacteria were unable to regulate their intracellular pH. Further experimentation with many treatment variations were not effective in producing labelled physiologically active *E.coli*. This technique does not appear to be readily applicable to the measurement of intracellular pH of Gram-negative organisms and so cannot be used to explore the physiology of pH stress. However, greater knowledge of the physiology of bacteria would enable a better understanding of the basis of modelled responses and could lead to the development of new ways of controlling bacterial growth.

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Table of Abbreviations

[AAC]	total concentration of acetic acid
A.S.E	Asymptotic Standard Error
a.u.	arbitrary units
a_w	water activity
a_{wmin}	notional minimum water activity for growth
BCECF	biscarboxyethyl-carboxy-fluorescein
cFSE	5 (&6) carboxyfluorescein succinimidyl ester
cFDASE	5 (&6) carboxyfluorescein succinimidyl diacetate ester
cfu	colony forming unit
[D]	concentration of dissociated acid
D_{min}	minimum dissociated acid concentration that prevents growth
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
GRAS	generally regarded as safe
GT	generation time
k	growth rate (defined as 1/generation time in minutes)
$\sqrt{k}$	square root of growth rate (as defined above)
[LAC]	total concentration of lactic acid
O.D.	optical density
pH_i	intracellular pH
pH_{min}	notional minimum pH for growth
pK_a	dissociation constant for organic acids
$\sqrt{M.S.E.}$	Root Mean Square Error
S.E.	Standard Error
T	temperature
T.G.I.	temperature gradient incubator
T_{min}	notional minimum temperature for growth
T_{max}	notional maximum temperature for growth
[TAC]	total concentration of an organic acid
%T	percent transmittance (measure of optical density of broth culture)
[U]	concentration of undissociated acid
U_{min}	minimum undissociated acid concentration that prevents growth

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Papers Produced in Part from this Thesis

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1 INTRODUCTION

1 Introduction

1.1 Food Poisoning

Food poisoning has occurred throughout history. Many food preparation techniques such as fermentation, salting, cooking and chilling were developed, at least in part, to kill or control the growth of pathogenic bacteria as well as those that cause spoilage. Despite recent advances in sanitation and increases in food hygiene standards foodborne diseases are still an increasing problem in the industrialised world (Maurice, 1994; Altekruse and Swerdlow, 1996; Kaferstein *et al.*, 1997).

Specific modern lifestyle factors contribute to the continuing prevalence of food poisoning (Collins, 1997). These include the consumption of more meals prepared outside the home, larger retail supply and distribution chains, the scaling up of food production in factories and a lower level of understanding of hygiene issues in the community. Also there are an increasing number of individuals in the population who are at greater risk of disease due to being elderly or immunocompromised (Morris and Potter, 1997).

One of the most important factors in the increase of foodborne disease is the emergence of new and more virulent pathogens. Novel pathogens have emerged which have enhanced virulence through acquisition of new virulence factors or combinations of factors acquired from other bacteria. New pathogens can result from physiological adaptations such as psychrotrophic strains able to grow under refrigeration or thermophilic strains better able to survive cooking. Emerging pathogens include *Campylobacter* spp., *Listeria monocytogenes*, *Salmonella* spp., *Vibrio* spp., *Yersinia enterocolitica* and a number of *Escherichia coli* strains (Doyle, 1994; Tauxe, 1997).

The cost of foodborne disease is great. Food trade between countries can be adversely affected by contamination with foodborne pathogens (Kaferstein *et al.*, 1997). Due to the widespread nature of low level disease, accurate determination of the amount of foodborne illness is difficult as it is often not reported to a doctor. Even less frequently are samples taken and a positive identification made of the causative agent. However, with new pathogens, the consequences of illness can be much more severe (Lindsay, 1997). Vulnerable individuals may be killed by foodborne pathogens. There is increasing consumer pressure for fresh healthy foods with less preservatives and additives (Zink, 1997). Many foods have come to depend on high levels of preparation hygiene, minimal processing (Manvell, 1997) and refrigeration for their microbial safety. In order to safeguard the consumer while meeting consumer demands, more knowledge of new foodborne organisms is required in order to prevent food poisoning.

1.2 *E. coli* : History of a Pathogen's Emergence

E. coli is a Gram negative, mesophilic, asporogenic straight rod that may be petrichously flagellated or nonmotile, from the family Enterobacteriaceae (Orskov, 1984; Doyle and Padhye, 1989). It is an important component of the facultatively anaerobic biota in the intestine of warm-blooded animals (Pelczar *et al.*, 1993). It has been used as an indicator of faecal contamination (Ashbolt and Veal, 1994) and, as a highly studied microorganism, is frequently used as a tool in genetic engineering (Maniatis *et al.*, 1982), biotechnology (Walker and Gingold, 1988) and molecular biology (Ausubel *et al.*, 1998). The following section will describe this emerging pathogen and the various factors, microbiological, medical and environmental, which combine to make *E. coli* infection a difficult and continuing problem.

1.2.1 Types of pathogenic *E. coli*

Until recently, *E. coli* was primarily known to medicine as a cause of urinary tract infections (Jawetz *et al.*, 1968). Some serotypes had been linked with infantile diarrhoea outbreaks though it was not regarded as an important pathogen. Pathogenic *E. coli* were associated with poor hygiene and diarrhoea in developing countries and mainly occurred in children (Robins-Browne, 1990). During the last two decades, strains of *E. coli* have emerged as a cause of major foodborne disease outbreaks. The several types of pathogenic *E. coli* are defined by the differences in the mechanisms of pathogenicity and consequently types of disease caused (Table 1.1). Of these new pathogenic strains EHEC (Table 1.1) are considered the most important as they can cause serious even fatal illness often requiring hospitalisation.

Table 1.1 Pathogenic *Escherichia coli* as defined by Doyle *et al.* (1997)

Name of Type	Mechanisms of Pathogenicity	Type of Disease
Enteropathogenic *E. coli* (EPEC)	no toxins produced mechanisms unclear	diarrhoea in children
Enterotoxigenic *E. coli* (ETEC)	heat labile or heat stable enterotoxins	traveller's diarrhoea, diarrhoea in children
Enteroinvasive *E. coli* (EIEC)	cellular invasion & tissue destruction	bloody diarrhoea, poor hygiene
Diffuse-adhering *E. coli* (DAEC)	diffuse adherence to cells no toxins or invasion	diarrhoea in children
Enteroaggregative *E. coli* (EAggEC)	aggregative adherence to endothelial cells	diarrhoea in children
Enterohaemorrhagic *E. coli* (EHEC)	haemolysin, attachment, two toxins (Shiga-like/Verotoxins)	haemorrhagic colitis, haemolytic uraemic syndrome, thrombotic thrombocytopenic purpura

1.2.2. Syndromes of EHEC Infection

E. coli O157:H7 is the most commonly studied organism within the EHEC group (Johnson *et al.*, 1983). It is prevalent in outbreaks in the USA (Johnson *et al.*, 1983; Griffin and Tauxe, 1991) but not in some other countries such as Australia (Desmarchelier, 1997). Verotoxins or Shiga-like toxins produced by EHEC act on the ribosomes of eucaryotic cells to prevent protein synthesis and are toxic to cells at very low concentrations (Paton and Paton, 1995). There are three syndromes linked with EHEC: haemorrhagic colitis, haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura.

Haemorrhagic colitis (HC) is the main disease associated with EHEC infection. It is characterised by diarrhoea that becomes severely bloody in the absence of conventional enteric pathogens (Griffin & Tauxe, 1991). Half to a third of those with EHEC infection are admitted to hospital (Lansbury and Ludlam, 1997). Haemolytic uraemic syndrome (HUS) is a sequela (complication) of haemorrhagic colitis occurring in 2-21% of cases depending on the susceptibility of the patients (Lansbury & Ludlam, 1997). It occurs most often in higher risk groups, such as the young and the elderly (Griffin & Tauxe, 1991). The disease causes a deficiency in the red blood cells due to lysis and a decrease in the number of platelets in the blood. Toxin damage to the endothelial cells triggers the clotting mechanism and small clots can block capillaries in the kidneys and other organs causing tissue damage. Patients often require blood transfusions, dialysis and intensive care (Doyle, 1991). It is the most common cause of acute renal failure in children in the UK and America (Lansbury & Ludlam, 1997). Thrombotic thrombocytopenic purpura (TTP) is an extension of the symptoms of HUS to include the central nervous system, and it is a disease that occurs more commonly in adults. Death results from blood clots in the brain (Doyle, 1991).

The use of pharmaceutical agents that decrease bowel motility in cases of haemorrhagic colitis has been found to lengthen the course of the disease and lead to increased likelihood of developing haemolytic ureamic syndrome (Bell *et al.*, 1997). The use of an antibiotic was also found to be counterproductive (Yoh and Honda, 1997). It has been concluded that no current intervention strategies were successful at controlling haemorrhagic colitis or preventing the continuation of *E. coli* O157 infections into haemolytic ureamic syndrome (Bell *et al.*, 1997; Lansbury & Ludlam, 1997).

Diarrheal symptoms usually resolve in about a week, unless the patient progresses to haemolytic ureamic syndrome or thrombotic thrombocytopenic purpura (Lansbury & Ludlam, 1997). Mortality rates are typically on the order of 3%, except at the extremes of age where rates up to 35% have been reported (Lansbury & Ludlam, 1997).

1.2.3 Characteristics of EHEC

E. coli O157:H7 was found to have biochemical characteristics distinct from those of other *E. coli,* such as the inability to ferment sorbitol within 24 hours (Johnson *et al.*, 1983) as well as a lack of β-glucuronidase activity (Doyle and Schoeni, 1984), both are factors that affect its detection in normal *E. coli* isolation procedures.

Certain *E. coli* O157:H7 strains did not grow well at temperatures above 44.5 (Doyle & Schoeni, 1984; Raghubeer and Matches, 1990). This significantly lower maximum temperature was suspected to cause a lack of isolation of these strains using normal procedures for *E. coli.* Interestingly in these two studies minimum temperatures were also significantly higher than the minimum growth temperature for *E. coli* (Shaw *et al.*, 1971). This suggests their studies were limited by the short time frame over which the measurements were taken. In later studies by Palumbo *et al.* (1995) standard isolation procedures were shown to detect 78% of *E. coli* O157:H7 strains (18 of 23 tested) at 45°C with gas and turbidity.

The responses of various strains of *E. coli* to temperature can be compared by creating growth rate models. Overall the temperature growth curve of these pathogenic strains of *E. coli* shows a significant displacement to higher temperatures compared to a non pathogenic strain, with a higher optimum temperature and possibly a higher maximum growth temperature (Salter *et al.*, 1998). This is in contrast to other studies (Doyle & Schoeni, 1984; Raghubeer & Matches, 1990) and demonstrates that, although some specific strains of *E. coli* studied show slow growth at higher temperatures, interstrain variability is significant (Palumbo *et al.*, 1995).

EHEC's mechanism of pathogenesis involves, first, adhesion to the host cell membrane by forming an attaching and effacing lesion. This involves destruction of the protruding fingershaped villi on the surface of the gastrointestinal cell. Then the attached bacteria produce one or both of the two types of toxin. These toxins are alternatively called Shigalike toxins 1 and 2 or Verotoxins 1 and 2. The subgroup of *E. coli* that produces these toxins has been called Shigalike toxin producing *E. coli* (SLTEC) or Verotoxin producing *E. coli* (VTEC) respectively. Recently it was proposed to rename the toxins "Stx" after the *Shigella dystentarie* type 1 toxin to which is it very similar. Considerable work has been done and much is now known about the genetics, structure, receptors and mode of action of these virulence factors (Doyle *et al.*, 1997). In fact, much more is known about the molecular biology of these organisms than is known about their clinical or epidemiological aspects (Neill, 1997). This is especially true of the non-O157:H7 serotypes (Neill, 1997).

1.2.4 Epidemiology of EHEC Infections

The EHEC group was first conclusively linked to illness when *E. coli* O157:H7, a previously rare serotype, was reported to cause bloody diarrhoea (Riley *et al.*, 1983). Serotypes of *E. coli* are differentiated by two main antigenic determinants: the cell wall lipopolysaccharide O antigen and the flagellar H antigen (Varnam and Evans, 1991). The EHEC includes other serotypes such as O26 and O111. Pathogenic serotypes appear frequently in the stools of haemorrhagic colitis patients and do not appear in those of healthy people (Griffin & Tauxe, 1991). The pathogenic nature of these organisms and toxins have been demonstrated in animal models (Padhye *et al.*, 1987).

Non-O157:H7 EHEC have been isolated more frequently than O157:H7 from foods and food animals (Neill, 1997). The assumed significance of *E. coli* O157:H7 as the primary cause of HUS has lead to an emphasis on studies of this organism and not other pathogenic serotypes (Johnson *et al.*, 1996). Other *E. coli* serotypes such as *E. coli* O111 have also been responsible for outbreaks of HUS (Table 1.2). From studies in Australia, the clinical incidence of *E. coli* O157:H7 is not as high as that found in American studies, but this could be due to the respective strategies of testing employed. In the USA, most screening is specific for *E. coli* O157:H7 using unique biochemical characteristics of the serotype (Padhye and Doyle, 1992; Easton, 1997). In Australia, the screening is for toxin production rather than *E. coli* O157:H7 itself (Goldwater and Bettelheim, 1995).

E. coli O157:H7 and other EHEC have emerged as important new pathogens that cause both large scale outbreaks involving hundreds of cases (Table 1.2) in addition to many smaller outbreaks and sporadic cases. Although the outbreaks attract the most attention from the media and the public, 90% of the cases of verotoxin producing *E. coli* O157 infections are sporadic (Wilson *et al.*, 1997; Parry *et al.*, 1998).

E. coli O157:H7 has a low infective dose (Griffin & Tauxe, 1991; Doyle *et al.*, 1997). This implies that the organism is capable of surviving the gastric barrier and can cause disease when small numbers of organisms are present in food. The occurrence of person to person transmission has occurred in groups of people at high risk of infection. These groups include children in kindergartens (Borzczyk *et al.*, 1987) or day care centers and the elderly in nursing homes (Carter *et al.*, 1987). Person to person transmission has also been reported from a child with HUS to a nurse (Karmali *et al.*, 1983). Person to person transmission is evidence that EHEC has a low infective dose (Griffin & Tauxe, 1991).

Table 1.2 Examples of Outbreaks of EHEC

Year	Serotype	Cases (deaths)	Suspected Vehicle	Population	Location
1982	O157:H7	26	Ground Beef	Community	Oregon[A]
1982	O157:H7	21	Ground Beef	Community	Michigan[A]
1982	O157:H7	31	Ground Beef, P to P*	Nursing Home	Ontario[A]
1984	O145:H-	100	-	School	Japan[B]
1984	O157:H7	34 (4)	Ground Beef	Nursing Home	Nebraska[A]
1984	O157:H7	36	Person to Person	Day Care	N. Carolina[A]
1985	O157:H7	73 (19)	Sandwiches, P to P*	Nursing Home	Ontario[A]
1985	O157:H7	24	Handling potatoes	Community	England[A]
1986	O157:H7	46	Raw Milk	Kindergarten	Ontario[A]
1986	O111:H-	22 (1)	-	Orphanage	Japan[B]
1987	O157:H7	26	Turkey Sandwiches	Community	England[A]
1987	O157:H7	51 (4)	Ground Beef	Half-way house	Utah[A]
1988	O26:H11	5 (1)	Tap water	Community	Czech. Rep.[B]
1988	O157:H-	6	-	Community	Germany[B]
1990	O157:H7	174	Water	Kindergarten	Japan[C]
1990	O157:H7	243 (4)	Drinking Water	Community	Missouri[A]
1991	O111:H-	234	-	School	Japan[B]
1992	O157:H7	41(1)	Person to Person	Day Care	Germany[D]
1991	O?:H19	89	-	School	Japan[B]
1992	O111:H-	**9**+ (1)	-	Community	Italy[B]
1992/3	O157:H7	732 (4)	Ground Beef	Community[K]	USA[E]
1993	O157:H7	84	Person to Person	Inuit community	Canada[F]
1994	O157:H7	45	Milk	Community	Scotland[G]
1994	O104:H21	18	Milk	Community	Montana[B]
1994	O157:H7	17	Salami	Community	Washington[H]
1995	O111:H-	>100 (1)	Mettwurst	Community	Australia[B]
1996	O157:H7	10,000 (11)	Water Cress	Primary Schools	Japan[G]
1996	O157:H7	6333 (13)	Radish sprouts	Community	Japan[I]
1996	O157:H7	69 (1)	Fresh Apple Juice	Community[L]	USA[F]
1996	O157:H7	408 (18)	Meat	Community	Scotland[I]
1998	0118:H2	126	Salads	School	Japan[J]

+ No of cases in bold indicates recorded numbers of HUS rather than HC or diarrhea; P to P* = Person to person transmission; where the number of deaths is not given because it is unknown not because deaths were zero, A - (Doyle, 1991), B - (Johnson *et al.*, 1996), C - (Akashi *et al.*, 1994), D - (Karch *et al.*, 1997), E - (Desmarchelier and Grau, 1997), F - (Rowe *et al.*, 1994), G - (Anon, 1996a), H - (Tilden, 1996), I - (Lansbury & Ludlam, 1997), J - (Hashimoto *et al.*, 1999), K - Washington, California, Idaho and Nevada, L - Connecticut and New York.

The increasing incidence of foodborne outbreaks of this disease may be due to changes in farming and food manufacturing practices. Cattle have been shown to be the main reservoir for this pathogen with a carriage rate, for example, in the UK of 1 - 8% (Lansbury & Ludlam, 1997), though it has also been isolated from sheep and deer (Tarr *et al.*, 1997) at rates that vary widely between countries (Desmarchelier, 1997). Current animal husbandry practices such as feed lots and the housing of cattle indoors over the winter allows for greater spread of the pathogen between animals (Borzczyk *et al.*, 1987; Zhao *et al.*, 1995; Dargatz *et al.*, 1997). Calves are more likely to carry EHEC than older cattle and in most cases carriage of EHEC strains is transient and short term with strain types carried by a herd changing frequently (Wilson *et al.*, 1997). There is also an increasing incidence of EHEC carriage when new cattle are introduced into a herd. *E. coli* has been shown to survive in water or manure over a period of 70 days (Rice *et al.*, 1992)

There is an apparent summer peak in the incidence of HUS infections in the UK and the USA (Easton, 1997; Lansbury & Ludlam, 1997). This has a variety of possible causes including a higher environmental temperature and, therefore, a greater risk of temperature abuse of foods (Bryant *et al.*, 1989) or increases in the carriage of EHEC by cattle (Lansbury & Ludlam, 1997). The greater incidence of EHEC infections in summer has also been found in Australia (Desmarchelier, 1997) and several other countries such as Argentina (Lopez *et al.*, 1997). Epidemiological studies by Bryant *et al.* (1989) determined that exposure to hamburger meat, or barbecued food, or eating out were not risk factors for *E. coli* O157:H7 infection suggesting that bad food handling procedures are necessary to give rise to EHEC infection. Other studies found that consumption of undercooked ground beef was a significant risk factor (Wilson *et al.*, 1997) but that other factors must also contribute significantly. Recent cases have suggested a link between exposure to cattle on dairy farms and infections (Wilson *et al.*, 1997).

There is a large increase in the incidence of EHEC infection at the extremes of age. For example in Scotland there is an incidence of 11.07/100,000 for 0-4 years old, 2.96 / 100,000 for 5-14 years old and 2.30/100,000 for over 65 year olds (Simmons, 1997). This compares to an overall incidence of 0.80/100,000 in England and Wales (Lansbury & Ludlam, 1997). In Australia, the incidence of HUS is 0.62/100,000 children under 16 years (Desmarchelier, 1997), compared to an incidence of 22/100,000 children under 5 years in Argentina (Lopez *et al.*, 1997). There is a strong geographical aspect to the incidence of infections that is yet to be fully explained. Areas such as Alberta have very high incidences of 12.1/100,000 (Lansbury & Ludlam, 1997) compared to the incidence in the country overall, 3 - 5.3/100,000 (Wilson *et al.*, 1997). In Oregon and Washington, where there is a history of rigourous surveillance, the number of reported cases (excluding outbreaks) does not appear to be increasing (Tarr *et al.*, 1997).

Other *E. coli* types primarily follow a pattern of human to human transmission (Doyle & Padhye, 1989). In contrast, the suspected original source of *E. coli* O157:H7 infections is commonly a bovine source, such as meat or milk, or contamination of uncooked food types such as fresh produce with milk or meat products or manure (Table 1.3). Pathogenic *E. coli* has been isolated from foods implicated as vectors in outbreaks of *E. coli* infections (Zhao *et al.*, 1995). There have also been waterborne outbreaks where the suspected source has been fecal contamination from a human or bovine source (Dev *et al.*, 1991; Rice *et al.*, 1992). Details of a recent waterborne outbreak can be found online (Promed Archive, 2000). Person to person transmission has been found to occur secondarily (Carter *et al.*, 1987).

Meat products such as ground beef have been recognised as the most important vehicle for transmission of EHEC (Table 1.3) since HUS was first recognised in 1983 as being associated with *E. coli* O157:H7 (Wells *et al.*, 1983; Willshaw *et al.*, 1994). The grinding of beef can spread the pathogen from the surface into the rest of the beef thus increasing the risk of infection. *E. coli* can survive longer in the cooler centre of a beefburger and may not be killed if the beefburger is undercooked (Doyle & Schoeni, 1984). Additionally, the mixing of meat from various carcasses to create ground beef may facilitate the spread of *E. coli* throughout the final meat product.

Unpasteurised milk has a high risk of contamination with pathogenic strains carried by the dairy cattle. The consumption of unpasteurised milk has been linked to outbreaks (Martin *et al.*, 1986; Borzczyk *et al.*, 1987; Wells *et al.*, 1991) and is ranked fifth as a vehicle of infection (Table 1.3). The use of unpasteurised milk in the manufacture of cheese was implicated in outbreaks of other pathogenic *E. coli* types (Marier and Wells, 1973; Fantasia *et al.*, 1975). Pasteurised milk can be a vehicle if post-pasteurisation re-contamination occurs (D'Aoust *et al.*, 1988). Post-pasteurisation re-contamination was believed to be the cause of an outbreak in Scotland where *E. coli* O157 was isolated from bottling equipment. Contaminated equipment could have re-introduced the pathogen into the milk after it was pasteurised (Upton and Coia, 1994).

Table 1.3 - Leading Food Vehicles or Mode of Spread for *E. coli* O157:H7 outbreaks in the USA (1982-1994) (Doyle *et al.*, 1997)

Rank	Vehicle	No. of Outbreaks
1	Ground Beef	22 (32.4%)
2	Person to Person	9 (13.2%)
3	Vegetables, Salad Bars	4 (5.9%)
4	Water, Swimming Water	3 (4.4%)
5	Roast Beef	2 (2.9%)
5	Raw Milk	2 (2.9%)
5	Apple Cider	2 (2.9%)
	Unknown	19 (27.9%)

Another food that has been linked with *E. coli* outbreaks (Table 1.3) is apple cider (Besser *et al.*, 1993) where the source was considered to be contamination of "dropped" apples with manure from cattle grazing in the orchards. In such cases the apples were not washed nor the cider pasteurised nor was any preservative added. Another acidic food type, implicated in an outbreak of HUS in 1993, was mayonnaise (Zhao and Doyle, 1994; Hathcox *et al.*, 1995). In this case it was believed that raw beef juices caused cross contamination of the mayonnaise type dressings at a franchise restaurant chain (Erickson *et al.*, 1995). *E. coli* can form biofilms in food processing plants (Dewanti and Wong, 1995) although this mechanisms has not yet been implicated as the cause of *E. coli* infections.

1.2.5 Detection of EHEC

The increase in recorded incidence of HUS from *E. coli* O157:H7 can be partially explained by an increase in the awareness of the organism and the availability of specific tests for it in clinical samples (Bettelheim, 1995) as well as in food products (Tortorello and Stewart, 1994). Regular screening of possible cases will likely lead to an increase in the detection of sporadic or geographically scattered infections. However, the symptoms associated with HC are distinctive enough to demonstrate that the increase is real, and shows that the disease is spreading and increasing in incidence, especially the number of large foodborne outbreaks (Griffin & Tauxe, 1991). Many new techniques have been developed for the detection of EHEC and some are listed below (Table 1.4). Comprehensive reviews were given by Padhye and Doyle (1992) and Desmarchelier and Grau (1997). A simple, fast test for all pathogenic types and for all foods is still lacking despite large scale efforts in development of detection methods using the latest molecular and enzyme technologies (Anon., 1996 a or b). This is in part due to the low infective dose. Food containing low levels of EHEC, with a low probability of detection of the organism, appears normal and may show no sign that is dangerous to eat.

Table 1.4 - Methods for Detection of EHEC

Method	Reference
Monoclonal antibodies	(Padhye and Doyle, 1991a)
Enrichment sandwich ELISA procedures	(Padhye and Doyle, 1991b)
Antibody-direct epifluorescent filter techniques	(Tortorello & Stewart, 1994)
Modified fluorescent-antibody techniques	(Pyle *et al.*, 1995)
Visual immunoassay procedures	(Flint and Hartley, 1995)
Enrichment plating procedures	(Flint and Hartley, 1995)
Pulsed field gel electrophoresis	(Johnson *et al.*, 1995a)
Immunomagnetic separation and PCR	(Weagent *et al.*, 1995)
Detection of enterohaemolysin production	(Bettelheim, 1995)
EHEC-Tek (Organon Tecnika Corp.)	(Johnson *et al.*, 1995b)
Petrifilm™ Test Kit-HEC	(Bennett *et al.*, 1995)

1.2.6 Control of EHEC Infection

The prevention of outbreaks of *E. coli* infection needs to involve various strategies at various levels of the "farm to fork" continuum. Measures should be in place to prevent outbreaks of enteric pathogens with an animal reservoir, such as preventing the contamination of food with manure which would remove the main source of these organisms. However, many problems exist with implementing complete elimination. It has been shown that *E. coli* can survive for long periods in manure (Wang *et al.*, 1996) so aging the manure before use as a fertiliser does not assure safety. Contamination of fresh produce in this way with *E. coli* is especially hazardous, as often it will not receive a heat treatment prior to consumption. Also bacteria can be protected within the plant's tissue from washing and disinfection (Itoh *et al.*, 1998). Ideally, animals should be slaughtered with minimal contamination of the carcasses. *E. coli* O157:H7 has been declared an adulterant of meat in the USA, and its presence therefore considered "unacceptable" (Nicholls, 1995). However, as the risk of initial contamination cannot be totally eliminated, a sanitisation step in the slaughtering process is necessary to improve the microbial safety of the meat.

Various methods of meat carcass decontamination are in use and have been shown to reduce the population of microorganisms on the carcass to varying degrees (Dickson and Anderson, 1992). These include: i) water rinsing (Dorsa *et al.*, 1997b); ii) chlorine (Bautista *et al.*, 1997); iii) organic acids (Anderson and Marshall, 1990; Dickson, 1991; Hardin *et al.*, 1995; van Netten *et al.*, 1995); iv) electrical shock (Bawcom *et al.*, 1995) and v) antimicrobials, for example trisodium phosphate (Dickson *et al.*, 1994) and glucono-delta-lactone (Qvist *et al.*, 1994; Zepeda *et al.*, 1994). There are also methods that use a combination of techniques (Corry *et al.*, 1995; Cutter *et al.*, 1997; Patterson and Kilpatrick, 1998). There is still debate over the effectiveness of these various techniques in order to achieve the best chance of microbial safety (Dorsa *et al.*, 1997a).

Adequate cooking of primary food sources and good food handling practices (to prevent cross contamination) are necessary to provide safety in all foods. Food handlers and the general public need to be educated about the risks associated with foodborne diseases and preventative measures such as hygiene, adequate cooking and good food handling practices. If possible, foods should be formulated which do not support the growth or survival of *E. coli*. Many foods have been shown to support at least the survival of *E. coli* for sufficiently long periods to cause infection. If the conditions of the food itself do not sufficiently inhibit *E. coli*, preservatives and other processes such as pasteurisation need to be considered as additional barriers to infection. In the future oral immunisations could be developed for animal reservoirs (Robins-Browne, 1995). However the sporadic nature of carriage of the organism and the numerous serotypes that are potentially pathogenic would make this method of control very difficult.

There is an increased risk of foodborne *E. coli* disease from only mild contamination of foods and from foods where the organisms cannot grow, but can survive because small number of organisms are required to cause disease (Easton, 1997). It is probable that this low infective dose has led to new origins of disease such as through animal contact on farms (Wilson *et al.*, 1997). Host animals, such as cattle or calves, which make up the animal reservoir of EHEC, do not necessarily get sick with strains that are pathogenic to humans such as O157:H7 (Johnson *et al.*, 1996). Consequently animals carrying EHEC are not always recognisable and thus able to be separated from other animals. Carriage in cattle herds is often present at a low levels but there have been a number of cases associated with direct contact with cattle during farming or slaughter (Easton, 1997). There is a high public awareness of this disease and the potential of litigation against food production companies for any of their actions or lack of action that would increase the risk of disease. There is also a lack of successful control strategies due to the sporadic nature of infection in the worldwide animal reservoirs of various ruminant species and the ability of the organism to survive for long periods in water, the environment and some foods.

The seriousness of new foodborne diseases has focused the attention of many different areas of microbiology on controlling and preventing them. There has been a large effort to understand the epidemiology and molecular biology of EHEC and to develop new detection techniques, as well as clinical studies of the diseases and how toxins act in the human body. Some of the most promising work to prevent this disease has focused on understanding EHEC physiology and how growth and survival is affected by inhibitory factors such as pH and organic acids. It is in this area that Predictive Microbiology studies such as those described in this thesis can help synthesise the current knowledge into a useful mathematical form that can be used in the food industry to help control and prevent this disease.

1.3 Predictive Modelling

Predictive microbiology was proposed as a potential solution following concern over the increasing incidence of food poisoning and pressure from the public on the food industry to develop new food products that were lower in salt, cholesterol and preservatives (McMeekin *et al.*, 1993). It provides an alternative way of addressing the problems of microbial food spoilage and the problems caused by the presence or growth of pathogens in food. Predictive microbiology facilitates the safer production and handling of food products.

The strategy of end product (challenge) testing, traditionally used to assess microbial food contamination, is expensive and time consuming especially for the development of new product formulations. The results can determine if something has gone wrong in the process, but do not indicate where or how the problem occurred. Predictive microbiology has more wide-ranging applicability and usefulness and may provide a better understanding of the underlying physiological processes controlling the growth of microorganisms in food (McMeekin *et al.*, 1993).

There are four stages in predictive microbiology to enable the development of models to predict microbial growth under known conditions (Ross and McMeekin, 1994). The stages are:

i) Identifying the organisms and conditions of interest.
ii) Collection of data over total range of interest for all variables.
iii) Development of mathematical models that describe the data and validation of these models in foods.
iv) Prediction of growth under new conditions by interpolation by using the mathematical model.

The same approach was used to develop the time and temperature combinations necessary to kill different types of organisms, for example in pasteurisation or canning. It has also been used in the fermentation industry where exact knowledge of the conditions of growth is necessary for optimal production (McMeekin *et al.*, 1993).

Predictive microbiology works because, despite the apparent complexity of foods, many factors in the food environment have little effect on the growth of microorganisms. Nutrients are rarely limiting (McMeekin *et al.*, 1993) so the main influences on microbial growth are temperature, water activity (the amount of available water) and pH of the food. Temperature, water activity and pH vary depending on the food type and storage conditions. In most foods one or more of these factors is inhibitory to microbial growth. Microbial interactions in food are generally not significant.

In some food types, such as fermented food, microorganisms grow to high numbers and microbial interactions are important. For example, lactic acid production by lactic acid bacteria inhibits growth of other bacteria in cheese. Processes such as cheese ripening and fermentation inhibit the growth of unwanted organisms by changing the food environment. These changes can occur by the production of organic acids, lowering the pH, and by production of other inhibitory metabolites (Barbosa *et al.*, 1993).

The increasing development and use of mathematical modelling and predictive microbiology in the food industry has been facilitated by the advent of computers and ready access to this new technology. Large cooperative groups of microbiologists have been formed to create data and models for computer programs describing many different organisms. This work provides modelling products for the food industry, some of which are commercial, such as Food Micromodel and others such as the Pathogen Modelling Program which are available free of charge (Ross, 1999). Such software, which incorporates predictive models, enables predictions about shelf-life and safety of the products given sufficient information about the product and its storage conditions. However, care must be taken that these programs are used correctly and are not used to give false predictions. There are specific rules that need to be obeyed to ensure that predictive modelling predictions are valid. For example, predictions are only possible within the range of values of data from which the model was developed (McMeekin *et al.*, 1993). Extrapolations outside the area of data are not valid. Models developed in laboratory media need to be rigorously tested for applicability and validity in foods.

The pressures to overcome the increasing levels of food poisoning incidents across the developed world has lead to new challenges. There are specific characteristics of pathogens such as *E. coli* which require a change in the traditional approach of predictive microbiology. These pathogenic organisms are characterised by a low infective dose (Griffin and Tauxe, 1991). This is in comparison to other pathogens, such as *Bacillus cereus*, that require growth to a particular level before toxin production and pathogenicity are achieved (Foegeding and Berry, 1997). It is much less informative to find out how fast the organism can grow where less than a hundred organisms are necessary to cause disease (Doyle *et al.*, 1997) as this number will be achieved quickly by cell doublings at any growth rate. In such cases the conditions of interest are those that prevent the growth of the organism and, where possible, decrease the population.

Hazard Analysis and Critical Control Point (HACCP) schemes have been developed for food processing and are achieving a high level of importance in food industry standards and regulations. HACCP aims include the identification of the risks of microbial growth occurring. This involves identifying which stages of the process are critical to control, and the ranges of conditions required to achieve control.

Predictive microbiology enables both adequate monitoring and confidence in the safety of the products through objective assessment of Critical Control Points (CCP) (Harrigan and Park, 1991; Ross & McMeekin, 1994). Quantitative Microbial Risk Assessment is another new field being developed to help determine what measures can be taken to reduce food poisoning (Lammerding and Paoli, 1997). However, as in HACCP, the ability of Predictive Microbiology to aid the production of more realistic and useful Risk Assessments and to add valuable knowledge to these processes is currently underrecognised and underutilised (Miles and Ross, 1999; Ross, 1999).

Specific information is required in order for predictive microbiology to be successful. First, collection of detailed and rigorous data: second, the use of expert mathematical knowledge to fit the appropriate mathematical model to the data; and, then, validation of the model in real food. The data collected for predictive microbiology is also helpful for the investigation of physiological mechanisms underlying bacterial processes. Determination of the conditions leading to growth, survival and death of microorganisms is important where that data is novel for organisms or conditions or can be used to clarify conflicting information. Such information is needed to design food processing and handling systems and is rare in comparison with studies on the genetic aspects of these pathogenic organisms, despite the fact that knowledge of the physiological mechanisms can be a powerful tool for increasing food safety. New ways of inhibiting the growth of microorganisms can be developed if the underlying physiological mechanisms are known.

1.3.1 Kinetic Models

Kinetic modelling is where the model describes the growth rate of a microorganism. Most foods are a new environment containing nutrients that the microorganisms are able to utilise. Therefore, the growth is equivalent to that of batch culture with the increase in bacterial numbers, the growth curve, showing a lag, exponential growth, stationary and, eventually, a death phase (Figure 1.1). The 'modified-Gompertz' equation shown below has been used to define this type of bacterial growth curve. It is fitted by non-linear regression to the data, and information about the growth rate and lag times may be derived (McMeekin *et al.*, 1993). This equation is written:

$$\log N_t = A + D\exp\{-\exp[-B(t-M)]\} \qquad (1.1)$$

where t = time
N_t = population density at time t
A = value of lower asymptote
D = difference in value of upper and lower asymptote
M = time at which exponential growth rate is maximal
and B is related to the slope of the curve at M such that
BD/e is the slope of the tangent to the curve at M

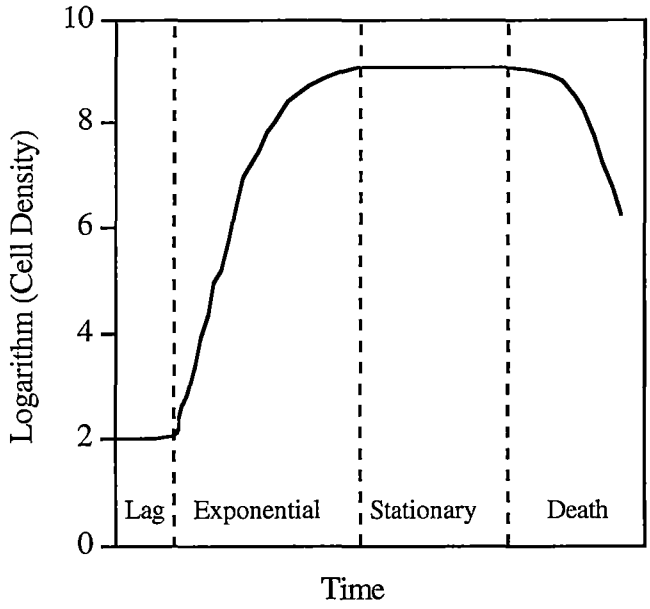


Figure 1.1 - A typical bacterial growth curve showing the increase in population over time in a batch system.

Environmental conditions that have an effect on bacterial growth in high moisture foods are, in order of practical utility, temperature, water activity, pH, gaseous atmosphere, addition of preservatives or antimicrobials. In dried or low moisture foods, water activity is usually the most important. The level of growth rate inhibition can be determined for each of these conditions. Thus, the effect of the total environment of the organism on the growth of the organisms in the food can be quantified. In this sense predictive microbiology is a type of microbial ecology (Ross & McMeekin, 1994; McMeekin *et al.*, 1997).

Systematic variation in a factor can be used to build up a picture of the extent to which that factor affects the growth rate and the interaction, if any, between factors. An effective description of the growth of an organism can be given by a mathematical description or model of the relationship between the growth rate and the factors once sufficient data has been obtained (McMeekin *et al.*, 1993). The main regulating factor affecting the growth of microorganisms is temperature where nutrients are not limiting. This is because temperature is commonly and easily used to control bacterial growth, as in refrigeration. It is also important physiologically as it cannot be externalised or avoided by maintaining a different internal environment to the exterior. The only bacterial growth strategies possible are those that compensate for the effects of temperature. Many mathematical equations have been developed to describe how temperature affects microbial growth rate, some of these are described below. Temperature is a good example to compare the different types of approaches and equations used to create kinetic models which include Arrhenius, square root (Bě lehrádek) and polynomial models (Ross & McMeekin, 1994).

1.3.1.1 Simple Arrhenius Model

The Arrhenius equation describes the rate of chemical reactions and their variation with temperature. Arrhenius's ideas were adopted by microbiologists to describe the growth of microorganisms. The theoretical basis of the mathematical model is the assumption that basic physiology of a microorganism is equivalent to a complex series of chemical reactions. The Arrhenius equation can be expressed as:

$$\ln k = \ln A - {}^{E_a}/_{RT} \qquad (1.2)$$

where k = the specific reaction rate constant

A = "collision" factor

E_a = activation energy

R = universal gas constant

T = absolute temperature (Kelvin)

This equation gives a good description of the microbial growth rate's response to temperature for part of the organism's temperature range. Above and below the "normal physiological range" of growth temperatures for the organism the observed growth rate becomes less and less than that predicted by the Arrhenius equation (Figure 1.2).

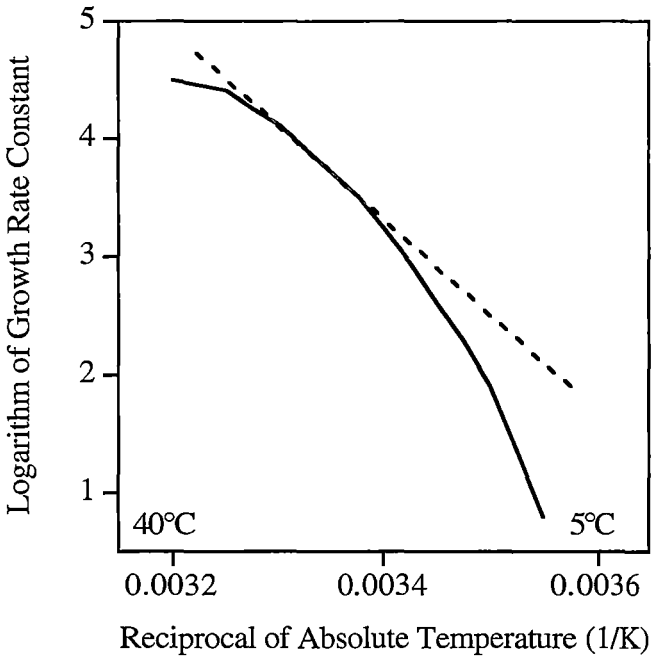


Figure 1.2 - Arrhenius plot for *E. coli* data (solid line) adapted from (McMeekin *et al.*, 1993) showing the Arrhenius straight line (dotted line)

The deviation from the straight line Arrhenius relationship occurs because enzymes act as biological catalysts in microorganisms for most reactions within the cell. Enzymes are inactivated by temperature extremes. They are also synthesised by biological processes that are affected by temperature. Microbial physiology is under complex biochemical control to maintain life under less favourable conditions. Therefore the simplification of microbial growth rate to the rate of a simple chemical reaction does not apply except within the limited range of optimal conditions (Neidhardt *et al.*, 1990).

1.3.1.2 Schoolfield Model

The simple Arrhenius model has been modified by the addition of terms added to overcome the deviation from linear at low and high temperatures. This has resulted in models such as the Schoolfield model (Schoolfield *et al.*, 1981). The model was mathematically reworked with extra terms added to take into account the high and low temperature inactivations that occur in bacterial growth response. The form of the Schoolfield model is given below:

$$\frac{1}{k} = \frac{\rho(25°C)\frac{T}{298}\exp\left[\frac{\Delta H_A}{R}\left(\frac{1}{298}-\frac{1}{T}\right)\right]}{1+\exp\left[\frac{\Delta H_L}{R}\left(\frac{1}{T_{1/2L}}-\frac{1}{T}\right)\right]+\exp\left[\frac{\Delta H_H}{R}\left(\frac{1}{T_{1/2H}}-\frac{1}{T}\right)\right]} \qquad (1.3)$$

where k = kinetic parameter (e.g. time)
R = universal gas constant
T = absolute temperature (K)
$\rho(25°C)$ = inverse of the fitted kinetic parameter at 25°C
H_A= constant describing the enthalpy of activation for microbial growth
H_H = constant describing the enthalpy of high temperature inactivation of growth
$T_{1/2H}$ = constant describing the high temperature inactivation of growth rate
$T_{1/2L}$ = constant describing the low temperature inactivation of growth rate
H_L = constant describing the enthalpy of low temperature inactivation of growth

The modification of the Arrhenius model is an attempt to create a mechanistic model based on an underlying theory, rather than the use of an empirical model using a convenient mathematical form without a theoretical basis. The adjustment of the equation which is necessary to create a model that will fit real data creates a model that is mathematically very complex. Yet, despite this complexity, it does not totally describe the enzymatic control of bacterial growth. The parameters added describe an overall whole cell "inactivation" which is not based on theory. Taking this into account the model is no longer strictly mechanistic as only part is based on an underlying theory.

As empirical models, modified Arrhenius models give a good description of the data. However they are complex to use and have a high number of parameters. This complexity leads to both a barrier to understanding and lack of parsimony which are undesirable for ease of use and mathematical reasons respectively. Other models can describe the data equally well with fewer parameters.

1.3.1.3 Polynomial Models

Other types of empirical model are polynomial models or response surface models. These use the form of a polynomial function in the parameters being modelled. This involves using each parameter and every combination of the parameters multiplied, squared and so on. Multiple linear regression techniques are used to give the best fit values for the parameters. Second order polynomial regressions have the general form (Ross, 1993):

$$Y = a + b_1X_1 + b_2X_2 + ...+b_iX_i+ ... +b_nX_i^2+ ...+b_vX_1X_2+ ..b_zX_iX_j \qquad (1.4)$$

where a, $b_{1,2,...z}$ = parameters to be estimated
$X_{1,2\ ...i,j}$ = variables
Y = response variable e.g. Log (rate)

The growth responses of an organism to many different types of stress, for example temperature, water activity, pH, modified atmospheres as well as antimicrobials such as nitrite can be described in one model. However as they have such a large number of terms, these models are awkward to use. Often these models can be very effective at describing their own data set but less able to describe other data sets (Ross, 1999).

Groups such as the Predictive Modelling Programme funded by the UK Ministry of Agriculture, Fisheries and Food (MAFF) use polynomial models for the modelling of their data for consistency (Sutherland *et al.*, 1995). However these model are essentially exercises in curve fitting and they do not describe an underlying hypothesis for the inhibitory effects. As such they do not determine values of practical and physiological significance as do square root models. Square root models have an equal or better goodness of fit. The fitted model parameter values produced by square root modelling help to understand the response and these values also allow comparison between different strains and organisms for these values (McMeekin *et al.*, 1993; Ross, 1999). They reflect the existing knowledge and provide a framework for building further understanding.

1.3.1.4 Square Root Models

The development of square root or Bě lehrádek type models was based on an observation (Ohta and Hirahara, 1977) of the response of nucleotide breakdown, which was comparable to the response of bacterial growth to temperatures in the sub-optimal growth region. Ratkowsky *et al.* (1982) determined that the response was well described by a mathematical equation of the form below. The square root model is :

$$\sqrt{k} = b(T - T_{\min}) \qquad (1.5)$$

where k = growth rate
b = slope of regression line, a fitting parameter
T = temperature (Kelvin)
T_{min} = a notional value of temperature where growth rate is zero

T_{min} is estimated by extrapolation of the regression line derived from a plot of $\sqrt{k}$ versus temperature to the temperature axis (McMeekin *et al.*, 1993). Later it was discovered that this square root equation is a specific case of an equation used by Bě lehrádek in the early part of this century to describe the rate of biological reactions as shown below:

$$k = a(t - \alpha)^b \qquad (1.6)$$

where k = rate
t = temperature
α = a "biological zero"
a, b = fitted parameters

Further work on this model type allowed it to be extended to the full temperature range of an organism (Ratkowsky *et al.*, 1983). This approach of using a minimum or limiting value has been used in the modelling of other factors, such as water activity and pH, in a similar way to temperature. The square root model for sub-optimal temperature and water activity is shown below (Chandler and McMeekin, 1989).

$$\sqrt{k} = b\sqrt{(a_w - a_{w_{\min}})}(T - T_{\min}) \qquad (1.7)$$

where a_W = water activity
a_{Wmin} = theoretical minimum water activity for growth
and other terms are as defined above for square root models

Additional factors such as water activity (Eqn 1.7), pH and antimicrobial additives which also affect bacterial growth rates have been included as parameters in some mathematical models. Temperature, and even water activity are well studied and modelled inhibitory factors in foods. In contrast, pH and the presence of organic acids is a more complex situation that has been less frequently and satisfactorily modelled.

1.3.1.5 Square Root Models for pH

Adams *et al.,* (1991) described a square root type pH and temperature model for the growth rate of *Yersinia enterocolitica* for sulfuric, citric, lactic and acetic acids in the range of pH 4 - 6.5 and temperature 0 - 25°C (Eqn 1.8). They used the water activity square root term described previously as a prototype for a term for the effect of pH. The magnitude of the response is proportional to the difference between the measured value and a threshold value. The fit of the model was good. However, their use of different acids required the use of acid-specific pH_{min} values though even then acetic acid inhibition was not well described by the model. Also Little *et al.*, (1992b) attempted to define a pH, acidulant and temperature model for the survival (not growth) of *Yersinia enterocolitica* in the pH range 3.0 - 4.5 using this model and they were not able to find a good fit.

$$\sqrt{r} = c\sqrt{(pH - pH_{\min})}(T - T_{\min}) \qquad (1.8)$$

where r = growth rate
c = constant
pH_{min} = theoretical minimum pH for growth
and other terms are as defined above for square root models

Wijtzes *et al.,* (1993) described a water activity, pH and temperature model for *Listeria monocytogenes* in the pH range 4.6 - 7.4, water activity 0.95-0.997 and temperature 5 - 35°C (Eqn 1.9). They used the term covering the whole temperature range (Ratkowsky *et al.*, 1983) as a prototype for a term covering the whole range of pH. They also used the water activity term and the sub-optimal temperature terms characteristic of square root models (Eqn 1.9). While they found a good fit to their data, other data sets exist for which the model does not give a very good fit (Ross, 1993; Miles, 1994).

$$\sqrt{\mu}_m = b\sqrt{(a_w - a_{w_{\min}})}(pH - pH_{\min}) \times \{1 - \exp[c_{pH}(pH - pH_{\max})]\}(T - T_{\min}) \qquad (1.9)$$

where μ_m = maximum specific growth rate
c_{pH} = regression coefficient
b = regression coefficient
pH_{max} = theoretical maximum pH for growth
and other terms are as defined above for square root models

1.3.1.6 Other Models for pH

Wijtzes *et al.,* (1995) found that both the pH_{min} and pH_{max} were independent of temperature. They used a parabolic equation to described the growth rate response to pH and a square root model temperature term and found it gave a good fit. Their acidity and temperature model for *Lactobacillus curvatus* growth rates and lag times over the pH range of 4.5 to 9.0, and temperatures from 6 to 30°C is shown below.

$$\mu_m = b(T - T_{\min})^2 (pH - pH_{\min})(pH - pH_{\max}) \qquad (1.10)$$

where all terms are as defined previously. However there are other data sets (Ross, 1993; Miles, 1994) where the overall shape of the growth response to pH is not parabolic but has a plateau, a range of near-optimal pH values, which has the same optimal growth rate. For these data sets this model also does not give a good fit .

An examples of a polynomial model for *E. coli* O157:H7 growth that includes pH is Buchanan *et al.,* (1993). They developed models for the *B* and *M* values of the modified Gompertz equation, with both logarithmic and square root transformations, under both aerobic and anaerobic conditions giving a total of eight models containing 10 terms each. They found that the majority of the response was described by the primary terms and that the cross product terms were less important, suggesting that like previous square root models, the effects of temperature, pH and water activity were independent. As an example the square root transformation for B under aerobic conditions is shown below:

$$\begin{aligned}\sqrt{B} = {} & -1.2299 + 0.0223(T) - 0.3506(pH) - 0.00416(NaCl) \\ & +0.00182(T)(pH) - 0.000171(T)(NaCl) + 0.000315(pH)(NaCl) \\ & -0.000316(T)^2 - 0.0287(pH)^2 - 0.000055(NaCl)^2\end{aligned} \qquad (1.11)$$

where T = temperature 5-42°C
pH = pH 4.5-8.5
$NaCl$ = NaCl 5-50 g l^{-1}

Sutherland *et al.* (1995) also used a similar model for *E. coli* O157:H7 growth containing the same 10 terms but did not publish their fitted model values. Other examples of organisms that have been modelled for pH as well as other factors using polynomial models include the pathogens *Yersinia enterocolitica* (Little *et al.*, 1992a; Dengremont and Membre, 1994; Bhaduri *et al.*, 1995), *Aeromonas hydrophila* (Palumbo *et al.*, 1992) and salmonellae (Gibson *et al.*, 1988) and the meat spoilage organism *Brocothrix thermospacta* (McClure *et al.*, 1993).

There have been many different strategies to model the effect of pH on the growth response of various organisms. Eklund (1983) was the first to model the different effect of the dissociated and undissociated forms of sorbic acid and calculated the minimum inhibitory concentrations of both. It was found that sensitivity to the acid varied according to the organism but both the dissociated and undissociated forms had an inhibitory effect on *Bacillus subtillus, B. cereus, E. coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans*. Cole *et al.* (1990) used a simple experimental design with three temperatures and modelled the pH at each with different quadratic equations. They found that the effects of the inhibition of high salt and low pH were additive and not synergistic and that there was a linear relationship between growth rate and hydrogen ion concentration.

Models have been developed which describe the transition between growth rate and death rate in a combined rate model. These primarily describe growth rate and death rate due to high temperature, rather than other factors (Van Impe *et al.*, 1992; Peleg, 1995). Some models have been developed for the transition between growth rate and death rate due to factors such as sodium chloride concentration (Whiting and Cygnarowicz-Provost, 1992; Walker and Jones, 1994; Membré *et al.*, 1997).

Rosso *et al.* (1997) developed a novel type of model that predicts the minimum pH at which growth will occur for different organic acids depending on their pK_a (chemical dissociation constant) values. However this model is restricted to when the acid is used as the sole acidulant and gives no indication of the effect of the acid on growth rate above the minimum pH. It is interesting to note, however, that a mathematical relationship exists that relates the pK_a and the minimum pH for a variety of acids that are quite different chemically and biologically. Hsiao and Siebert (1999) also modelled seventeen different organic acids for their minimum inhibitory concentrations, at pH 5.25, for a variety of bacteria. They found that the physical and chemical properties of an acid could be used to predict its inhibitory effects.

Overall, pH and specific organic acid inhibitory effects are apparently more complex than those of temperature and water activity. Although pH has been modelled in the same way as these other factors, it may be an oversimplification of the true situation and further factors may need to be taken into account within the model before the effects can be comprehensively described.

Recently a new type of pH model that allows the modelling of the inhibition by organic acids separately to pH inhibition, in terms of hydrogen ion concentration, has been developed (Presser *et al.*, 1997). Further adaptation and use of this model is described in this thesis.

1.3.2 Probability Models

Probability models are perhaps the oldest predictive models in use in food microbiology, dating back to the elucidation of thermal death kinetics of microorganisms and their spores in the early 1920s and the use of these calculations to achieve safety in the canning process (Stumbo *et al.*, 1983). More recent activity in the 1970s developed models to predict the likelihood of growth, toxin production or death within a given time and often are developed for the outgrowth of spore forming bacteria, such as *Clostridium botulinum.* Polynomial probability models are the most common type (Genigeorgis *et al.*, 1971; Lindroth and Genigeorgis, 1986; Lund *et al.*, 1987; Gibson and Roberts, 1989; Lund *et al.*, 1990). However, logistic functions have also been used by others (for example, Whiting and Oriente (1997)).

Genigeorgis *et al.* (1971) used a probability model to describe the decimal reduction of *Staphylococcus aureus* as a function of environmental factors (% NaCl and pH). The probability of a single cell growing was modelled as $P=R_G/R_I$ where R_I is the number of cells inoculated and R_G is the number of cells able to grow over a specific time period. This polynomial expression has terms for each factor as well as squared and multiplicative terms. The number of decimal reductions resulting from environmental factors was expressed as

$$\begin{aligned} Log(R_G / R_I) = a + b_1(\%NaCl) + b_2(pH) \\ + b_3(\%NaCl)^2 + b_4(pH)^2 \\ + b_5(\%NaCl)(pH) \end{aligned} \qquad (1.12)$$

A problem with this type of model is that P is not constrained to values between 0 and 1. Therefore the model can predict probabilities that are negative or greater than one.

A new type of probability modelling has emerged in this laboratory with the potential to improve the ability of predictive microbiology to describe the interface between conditions were growth is possible and those where growth is not possible.

1.3.3 Growth/No Growth Interface Modelling

In modelling the growth of microorganisms it is sometimes of most interest to determine where the boundary or set of conditions occur which first prevent the growth of the organism. This is the case in situations such as the presence of *E. coli* in food, where the pathogen has a low infective dose and formulations that prevent the growth of the organism, or preferably cause its decline, are sought.

Growth/no growth (or growth limits) models are a new type of a probability model first developed by Ratkowsky and Ross (1995). They proposed a logistic regression model to describe the growth/no growth interface for conditions including temperature, pH and additives such as sodium nitrite. They were able to show that the proposed model (shown below) gave a good fit for *Shigella flexneri* data (Zaika *et al.*, 1989; Zaika *et al.*, 1991; Zaika *et al.*, 1992).

$$logit(P) = b_0 + b_1 \ln(T - T_{\min}) + b_2 \ln(pH - pH_{\min}) + b_3 \ln(a_W - a_{W_{\min}}) + b_4 \ln(NO2_{\max} - NO_2) \quad (1.13)$$

where NO_2 = concentration of sodium nitrite (ppm)

$NO2_{max}$ = upper notional NO_2 value at which growth rate is predicted by extrapolation to be zero

logit $(P) = \ln\{P/(1-P)\}$

and other terms are as previously defined

The model differs from previous probability models because it uses the form of a growth rate model, specifically the structure of a square root type kinetic model. It models the probability of growth of a non sporeforming organism as did Genigeorgis *et al.* (1971). This is in contrast to most probability models which describe the rate of death or probability of toxin production or outgrowth of spores within a certain time (Lindroth & Genigeorgis, 1986; Lund *et al.*, 1987; Gibson & Roberts, 1989; Lund *et al.*, 1990; Whiting & Oriente, 1997).

The data used by Ratkowsky and Ross (1995) was time-limited with growth or no growth only being observed after 24 hours and not over longer periods. The treatment of the data was probabilistic, i.e. it modelled the presence or absence of growth (Ratkowsky & Ross, 1995). There is a possible limitation of using time-limited data, as growth/no growth modelling aims to predict the "absolute" probability of growth given infinite time, rather than within a specified time limit.

This new type of model may be used to give the probability of growth for given conditions. Conversely, it may be used to predict the set of conditions that give a particular probability of growth, i.e. the shape of the boundary between growth and death. This is advantageous in determining the abruptness of the boundary between growth/no growth conditions. The model also allows the prediction of probability of growth to be varied according to the level of stringency required.

The division between growth rate and probability of growth modelling is somewhat artificial due to the fact that, as growth rates decrease, the cell's growth response becomes more variable (Ratkowsky *et al.*, 1991; Ross & McMeekin, 1994; Graham *et al.*, 1996). Under extreme stress there is a growth region where the use of both models is possible. The information given by a probability model becomes increasingly relevant as conditions become less and less favourable for growth. Predictions by a growth rate model are not possible (Ross & McMeekin, 1994; Graham *et al.*, 1996) where there are observations of no growth. Also information about the conditions under which no growth is observed is not able to be used in most growth rate modelling (Ross, 1993; Graham *et al.*, 1996).

Specific no growth data are less frequently collected or published compared to compilations of growth rate data (Sutherland *et al.*, 1995). Data used in growth rate modelling can also be used in growth limit modelling, although the amount of information from each piece of data is reduced to only a binary observation i.e. growth or no growth (Appendix 3.1). However, this information can still be used to target the sets of environmental conditions under which the growth response is variable and where useful growth/no growth data could be collected.

In order for mathematical models to describe the responses of bacteria in a simple but meaningful way a greater understanding of the mechanisms underlying the responses to different conditions, why inhibition occurs and how it limits growth is needed. There is still much to be understood about how organic acid and pH inhibition occurs. A greater knowledge of the physiology of microorganisms in response to acid can help design better models and guide their use. In order to understand the physiological responses of bacteria to acid, we must first understand the basic chemistry of organic acids.

1.4 Chemistry of Acids

There are two main kinds of acids. The first, called mineral acids or strong acids, dissociate totally in aqueous solution to form their constituent ions as shown below (1.15). The addition of H^+ lowers the pH of the solution. Examples of this kind of acid are hydrochloric acid, sulfuric acid and nitric acid.

$$HA \Rightarrow H^+ + A^- \qquad (1.14)$$

where HA = undissociated form of the acid
A^- = dissociated (ionised) form of the acid e.g. chloride ions, Cl^-
H^+ = hydrogen ions

In contrast to strong acids the other type of acid, called organic or weak acids, only partially dissociate and so exist in aqueous solution as both the dissociated (ionised) form and the undissociated form as shown below:

$$HA \leftrightarrow H^+ + A^- \qquad (1.15)$$

The equilibrium of the dissociation of a weak acid is dependent on pH and can be described by the Henderson-Hasselbalch equation shown here

$$pH = pK_a + \log\frac{[A^-]}{[HA]} \qquad (1.16)$$

The pK_a of an acid is a constant although it varies slightly with temperature. It is equal to the pH at which half of the total concentration of acid is in the dissociated (ionised) form and half is undissociated. The relative amount of acid in each form changes very rapidly at pH values around the pK_a and near this pH very small changes in pH will significantly change the amount of acid in dissociated and undissociated forms. When the pH is much lower than the pK_a, almost all the acid will be in the undissociated form. When the pH is much higher than the pK_a, almost all the acid will be in the dissociated form. The pK_a of an acid is also an indication of how "strong" the acid is or how able it is to donate hydrogen ions to an aqueous solution. The lower the pK_a the stronger the acid. The ability of an organic acid to donate its hydrogen ion depends on the complex details of the types of organic groups and bonding that makes up the molecule. Organic acids were so named because they were commonly found in organic tissue.

1.5 Organic acids in Foods

Foods exhibit a wide variety of pH, as shown in Table 1.5 and Figure 1.1, although most are neutral or slightly acidic. Organic acids are important constituents of many types of food. Foods vary in the types of acids present and the relative concentrations of each acid. Lactic acid is present in meat and fish due to the metabolism of the muscle cells. It is also produced by bacteria in fermentation processes and is an important inhibitory factor in fermented foods. Other processed foods have organic acids such as vinegar (acetic acid) or lemon juice (citric acid) added as they are made, for example condiments and sauces. Fruit and vegetables intrinsically contain significant levels of organic acids.

Table 1.5 Normal pH range of general food types adapted from Jay (1992)

Food	pH	Organic Acid (mM)
Beef Meat	5.1-6.4	65-130[A]
Fish muscle (most species)	6.6 - 6.8*	65-130[B]
Dairy Products	4.5 - 6.5	10-20[C]
Vegetables	4.8 - 7.3	5-40[C]
Fruit	2.8 - 6.7	50-300[C]
Vinegar	2.4-3.4[D]	800[C]

A - (Grau, 1981), B - (Sikorski *et al.*, 1990), C - (Holland *et al.*, 1991), D - (Weiser *et al.*, 1971)
* - Much wider variations in pH may occur even within a species depending on season and feeding (Love, 1980)

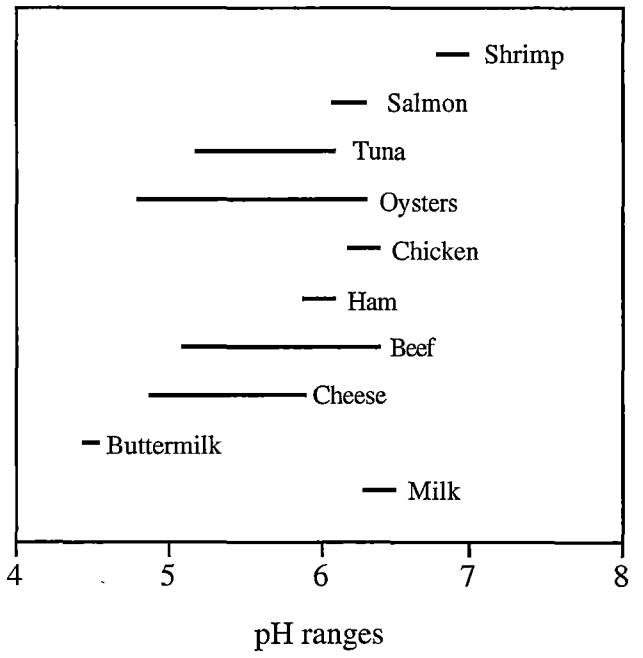


Figure 1-3 - pH range of various food taken from Jay (1992)

Organic acids are produced as byproducts of metabolism and are important in flavour and ripening in fruits and vegetables (Duckworth, 1966). Citric acid is the most abundant acid in many fruits including citrus fruits such as lemon, orange and lime, as well as black and red currants, raspberries, loganberries, strawberries, cranberries, blueberries, pineapples, pomegranates and pears. Lemons can contain over 3% citric acid. Malic acid is the principal acid in apples and most other stone fruit (such as plums, cherries and apricots) and also in bananas. In some fruits citric and malic acids are in equal amounts such as peach and gooseberry. Other acids also important in fruit are isocitric acid in blackberries, tartaric acid in grapes and citramalic acid in apple peel. Levels of acids in fruit vary during ripening mostly decreasing as fruit sweetens. However quinic and shikimic acids increase in cherries and strawberries during ripening (Duckworth, 1966).

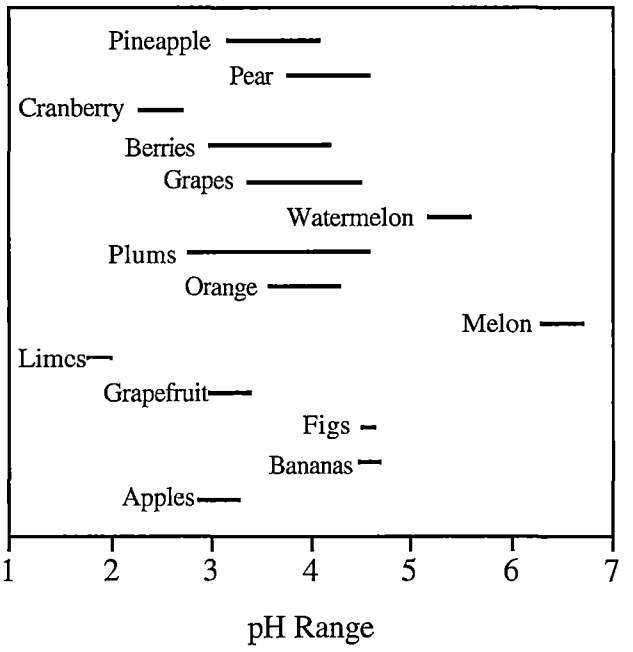


Figure 1-4 - pH range of fruits adapted from Jay (1992) and Weiser *et al.* (1971)

Vegetables also contain these same acids but usually in lower amounts and they also tend to have a higher pH (Duckworth, 1966). Another important acid in vegetables is oxalic acid which is present in large amounts in spinach and rhubarb and which can be toxic to humans due to its ability to chelate iron (Eskin *et al.*, 1971). Other organic acids are also able to chelate minerals from the diet so that they are not absorbed as well in the intestine. Although with most acids, such as lactic and citric, the effect is minor, phytic acid and oxalic acid can have significantly detrimental effects if consumed in large quantities (Ferrando, 1981).

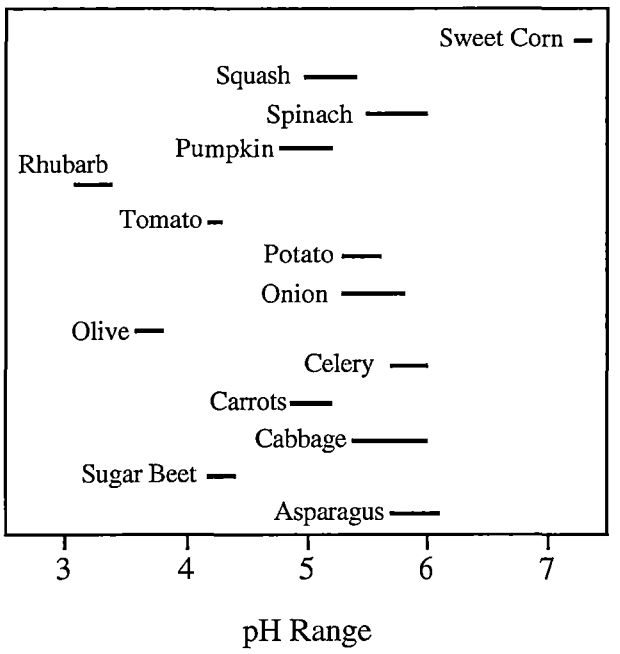


Figure 1-5 - pH range of vegetables taken from Jay (1992)

1.5.1 Types of Organic Acid in Food

Organic acids can have varying numbers of carbons and other functional groups such as hydroxyls (OH) or double bonds. The chemical properties of the common acids are shown in Table 1.6. Monocarboxylic acids are those with only one carboxylic acid (COOH) group and several are described below.

Acetic acid is used as an acidity regulator or food acid (Hanssen and Marsden, 1986). Commercial strength vinegar is usually less than 5% acetic acid. A normal solution of acetic acid is 6% with a pH of 2.36 (Weiser *et al.*, 1971).

Formic acid has been used as food preservative (Weiser *et al.*, 1971). It is produced by ants and has a characteristic smell. It is not permitted for use in Australia due to toxic effects (Hanssen & Marsden, 1986).

Propionic acid is used as a preservative against molds and fungi (Hanssen & Marsden, 1986) at levels of 2g/kg in flour products other than bread. It is considered to be nontoxic because it is a normal constituent present in the body from the metabolism of fatty acids (Weiser *et al.*, 1971).

Table 1.6 Chemical Properties of Different Organic Acids

Name of Acid	Molecular weight	pKa	[Undissociated] at pH 5 (%)	Structure
Acetic	60.1[C]	4.76[A]	35.5	CH_3COOH
Adipic	146.1[B]	4.4 / 5.6[B]	20.1	$HOOC(CH_2)_4COOH$
Benzoic	122.1[C]	4.19[C]	13.4	C_6H_5COOH
Citric	192.1[C]	3.13[D] 4.76[D] 6.40[D]	1.3	CH_2COOH (OH)-C-COOH CH_2COOH
Formic	46.0[C]	3.75[C]	5.3	HCOOH
Fumaric	116.1[C]	3.03 / 4.38[D]	1.1	HOOCCH=CHCOOH
Lactic	90.1[C]	3.86[C]	6.8	$CH_3CH(OH)COOH$
Malic	134.1[C]	3.40 / 5.05[D]	2.5	$HOOC(OH)CHCH_2COOH$
Propionic	74.1[C]	4.87[C]	42.6	CH_3CH_2COOH
Sorbic	112.1[C]	4.76[C]	36.5	$CH_3CH=CHCHCH=CHCOOH$
Succinic	118.1[C]	4.16 / 5.64[D]	12.6	$HOOCCH_2CH_2COOH$
Tartaric	150.0[C]	3.04 / 4.37[D]	1.1	$HOOC(OH)CHCH(OH)COOH$

A - (Budavari, 1989), B - (Gardner, 1972), C - (Hsiao and Siebert, 1999), D - (Dawson *et al.*, 1969)

Lactic acid is used in a wide variety of foods such as cheese, butter, egg, beer, bread, olives and relishes as an acidity regulator or food acid (Hanssen & Marsden, 1986). It is also used to enhance flavour (Shelef, 1994). Lactic acid is produced in milk by lactic acid bacteria. A Normal solution of lactic acid is 9% and has a pH of 1.9 (Weiser *et al.*, 1971).

Sorbic acid is used as a preservative against fungi (Weiser *et al.*, 1971), yeasts and moulds (Hanssen & Marsden, 1986). It occurs naturally in some fruits and it is allowed for use at varying levels (50mg/kg to 3g/kg) depending on the food type (Hanssen & Marsden, 1986).

Dicarboxylic acids have two carboxylic acid (COOH) groups and several are described below. Succinic acid, malic acid, tartaric acid, fumaric acid, adipic acid all occur naturally in various foods. All dicarboxylic acids except succinic acid are permitted to be added to foods in Australia as acidity regulators or food acids (Hanssen & Marsden, 1986). Adipic acid is also used as a nonhydroscopic raising and flavouring agent in foods (Hanssen & Marsden, 1986).

Tricarboxylic acids have three carboxylic acid (COOH) groups and the common example in foods is citric acid. This acid naturally occurs in food (lemons & oranges) and is also used as an acidity regulator and food acid in many foods (Hanssen & Marsden, 1986). A normal solution of citric acid is 6% and gives pH 1.73 (Weiser *et al.*, 1971).

Aromatic organic acids have an aromatic benzene ring as well as one or more carboxylic acid (COOH) groups. They can have sidechains off the ring with varying numbers of carbons and other functional groups such as hydroxyls (OH). Benzoic acid is an aromatic organic acid that naturally occurs in food (berries). It is used as a preservative against bacteria (Weiser *et al.*, 1971) and fungi in acidic foods (Hanssen & Marsden, 1986). It is allowed for use at varying levels (400mg/kg to 1.4g/kg) depending on the food type but may be a cause of hyperactivity (Hanssen & Marsden, 1986). Another example of an aromatic organic acid is salicylic acid. This acid is the active ingredient in aspirin. It has been reportedly used as a preservative but it is not approved for this type of use (Weiser *et al.*, 1971). Natural salicylates that are chemically similar occur in many fruits as well as cucumbers and tomatoes (Hanssen & Marsden, 1986). Cinnamic acid is another aromatic organic acid that is present in food.

Other types of acids include those that have a chlorine attached such as monochloracetic acid, previously used as a preservative, but now recognised as an irritant and prohibited by FDA (Weiser *et al.*, 1971)). Strong acids such as sulfuric and hydrochloric are also added to foods as preservatives, for example sulfuric acid is added to wines and dried fruits. Other complex organic acids such as pyroligneous acid are formed and absorbed by meat in the smoking process (Weiser *et al.*, 1971).

1.5.1.1 Chirality of Organic Acids

Some organic molecules are asymmetrical and can exists in two distinct forms, called stereoisomers, due to the presence of a chiral carbon atom in the molecule. These two forms which are nonsuperimposable mirror images called D(+) and L(-) are identical in their properties but can be distinguished by the rotation of polarised light. Chirality is important biologically as for some compounds, such as amino acids, only one form is produced. It is also important for some organic acids, for example in bacteria, where lactic acid isomeric composition can be used for classification. Different species produce different levels of lactic acid stereoisomers, either one form or the other or mixed (Benthin and Villadsen, 1995).

Normally, laboratory lactic acid is a mixture containing both stereoisomers (racemic mixture) which has a pK_a of 3.86 (de Wit and Rombouts, 1990; Shelef, 1994). There are small differences between the pK_a of the two forms D(+) and L (-) lactic acid but this variation is not large enough to be chemically significant (Budavari, 1989). However, in biological processes chirality is more important as enzymes may recognise chirality in organic molecules. Benthin and Villadsen (1995) reported a lactic acid bacteria which produces only the D-form and is more inhibited by the L-form of lactic acid. Also Huchet *et al.* (1997) reported that *Clostridium tyrobutyricum* preferentially utilises of the D form which enables a faster growth rate. The D and L lactic proportions in foods which are produced by microbial fermentation such as cheese and yoghurt (Benthin and Villadsen, 1995) can affect the taste of these foods.

1.6 Effect of Acid on the Growth of Microorganisms

The pH and acid content of a food type is important in determining the microorganisms normally present in the food (Booth and Kroll, 1989; Shelef, 1994) and is an important consideration in the growth or survival of spoilage or pathogenic microorganisms. Different types of microorganisms have distinct ranges of pH within which they are able to grow. Yeasts and moulds are able to grow at a much lower pH, (as low as 2), than most bacteria which are restricted to a pH range of 4 - 9 (VanDemark and Batzing, 1986; Booth & Kroll, 1989). Some moulds are able to grow up to a pH of 11 (Booth & Kroll, 1989). Specialised acidophilic and alkalophilic bacteria exist that can grow within a narrow range of very low (1-4) or very high pH (up to 12) beyond the range for other bacteria (VanDemark & Batzing, 1986). However these species rarely appear as spoilage or pathogenic organisms in foods.

Most types of bacteria produce organic acids as end products of their metabolism depending on the culture conditions (VanDemark & Batzing, 1986). Lactic acid bacteria, for example, are used to create many fermented food products such as cheese and yoghurt by their production of acid during growth (Abee *et al.*, 1994). They are either present originally in the food and allowed to grow (Feresu and Nyati, 1990) or, in more sophisticated food production, specific starter cultures of bacteria that have been selected for production of desirable flavours are added (Anon., 1998). Acetic acid is produced commercially from bacteria such as *Acetobacter* (VanDemark & Batzing, 1986). These bacteria have evolved specialised mechanisms of coping with high levels of acid in food (Hutkins and Nannen, 1993) and use their production of acids as a mechanism to outcompete the growth of other organisms (Anon., 1998). Some microorganisms can also use various organic acids as sole carbon sources. For example, citrate can be used by nonfecal coliforms but not *E. coli* (VanDemark & Batzing, 1986).

Organic acids are good preservatives in foods because they are effective at low concentrations, most are generally regarded as safe and can effectively inhibit the growth of many spoilage organisms. However, recent outbreaks of pathogens such as *E. coli* O157:H7 in shelf stable products have challenged the validity of presuming that prevention of spoilage also automatically gives sufficiently stringent conditions to ensure that any pathogens are not able to survive (Nicholls, 1995). This emphasises the need for a greater knowledge of the mechanisms of microbial inhibition by acids.

1.6.1 Mechanism of pH Inhibition on Microorganisms

Low pH or increased hydrogen ion concentration affects all parts of the cell. All cell components (nucleic acids, phospholipids and proteins) are pH sensitive and are only functional within a particular range of pH. DNA is reversibly damaged by low pH with growth possible following DNA repair (Booth & Kroll, 1989). It is not known whether synthesis of new macromolecular components is necessary for cell recovery from pH damage. The exterior of the cell (the outer membrane, cell wall, periplasm and the inner membrane) are more vulnerable to low pH as they are exposed to the exterior environment. These components are important for many transport systems for nutrients entering the cell and other enzymes are present in the periplasm (van Veen *et al.*, 1994). Low pH tolerant organisms (yeasts, moulds and acidophilic bacteria) have highly pH resistant enzymes on their cellular exterior (Booth & Kroll, 1989).

A large proton gradient, i.e. much lower pH outside than inside the cell, causes a passive influx of hydrogen ions. This is caused by an increase in the proton permeability of the membrane when the pH gradient across the membrane increases (Booth & Kroll, 1989). This is despite the relative impermeability of biological membranes to ions in general and protons in particular (Booth & Kroll, 1989). The influx of protons can also be counteracted somewhat by the buffering capacity of the cytoplasm of the cell. The buffering capacity varies between species but does not seem to depend on the natural environment of the organism (Krulwich *et al.*, 1985). The correct proton gradient across the membrane is necessary for many cellular processes such as ATP synthesis (Booth & Kroll, 1989). Membrane bound proton pumps help generate the protonmotive force by pumping protons across the membrane.

Hydrogen ion concentration can induce the transcription of specific proteins. Studies have shown certain proteins are necessary for growth at low pH but it is not known whether these are normal sites for growth inhibition (Booth & Kroll, 1989). The endproducts of metabolism are affected by the pH of the external medium to adjust the pH towards neutrality. There is a tendency to produce acidic end products if the medium is alkaline and alkaline end products if the medium is acidic (Gale and Epps, 1942). The membrane composition of bacterial cells is also dependent on the pH of the growth medium and may be an adaptation to low pH (Brown, 1996). Similar changes in the lipid ratio of the membrane occurs as a protective mechanism against low temperature. This is a possible explanation for the reporting of increasing survival rates under acidic rather than neutral conditions at low temperatures (Conner and Kotrola, 1995).

In the food environment low pH is not commonly present without organic acids but organic acids have their own inhibitory effects on bacteria that are separate to low pH.

1.6.2 Mechanism of Inhibition by Weak Organic Acids

Addition of organic acids lowers the pH of foods and prevents growth of bacteria (Gill and Newton, 1982). Organic acid inhibition is independent to pH because inhibition can occur at neutral pH (Houtsma *et al.*, 1994). Weak organic acids have additional growth inhibitory effects compared to strong acids because undissociated acid is lipophilic and can pass across the cell membrane (Ferreira and Lund, 1987; Young and Foegeding, 1993; Shelef, 1994). The undissociated acid should dissociate in the cell's interior if the intracellular pH is maintained at a higher level than the extracellular pH (Shelef, 1994). The dissociation of the acid inside the cell will lower the pH unless the cell removes the hydrogen ions to maintain the proton gradient across the cell membrane. If the cell does not maintain the correct proton gradient many processes, such as ATP formation, will be disrupted. The dissociated ion cannot easily diffuse out through the membrane again and so accumulates inside the cell (Houtsma *et al.*, 1994).

Undissociated acid is considered more toxic to the cell than dissociated acid (Ahamad and Marth, 1989) because antimicrobial activity increases at lower pH where undissociated acid concentration is highest (El-Shenawy and Marth, 1989). There is evidence that the dissociated acid has a lesser antimicrobial effect (Eklund, 1983; Salmond *et al.*, 1984).

Even in the presence of organic acids, organisms maintain an intracellular pH significantly higher than the surrounding medium (Young & Foegeding, 1993). The dissociation may not need to cause acidification of the cell's interior in order to inhibit growth. The inhibition may be due to the cell expending more energy to remove the extra protons and the dissociated acid from the cell (Young & Foegeding, 1993). Therefore the antimicrobial effect is not necessarily a decrease in the intracellular pH that denatures cell components such as proteins and DNA (Ita and Hutkins, 1991; Houtsma *et al.*, 1994). Greater inhibition occurs at the same intracellular pH in the presence of organic acids compared to strong acids (El-Shenawy & Marth, 1989; Glass *et al.*, 1992).

The amount of undissociated acid is a small proportion of the total acid at the near neutral pH of most foods as the dissociation constant's of most organic acids are in the range 3-4 (de Wit & Rombouts, 1990). However, small amounts (i.e. mM range) may still inhibit the growth of microorganisms.

There may also be specific metabolic effects of the undissociated acid molecule on cellular processes (Eklund, 1989; Ita & Hutkins, 1991; Houtsma *et al.*, 1994) such as respiration. Several TCA cycle intermediates are organic acids e.g. citric and malic acid. Lactic acid is also believed to alter the equilibrium of cellular reactions for which it is an end-product such as anaerobic respiration (Houtsma *et al.*, 1994; Shelef, 1994).

Organic acids have other antimicrobial effects including the ability to chelate metal ions such as iron (Shelef, 1994). The antimicrobial effect of sorbate was mitigated by additional magnesium ions suggesting chelation caused cell membrane damage (Statham and McMeekin, 1988). Organic acids can contribute to the lowering of water activity (de Wit & Rombouts, 1990; Shelef, 1994). For example, a lactic acid concentration of 100mM lowers the water activity of nutrient broth from 0.996 to 0.991. Different effects have been shown in broth and studies on food surfaces, indicating a possible effect of bacterial attachment to surfaces on the response to organic acids (Shelef, 1994). Many cell processes are affected by pH to differing degrees suggesting that pH inhibition involves a very complex system of biochemical interactions. Therefore, the fact that no single explanation for the inhibitory effects of organic acids has been found (Shelef, 1994) is not surprising.

1.6.3 Differences in Inhibition by Different Organic Acids

Different types of acid have greatly varying antimicrobial activities on bacterial cells. Antimicrobial activity is not purely dependent on the pK_a as different acids with similar pK_a values can have very different antimicrobial effects (Eklund, 1989). The growth rate is dependent on the intracellular pH and different acids lower the intracellular pH to different extents. This may be due to differences in : (i) the permeation on the undissociated molecule into the cell (de Wit & Rombouts, 1990) and (ii) the molecule's effect on transport or metabolism (Eklund, 1989).

Parabens, derivatives of para-aminobenzoic acids, are the most inhibitory organic acids followed by benzoic and sorbic acids (Eklund, 1989). The other common organic acids such as acetic, lactic, propionic and citric are all less inhibitory (Eklund, 1989), but in contrast they are "generally regarded as safe" (GRAS) in foods and are not linked to adverse human health effects. Therefore many recent studies have compared only the inhibitory effect of these GRAS organic acids (Rosso *et al.*, 1997).

The usual order of inhibition of these acids reported is acetic is more inhibitory than lactic which is more inhibitory than citric acid. This is for inhibition at the same total concentration of acid and using the organic acid alone to acidify the medium. This order has been found for *Clostridium botulinum* (McClure *et al.*, 1994), *E. coli* (Conner & Kotrola, 1995), *Listeria* (Ahamad & Marth, 1989; Sorrells *et al.*, 1989; Young & Foegeding, 1993), *Salmonella* (Perales and Garcia, 1990) and *Yersinia enterocolitica* (Brockelhurst and Lund, 1990; Adams *et al.*, 1991; Little *et al.*, 1992b). It is a shortcoming of those studies of the effect of organic acids on growth that they do not attempt to separate the effect of pH from

the effect of the organic acid. Another shortcoming is that inhibition is described merely as one measure of its inhibitory effect, for example the minimum pH at which growth occurs when the media is acidified with a particular acid (Rosso *et al.*, 1997). In contrast, this thesis and other studies (Tienungoon *et al.*, 1999) measure the maximum growth rate and minimum pH at which growth occurs over a range of total acid concentrations. This enables a complete and unambiguous picture of the pattern of inhibition to be developed.

Single measures of organic acid inhibition can be ambiguous or confusing. For example, the order of inhibition previously described is reversed when represented in terms of undissociated acid. This is because the dissociation constants of the three acids are in the reverse order (Table 1.6) while at the same pH there is less undissociated citric acid than undissociated lactic acid which is also less than undissociated acetic acid (Young & Foegeding, 1993). Similarly, Conner *et al.,* (1990) found that acetic acid was inhibitory at 44 mM undissociated which was greater than citric at 3 mM undissociated and lactic acid at 2 mM undissociated. However, using the value for the pK_a of lactic acid 3.86 (Dawson *et al.*, 1969; Budavari, 1989) rather than 3.1 (used by Conner *et al.*, (1990)), the undissociated concentration of lactic acid is 8 mM which gives the same order as Young and Foegeding (1993). In many studies of the effect on growth of organic acids the concentrations of acid are not calculated or recorded, which makes comparison difficult.

There have been contradictory results from studies to determine the effect of different organic acids on the intracellular pH of *Listeria.* Sorrells *et al.,* (1989) found that acetic was most effective at lowering the intracellular pH compared to citric acid. In contrast Ita and Hutkins (1991) found that citric acid lowered the pH_i more than acetic acid. Young and Foegeding (1993) suggested that the order of effectiveness of the extra inhibitory effect of acids (greater than pH lowering) was acetic greater than lactic greater than citric acid.

Molecular weight is also important in determining the relative inhibitory effect of organic acids (Table 1.6). As molecular weight increases there is a decreasing degree of permeability across the membrane (Abdul-Raouf *et al.*, 1993a). However, organic acids are lipophilic and it is not known whether this would significantly affect their transfer across the membrane (Booth & Kroll, 1989). For some bacteria there is evidence for regulated transport of organic acids such as acetic and lactic acid across the membrane (Russell, 1992). This may be an important method for maintaining cell growth and viability by removal of these acids when they reach toxic concentrations. Bacteria have many interconnected biochemical systems that can be used to regulate their internal environment for many conditions including pH.

1.6.4 pH homeostasis

Many cell processes are regulated and all cell processes are affected by the intracellular pH (pH_i) environment of the cell. The external pH environment which bacteria can experience varies to a degree that would be lethal if reflected by changes to the internal environment. In most bacteria the optimal pH of the interior of the cell is alkaline compared to the exterior (Booth, 1985) and maintenance of neutral or slightly alkaline intracellular pH (pH_i) is required for rapid growth (Harold and van Brunt, 1978). The impermeability of the cell's lipid bilayer to protons allows the cell to actively extrude protons from the cytoplasm to generate energy, without a large passive influx of protons back into the cell (Booth, 1985). The other main cellular processes that change the pH of the cytoplasm are the production of acids and bases by the cell's metabolism (Booth, 1985). Yeasts, moulds and other organisms can grow at very low pH because of their low optimal pH_i (Booth & Kroll, 1989).

Ionophores, which equalise the extracellular and intracellular pHs, can be used to determine the optimal pH_i range for a species. Gramicidin D allows exchange of monovalent cations (H+, Na+ and K+) thereby causing the ion gradient to collapse. In the presence of gramicidin growth occurred only when the external pH was maintained within the narrow neutral range that is equal to the pH_i range over which growth is normally observed (Harold & van Brunt, 1978). For example, *E. coli* a well studied example of a neutrophilic bacterium, has an optimal pH_i of 7.5 to 8.2 (Padan *et al.*, 1981; Booth & Kroll, 1989). High growth rates are still possible at pH_i as low as 6.5 although growth rate rapidly decreases to a lower limit at a pH_i of 6. This indicates a relatively high level of tolerance to changes in internal pH (Booth & Kroll, 1989). As the pH becomes more stressful, the cell must use increasing amounts of energy to maintain its internal environment. Eventually the pH conditions require so much energy to maintain homeostasis that growth is limited (Brown and Mayes, 1980). The optimal range of values of pH_i and its sensitivity to perturbation varies between species (Booth & Kroll, 1989).

Cells maintain internal pH as external pH rises until the difference in pH across the membrane (ΔpH) becomes so large it cannot be maintained. The ΔpH starts to collapse and cell viability is impaired (Hutkins & Nannen, 1993). Most bacteria can only tolerate small reductions in pH_i (e.g. 1 pH unit less than optimal) (Booth, 1985). The cell maintains a constant internal pH by a variety of mechanisms both passive and active (Booth, 1985). *E. coli* maintains its intracellular pH using proton pumping, sodium/hydrogen antiporters, other sodium dependent symporters and potassium/hydrogen antiporters (Padan *et al.*, 1981). This variety of interrelated and interdependent systems allows *E. coli* to tightly regulate its intracellular pH under the variety of environmental conditions it encounters.

Sodium/hydrogen antiporters have been linked to the regulation of intracellular pH of *E. coli* under alkaline conditions (Kroll and Booth, 1981). Potassium fluxes have been shown to be important for the maintenance of intracellular pH under acidic conditions. Weak organic acids have been shown to affect the rate of potassium uptake into potassium depleted cells (Bakker and Mangerich, 1983). In cells depleted of potassium, uptake of potassium via transport systems leads to a rise in intracellular pH, while the other component of the protonmotive force, the membrane potential, falls (Kroll & Booth, 1981). The decrease in the rate of potassium uptake was found to correlate to the intracellular pH and was independent of the type of organic acid used (Bakker & Mangerich, 1983). This implies that the intracellular pH of the cells, as part of the total proton motive force, is a factor controlling the rate of potassium uptake by *E. coli* cells (Bakker & Mangerich, 1983). It is suggested that in *E. coli* cells with sufficient potassium a cycling of potassium, closely linked with the hydrogen fluxes, is part of the system to maintain intracellular pH (Kroll & Booth, 1981). The main mechanism of inhibition of potassium uptake by weak organic acids was suggested to be the inactivation or lower turnover of the transport system carrier molecule caused by the low intracellular pH (Bakker & Mangerich, 1983). Potassium uptake is unlikely to be a limiting factor for growth of *E. coli* at low pH as induction of other transport systems occurs at low levels of potassium (Bakker & Mangerich, 1983).

At lethally low pH values, derangement of membrane structures of the cell and solute leakage from the cell occurs. Damage to the cell membranes leads to the release of magnesium ions, the degree of membrane damage caused by acidification appears to vary among organisms and is correlated with the degree of acid tolerance (Hutkins & Nannen, 1993). A reduction in growth rate of *Lactococcus lactis*, as a measure of cell damage, is correlated with the pH at which the specific activity of enzymes such as hexokinase and acetate kinase was reduced (Harvey, 1965).

Many aspects of the cell's interaction with the external environment are controlled by the pH. External parts of the cell, such as the transport and signaling proteins and the outer membrane or cell wall, must be tolerant to the low external pH or be rapidly replaced when damaged. There is evidence that the pH sensor of bacteria is external, as changes in pH can be "sensed" without a change in internal pH (Neely *et al.*, 1994). Exposure to acidic environments changes the synthesis of outer membrane porins (Foster and Spector, 1995) possibly leading to the increased resistance of the cell to the outer environment. The internal pH of the cell regulates many transport processes including ions such as sodium, potassium and phosphate, as well as uptake of amino acids and peptides (Hutkins & Nannen, 1993). Inhibition of the transport of essential nutrients could be a mechanism by which pH inhibits growth of some organisms.

1.7 pH Response of *E. coli*

E. coli is used in this thesis as an example of an important food pathogen with a low infective dose. The reported pH growth limits of *E. coli* vary, for example, from a lower limiting pH of 4.5 (Gale & Epps, 1942) to 4.4 (VanDemark & Batzing, 1986; Desmarchelier and Grau, 1997). Also, an optimum pH range for growth of 6-7 has been reported (VanDemark & Batzing, 1986). Literature values of the maximum pH permitting growth vary more widely from a pH of 9 (Gale & Epps, 1942; VanDemark & Batzing, 1986; Glass *et al.*, 1992) to 10 (Desmarchelier & Grau, 1997).

Most studies on the growth response of *E. coli* to pH and acid have been performed in food and few have been done in laboratory media. The exception often cited is a study by Glass *et al* (1992). They found for *E. coli* O157:H7 in tryptose soy broth, lactic acid was more inhibitory at each equivalent pH than was HCl. When lactic acid was the acidulant no growth was observed at pH 4.5. For hydrochloric acid growth was observed at pH 4.5 but not at pH 4.0.

Many foods involved with pathogenic *E. coli* strain outbreaks have been investigated in order to determine the potential growth, survival or decline of *E. coli.* Acidic foods are of particular interest, since studies have shown that *E. coli* is able to survive for longer periods than previously presumed in, for example, mayonnaise (Weagent *et al.*, 1994). The U.S. Food and Drug Administration (Anon., 1993) does not regard foods with pH below 4.6 to be potentially hazardous. Yet, because of its low infective dose and long survival time *E. coli* has been able to cause food poisoning in these high acid/low pH foods (Besser *et al.*, 1993; Morgan *et al.*, 1993).

As shown in Table 1.7 there is considerable variation in pH within samples of the same food types. The disadvantages of many of the food studies is that although they may describe exactly what happens in a particular food sample, they do not take into account the effect of variations and how these might affect bacterial growth and survival. Other variables in the food such as the difference between "real" mayonnaise and reduced calorie mayonnaise have been found to affect the survival rate of *E. coli* (Hathcox *et al.*, 1995). Using this type of study alone, separate studies on each different individual food would be necessary in order to give the necessary information covering the whole range of possible conditions for that type of food. These studies often only cover a narrow range of conditions so their results cannot provide information about growth limits (Abdul-Raouf *et al.*, 1993a).

Table 1.7 Studies on the Growth and Survival of *E. coli* in Foods

Food Vehicle	Food pH	Reported growth or survival of *E. coli*	Refs
Beef	5.7- 6.4	longer lag before growth at lower pHs at 25°C	1
Ground Roast Beef	6.1	survival at 10^5 cfu/ml for 72 hours at 5°C growth at 21°C & 30°C	2
Fermented Meat	4.8	decline 2 log cfu/ml from 5 x 10^4 cfu/ml during fermentation and 18 days drying and storage at 4°C for 2 months	3
Processed Cheese Slices	5.6-5.8	decline from 10^3 to 10^1 cfu/ml in 36 hours at 30°C (N.B. a_w 0.92-0.93)	4
Cheddar Cheese	4.9-5.2	survival from inoculum level 10^3 cfu/ml for 60 days at 6-7°C (N.B. a_w 0.95)	5
Mayonnaise/Dressings	3.2-3.9	survival from inoculum level 10^6 - 10^7 cfu/ml for <3 days at 25°C & for >35 days at 7°C for <4 days at 22°C & for 17 days at 4°C	6, 7, 8
		survival from inoculum level 6.5x10^3 cfu/ml for 21 days at 20°C & 55 days at 5°C	9
		survival from inoculum level 1x10^2 cfu/ml for <4 days at 30°C & <7 days at 20°C for <58 days at 5°C	10
Yoghurt	4.4-4.6 6.6 initial	decline from $10^{3.5}$ to $10^{2.7}$ cfu/ml in 7 days decline from 10^7 to 10^5 cfu/ml in 7 days	11
Apple Cider/Juice	3.4-4.1	survival from inoculum level 10^5 cfu/ml < 7 days at 25°C & for 7 to 31 days at 8°C	12, 13
		survival from inoculum level 3x10^4 cfu/ml for 14 to 21 days at 4°C	14
Salads Vegetables	7.0-7.4	growth at 12°C & 21°C survival at 5°C for 14 days from 10^5 cfu/ml	15
Cantaloupe/Melon	6-7	growth at 25°C survival at 5°C from 10^3 cfu/ml decline on rind at 5°C from 10^5 cfu/ml >3 log reduction in < 7 days	16
Soy Sauce	4.5	survival at 10^5 cfu/ml for 10 days at 8°C decline 1-3 log cfu/ml in 10 days undetectable from 10^5 cfu/ml in 9 days at 30°C	17

1 - (Grau, 1983), 2 - (Abdul-Raouf *et al.*, 1993a), 3 - (Glass *et al.*, 1992), 4 (Glass *et al.*, 1998), 5 - (Reitsma and Henning, 1996), 6 - (Erickson *et al.*, 1995), 7 - (Weagent *et al.*, 1994), 8 - (Raghubeer *et al.*, 1995), 9 - (Zhao and Doyle, 1994), 10 - (Hathcox *et al.*, 1995), 11 - (Massa *et al.*, 1997), 12 - (Besser *et al.*, 1993), 13 - (Zhao *et al.*, 1993), 14 - (Miller and Kaspar, 1994), 15 - (Abdul-Raouf *et al.*, 1993b), 16 - (Rosario and Beuchat, 1995) & 17 - (Masuda *et al.*, 1998).

Even within the same food there can be considerable variation in pH values. Many foods are not homogeneous but are mixtures of different separate ingredients (a sandwich or salad, for example) and even apparently homogenous foods such as mayonnaise are separated into aqueous and oil droplets much larger than the microorganisms within them. This requires compromises in order to attempt to study growth in these foods. Abdul-Raouf *et al.,* (1993a) inoculated *E. coli* into a roasted beef and mayonnaise slurry, a homogenised mixture that is very different in physiochemical conditions from the form of food which it was meant to represent.

The results of food studies can be summarised as follows. If conditions all favour growth of *E. coli*, i.e. pH > 4, temperature > 8°C and water activity > 0.950, growth will occur most rapidly at conditions close to optimal i.e. pH 7, temperature 40°C and water activity 0.995. As the conditions tend towards these optima, growth rate is increased and lag time decreased. If any condition is below these ranges, for example pH < 4, cells will not grow, some will survive, others will die. If there are organic acids present in the food, decline in cell numbers can occur at pH > 4 for example in yoghurt, cheese, fermented meats and soy sauce, at a rate depending on the concentration of acid and the other conditions such as water activity. Decline in cell numbers will occur more rapidly at higher temperatures (even within the growth range) (Shadbolt *et al.*, 1998) even if it is another condition, such as pH, organic acid or water activity that is below the growth range. Death can also occur at temperatures above the growth range (temperature >48°C) such as when a food is cooked.

The valuable information that cannot be determined from these studies is the effect of combinations of conditions, both within and outside the growth range, such as where neither water activity nor pH is lethal but through combined inhibition by each growth is not possible. Studies in laboratory media can provide a method of readily determining growth rate and its variation over a wide range of conditions so that generalisations about the trends in growth rate responses and the limits of growth can be rigorously determined.

Food studies can provide valuable information in specific cases, for example *E. coli* O157:H7 was found not to contaminate eggs by the transovarian route (the means by which *Salmonella* infects eggs) so eliminating the risk infection of eggs via poultry carrying the pathogen (Schoeni and Doyle, 1994). Proactive studies have been made of foods that have not yet been linked to *E. coli* O157:H7 infections (Abdul-Raouf *et al.*, 1993b; Rosario & Beuchat, 1995) but are presumed to be a risk due to previous outbreaks of salmonellae in these foods. Also studies within food can describe the interaction of different bacterial species within the food such as by using antibiotic resistant or bioluminescent strains. These artificially inoculated organisms can be selectively cultured or differentiated from other organisms that may be present on the food (Blackburn and Davies, 1994; Tomicka *et al.*, 1997).

In order to cover the full range of conditions, including pH, that may be encountered in food, studies on the growth rate and survival of organisms should be rigorously carried out in laboratory media. The results of these studies can then be validated in the different food types of concern much more easily once the growth responses under laboratory conditions are known (McMeekin *et al.*, 1993).

Fermentation processes occurring in meat products should be stringent enough to prevent the growth of pathogens and spoilage due to a combination of drying, lowering of pH and production of lactic acid. However several outbreaks of HUS have been linked to fermented meat products both in Australia (Eyles, 1995; Nicholls, 1995) and in the U.S. (Alexander *et al.*, 1995). *Salmonella* has also been linked to outbreaks from salami type products (D'Aoust and Evans, 1978; Taplin, 1982; Cowden *et al.*, 1989) and *E. coli* has been found to have similar or greater resistance to these conditions (Harrison and Harrison, 1996; Ellajosyula *et al.*, 1998). The reduction in water activity, due mainly to drying, is the most stringent condition for *E. coli* in fermented meats as it is below the growth permissive range for water activity (Lee and Styliadis, 1996). The pH of all salamis surveyed is within the growth range of *E. coli*, although depending on the concentration of lactic acid, this condition may also be lethal (Lee & Styliadis, 1996). Meat products such as jerky rely solely on drying (Faith *et al.*, 1998). Legislation has now been introduced in the U.S. to make fermentation and drying processes (such as those that make meat jerky) achieve a mandatory 5 log reduction in *E. coli* in order to be safe. However in practice this level of reduction can be hard to achieve (Hinkens *et al.*, 1996; Nickelson *et al.*, 1996; Shadbolt, 1998). An added complication to this situation is that organisms such as *E. coli* are not only strongly acid resistant, but can adapt during a mild stress to become much more resistant to a lethal acid stress as discussed in section 1.8 below.

1.8 Acid Resistance and Adaptation in *E. coli*

Acid resistance is defined as the survival of stationary phase cells at extreme low pH, lower than the minimum growth permitting pH (Small *et al.*, 1994). Gastrointestinal pathogens with a low infective dose are often found to be very acid resistant for example *E. coli* (Miller & Kaspar, 1994; Benjamin and Datta, 1995).

By contrast acid adaptation is the induction of mechanisms at moderately low pH that promote subsequent survival at extreme low pH. Acid adaptation occurs in *E. coli* (Goodson and Rowbury, 1989; Arnold and Kaspar, 1995; Benjamin & Datta, 1995; Leyer *et al.*, 1995), in *Salmonella* (Foster and Hall, 1991; Leyer and Johnson, 1992; Foster, 1993; Humprey *et al.*, 1993; Lee *et al.*, 1994) and in many other organisms (Brown, 1996).

Conditions in which bacterial metabolism is slowed promote acid tolerance. In *E. coli*, stationary phase cells are much more tolerant to low pH than rapidly growing cells (Arnold & Kaspar, 1995; Benjamin & Datta, 1995). Similarly, starvation (Arnold & Kaspar, 1995) and low temperatures (Miller & Kaspar, 1994; Raghubeer *et al.*, 1995) also increase acid tolerance. The presence of sodium or phosphate ions and the extent of aeration affect the acid sensitivity of cells (Rowbury, 1995). The attachment of bacterial cells greatly decreases their sensitivity to acid treatment (Poynter *et al.*, 1986; Rowbury, 1995). During meat decontamination, acid sprays were found to be effective if used within a short time of slaughter (Dickson, 1991) and ineffective if the decontamination procedure was delayed (Brackett *et al.*, 1994).

There are cross protection effects due to non specific global stress responses. Acid adapted cells have increased resistance to other stresses such as thermal and osmotic stress (Leyer and Johnson, 1993). At 4°C, there was greater survival rate of *E. coli* observed in acidified media than in a non-acidified control (Conner & Kotrola, 1995). This suggests that there are adaptations that are beneficial both as an adaptation to low temperature and as an adaptation to low pH. Possibly these are changes in cell membranes (Booth & Kroll, 1989).

Acid habituation in *Salmonella* involves the synthesis of new proteins under specific conditions (Foster, 1993; Humprey *et al.*, 1993). Additional mechanisms exist in *E. coli* and there is a wide variation in the acid tolerance of various strains (Cutter and Siragusa, 1994; Arnold & Kaspar, 1995; Brown, 1996).

The significance of acid adaptation in *E. coli* is myriad. Acid adaptation causes greater survival rates of *E. coli* in foods where pH is a significant lethal factor such as meat fermentation, apple cider and salami manufacture (Leyer *et al.*, 1995). This suggests the use of healthy growing exponential cultures as inocula in food challenge testing is inappropriate and may give falsely rapid death rates. The large number of influences on acid tolerance gives rise to a need for the use of consistent culturing conditions so that different studies can be directly compared (Rowbury, 1995).

There is also a clinical implication. If *E. coli* undergoes sub-lethal pH stress in acidic foods this will then allow it to survive the gastric acidity barrier in greater numbers and gives a greater chance of causing disease. The gastric barrier is one of the primary defenses of the body against infection by bacteria in food (Peterson *et al.*, 1989) and low pH is used by the phagocytes of the immune system to kill bacteria. Any mechanism of adaptation of bacteria to overcome these barriers in the host's immunity significantly increases the pathogenicity of such an organism (Gahan and Hill, 1999). *E. coli* is already a serious foodborne pathogen that causes significant disease and mortality. More knowledge about *E. coli* inhibition by organic acids and pH is needed to help combat the increase and spread of this problem.

2. GROWTH RATE EXPERIMENTS AND MODELLING

2. Growth rate experiments and modelling

2.1 Summary

Existing data (Presser, 1995) for *Escherichia coli* strain M23 were used to develop a new model for lactic acid inhibition of growth rate containing a term for inhibition by high pH. This model had a better fit than the previously published models without the high pH term (Presser, 1995; Presser *et al.*, 1997) despite almost all the data being at pHs less than 8. At most acid concentrations there was a decrease in growth rate at pH higher than 7, therefore the high pH term fitted this trend in the data. The high pH response of *E. coli* M23 was determined at 37°C. Little decline in growth rate was observed below a pH of 9.2 but complete inhibition of growth occurred at a pH of 9.5.

The growth rates of four pathogenic and six nonpathogenic strains of *E. coli* were determined under a range of pH conditions (3.8-8.1). Models were fitted for the response of each strain to low pH. Measurable differences were found for estimates of pH_{min} and for the model constant (c) which reflects the fitted maximum growth rate. For pathogenic strains R10, R31, R91, R172 and nonpathogenic strains BR and MJR the data showed a systematic deviation from the model; the model overpredicted at lower pH (pH < 5) but underpredicted at higher pH (pH 5-8). The addition of a constant factor into the term for pH gave a better fit of the model to the data. This was in contrast to other strains where addition of this factor did not provide a better description, for example M23. For strain BR high pH inhibition was observed and addition of a high pH term was needed to fit the data.

The growth rate response to pH in the presence of acetic acid was determined for *E. coli* M23. A model for the response of strain M23 to inhibition by acetic acid and low pH was developed. This model was similar to the model developed for lactic acid inhibition. However, the term for dissociated acid required adaptation and several difficulties were found modelling the new data. Several datapoints were found to have excessive influence in the modelling process and had to be removed in order to find the best fit. Also some growth rates had to be estimated from the data using linear estimation rather than fitting the modified Gompertz equation by non-linear regression. Growth rates measured at low water activity and pH combinations added a further dimension to the data and allowed more accurate modelling of the growth rate response.

2.2 Introduction

In foods, pH is one of the important environmental constraints that can reduce the growth rate of potential spoilage or pathogenic microorganism. In many foods low pH is due to the presence of organic acids, either present endogenously (such as malic acid in apples, lactic acid in meat or fish) or produced in the food by microorganisms by metabolic processes such as fermentation, which usually produces lactic acid. While temperature is most often the primary variable controlling growth rate in food, it is also important to be able to predict the effect that pH and organic acid content will have on the growth rate of microorganisms. There are a large variety of low pH foods such as cheese and dairy products, fruit and fruit juice, mayonnaise and other fermented products such as salamis. In some of these foods, low water activity is also an important constraint to microbial growth. Most food has a pH less than 7, the only common alkaline food being egg white.

Some studies have tested the growth rate of microorganisms within food. While this approach is a necessary final step to validate laboratory media results, it does not readily allow for the systematic variation of each environmental factor. Therefore it alone cannot be used to develop a complete description of how each environmental factor affects the growth rate. In contrast, experiments using laboratory media can measure the response to specific environmental factors over a range of environmental conditions. This information can then be used to develop mathematical models which provide a concise description of this response. Once a model has been obtained, the response to variation can be predicted and the results of the model validated in real foods. Using a mathematical model allows the prediction of the effects of variations in environmental factors, without large scale experimentation, and also defines the region over which such predictions can be safely made.

During the process of fermentation there is a slow gradual increase in organic acid content and a consequent slow decrease in pH. As yet models do not exist which will predict the effect on pathogenic microorganisms of a gradually decreasing environmental pH and an increasing acid content in a fermented food product. However, this is an important area for further study.

This chapter describes the development of models which describe the inhibition of growth rate by pH in the range 3.8 to 8.1 for individual strains of *E. coli*, both pathogenic and non pathogenic. Models for the inhibition by combinations of low pH and acetic acid (0-800mM) or lactic acid (0-500mM) are also developed similar to previous models for combinations of low pH and lactic acid (Presser, 1995). This includes the recent development and addition of terms for inhibition by high pH.

2.3 Materials and Methods

Growth media were prepared differently for each type of experiment (see 2.3.1). The remainder of the protocol (2.3.2 - 2.3.5) was the same for all experiments. Details of the media, equipment and bacterial strains used are listed in Appendix 1.

2.3.1 Media Preparation

Experiment 1 - Growth Rate at low pH for Ten Different Strains of *E. coli*

Overstrength Nutrient Broth was made, for example 6.5 g sufficient to make up 500ml was made up with only 400ml of distilled water. The broth was divided by weight into two flasks and adjusted to pH 3 or 4 and pH 7 or 8.5 using concentrated hydrochloric acid and sodium hydroxide solutions. The broths were made up to the correct final volume (e.g. 250ml) using distilled water. For experiments where some broths contained organic acids, all broths were filter sterilised. For experiments where broths did not contain organic acids the broths were able to be sterilised by autoclaving.

For each condition (1-4 per experiment) a series of broths representing a pH gradient were obtained by mixing different proportions of the two broths described above. A total of 15ml of broth was placed in each L-shaped spectrophotometer tube (L-tube - Appendix 1). Calibration curves were used to determine the volume of each broth needed to give the desired distribution of pH values for each condition. After vortex mixing the L-tubes, 0.5ml samples were taken from each, placed in a sterile well plate (well volume ~3ml) and the pH measured using a pH meter with a flat bottomed pH electrode. The L-tubes were placed in the temperature gradient incubator (TGI - Appendix 1) overnight at 20°C to help to determine if any were contaminated. Contaminated tubes were discarded prior to beginning the experiment. These growth rate datapoints are presented in Appendices 2.3 and 2.4.

Experiment 2 - Growth Rate with low pH and low water activity

The method for Experiment 1 was employed with the following modifications. Sodium chloride (NaCl) was added to broths at the beginning of the preparation to obtain the water activities listed in Table 2.1. Sets of broths (as described above) were made for each water activity being tested. These growth rate datapoints appear in Appendix 2.1.

Table 2-1 NaCl Added to Nutrient Broth and Water Activities

Water Activity (final 500ml nutrient broth)	NaCl Added (%wt/vol)
0.955	7.00
0.965	5.25
0.975	3.50
0.985	2.00
0.996	0 (0.50% present in nutrient broth)

Experiment 3 - Growth Rate with suboptimal pH and Acetic Acid

The method for Experiment 1 was employed with the following modifications. Acetic acid was added to broths at the beginning of the preparation to obtain the total acetic acid concentrations (AAC) listed in Table 2.2. Sets of broths (as described above) were made for each acetic acid concentration. The broths in each flask were individually filter sterilised. Calibration curves were determined and used for each concentration of acetic acid. These growth rate datapoints are given in Appendices 2.1 and 2.2.

Table 2-2 Acetic Acid added to 500ml Nutrient Broth and Final Total Acetic Acid Concentrations

Total acetic acid concentration (mM) (in final 500ml nutrient broth)	Acetic acid added to nutrient broth (g of 88%w/w)
0	0.000
5	0.148
10	0.296
15	0.444
20	0.592
25	0.740
50	1.480
100	2.960
200	5.920
400	11.800
800	23.700

Experiment 4 - Growth Rate with high pH at 37°C

The method for Experiment 1 was employed with the following modifications. The broth was adjusted to pH 7 or pH 11 using a concentrated sodium hydroxide solution (or hydrochloric acid solution if necessary). Calibration curves were determined and used as previously. These growth rate datapoints are presented in Appendix 2.5.

2.3.2 Preparation of Inoculum

A loopful of culture was transferred from a Nutrient Agar slope to 60ml of Nutrient Broth in a 200ml conical flask. This was incubated statically at 37°C for approximately 16 hours. A level of inoculum was chosen to give an initial Percent Transmittance (%T) of between 80 and 90%T. Typically this was between 0.5 and 1ml of the overnight culture added to 15ml of broth in each L-tube. Samples for pH determinations were taken aseptically from all L-tubes after inoculation for all experiments. For some experiments, pH readings were taken at intervals as growth occurred by aseptic removal of 0.5ml samples. For some other experiments, samples for pH readings were also taken at the end of growth.

2.3.3 Growth Rate Determinations

Most growth rate experiments were performed using a TGI operated isothermally at 21°C ± 1°C (or in one experiment at 37°C). In isothermal experiments 60 different combinations of other conditions were tested simultaneously. Growth was monitored by measuring %T of each tube at 540nm using a spectrophotometer (Appendix 1) with 100%T set using sterile Nutrient Broth. The calibration was checked at intervals throughout the experiment. Readings were taken for a total of 21 days at intervals of approximately 5%T change until the %T fell to 5% or stopped decreasing. The temperature of each tube was measured 3-5 times, at different times of day, after the completion of the experiment and the average temperature calculated.

2.3.4 Calculation of Generation Times and Growth Rate

The %T readings were recalculated as the change in %T since time zero, (Δ%T) and the time as that elapsed since inoculation (Δt). A SAS PROC NLIN (SAS Institute Inc., 1989) routine, written by D. G. McPherson (Mathematics Dept., University of Tasmania) was fitted to the modified Gompertz function (Eqn 1.1) with the following parameters redefined below.

A = lower limit of detection of the spectrophotometer or Δ%T of initial microbial load.

D = difference in value of upper and lower limits of sensitivity of the spectrophotometer.

M = time at which rate of change of Δ%T is maximal.

B = maximum rate of change of Δ%T.

The fitted values were used to calculate generation times (Ross, 1993) according to Equation (2.1) below where B and D are defined as previously.

$$Generation\ Time = \frac{66.59}{BD} \qquad (2.1)$$

As a rule of thumb consistent estimates of generation time by this method require that the converged estimates of B and D are obtained within 10 iterations (McMeekin *et al.*, 1993). If the growth curve data did not allow this, the generation time (G.T.) was estimated by linear regression of those points on a graph of Δ%T versus time considered to represent the exponential phase of growth (G.T. was determined as the minimum time taken for a change of 24.5 %T).

2.3.5 Analysis of Growth Rate Models

The following general square root model (Eqn 2.2) was developed for suboptimal temperature, suboptimal water activity, low and high pH and the presence of organic acid (Presser, 1995; Presser *et al.*, 1997). It is similar to another model (Ross, 1999) but does not contain terms for superoptimal temperature, superoptimal pH or superoptimal water activity. The square root of growth rate is used to homogenise the variance of the growth rate data.

$$\sqrt{k} = c(T - T_{\min})\sqrt{(a_w - a_{w\min})}\sqrt{1 - 10^{pH_{\min} - pH}} \times \sqrt{1 - \frac{TAC}{U_{\min}(1 + 10^{pH - pK_a})}}\sqrt{1 - \frac{TAC}{D_{\min}(1 + 10^{pK_a - pH})}} + e \qquad (2.2)$$

where:

k = growth rate (1/generation time in minutes).

c = constant of proportionality.

a_W = water activity.

$a_{W\min}$ = theoretical minimum water activity for growth.

T = temperature.

$T_{\min}$ = a notional lower value of temperature where growth rate is predicted to be zero.

$pH_{\min}$ = a theoretical minimum suboptimal pH which prevents growth.

TAC = total concentration ([]) of organic acid = [undissociated] + [dissociated].

$D_{\min}$ = theoretical minimum [dissociated organic acid] required to prevent growth.

$U_{\min}$ = theoretical minimum [undissociated organic acid] required to prevent growth.

pK_a = dissociation constant - lactic acid = 3.86, acetic acid = 4.76 (Budavari, 1989).

e = error term.

The model was developed using terms for suboptimal temperature $(T\text{-}T_{\min})^2$ (Ratkowsky *et al.*, 1983), suboptimal water activity $(a_W\text{-}a_{W\min})$ (Chandler and McMeekin, 1989) and more recently developed terms for low pH $(1\text{-}10^{pH_{\min}\text{-}pH})$, undissociated (term containing $U_{\min}$) and dissociated organic acid (term containing $D_{\min}$) (Presser, 1995; Presser *et al.*, 1997).

A new term for high pH inhibition ($1-10^{pH-pH_{max}}$) has been added (for derivation of new term see Appendix 5.2). The pH_{max} term was included in this study when it was necessary to describe the response of the data. The addition of the pH_{max} term was found to improve the fit for other data where the inhibition by high pH was less obvious. The new pH_{max} term is the converse of the low pH term, and describes inhibition under conditions of low concentrations of H^+ and high concentrations of OH^- as shown below.

$$\sqrt{k} = c(T - T_{min})\sqrt{(a_w - a_{w_{min}})}\sqrt{1 - 10^{pH_{min} - pH}}\sqrt{1 - 10^{pH - pH_{max}}} \times \sqrt{1 - \frac{TAC}{U_{min}(1 + 10^{pH - pK_a})}}\sqrt{1 - \frac{TAC}{D_{min}(1 + 10^{pK_a - pH})}} + e \qquad (2.3)$$

pH_{max} is a theoretical maximum superoptimal pH which prevents growth and other terms are as previously defined for Eqn 2.2

The growth rate models (Eqn 2.2 & 2.3) were fitted to data using SAS PROC NLIN (SAS Institute Inc., 1989), a procedure for non-linear regression modelling. During fitting, values for the parameters were estimated. These estimated values substituted in the equation were used to represent graphically the model's description of the growth rate response of *E. coli*. Various representative values of pH and/or acetic or lactic acid concentrations were chosen to compare to the experimental data. The parameters $a_{w_{min}}$, pH_{min}, pH_{max}, U_{min}, D_{min} were estimated from the data. There was sufficient variation in isothermal incubation temperature (up to ± 2C°) to require the inclusion of a temperature term. However, as all growth rates were measured at approximately 20°C, there was not enough variation in temperature to successfully estimate a T_{min} value. Using values obtained from previous temperature modelling of *E. coli* (Ross, *pers. comm.*) an integer value of 4°C was selected for the fixed T_{min} value.

Several criteria given by SAS PROC NLIN (SAS Institute Inc., 1989) were used for comparison of the model's ability to describe the data. Root mean square error (√M.S.E.) was used to compare the fit of the models. It is a measure of the average deviation of the predicted from the observed growth rates and its expected value is independent of the number of datapoints or degrees of freedom. In some cases the asymptotic standard error (A.S.E.) was used to determine the significance of differences in parameter estimates. It is an underestimate of error in comparison with the standard error (Ratkowsky, *pers. comm.*) and it is therefore more likely to predict that estimates are different than those predictions of similarity based on the standard error.

2.4 Results

2.4.1 Lactic Acid Growth Rate Modelling

Data previously obtained for *E. coli* M23 (Presser, 1995) contained 96 growth rates under varying conditions of pH (3.8-8.5) and lactic acid (0-500mM). This will be called Dataset 1. Dataset 1 was fitted to Eqn 2.2. The fitted equation is shown below:

$$\sqrt{k} = 0.0289(T-4)\sqrt{(a_w - 0.951)}\sqrt{1 - 10^{3.91-pH}} \times \sqrt{1 - \frac{LAC}{10.7(1+10^{pH-3.86})}}\sqrt{1 - \frac{LAC}{1036(1+10^{3.86-pH})}} \quad (2.4)$$

A subset of this data, Dataset 2 (Presser, 1995), containing 0-100mM lactic acid data only, was subsequently used to fit the growth rate model (Presser *et al.*, 1997) as below :

$$\sqrt{k} = 0.0248(T-4)\sqrt{(a_w - 0.934)}\sqrt{1 - 10^{3.90-pH}} \times \sqrt{1 - \frac{LAC}{10.7(1+10^{pH-3.86})}}\sqrt{1 - \frac{LAC}{823(1+10^{3.86-pH})}} \quad (2.5)$$

In this study Dataset 1 and Dataset 2 were also used to develop new models with a pH_{max} term. Dataset 1 fitted to Eqn 2.3 (with a pH_{max} term) is shown below:

$$\sqrt{k} = 0.0268(T-4)\sqrt{(a_w - 0.942)} \times \sqrt{1 - 10^{3.91-pH}}\sqrt{1 - 10^{pH-8.80}} \times \sqrt{1 - \frac{LAC}{10.5(1+10^{pH-3.86})}}\sqrt{1 - \frac{LAC}{1081(1+10^{3.86-pH})}} \quad (2.6)$$

Dataset 2 fitted to Eqn 2.3 (with a pH_{max} term) is shown below:

$$\sqrt{k} = 0.0229(T-4)\sqrt{(a_w - 0.922)} \times \sqrt{1 - 10^{3.91-pH}}\sqrt{1 - 10^{pH-8.84}} \times \sqrt{1 - \frac{LAC}{10.6(1+10^{pH-3.86})}}\sqrt{1 - \frac{LAC}{835(1+10^{3.86-pH})}} \quad (2.7)$$

Estimated values of the model parameters pH_{min} or U_{min} varied little from 3.90 ± 0.02 to 3.91 ± 0.03 and from 10.5 ± 0.6 to 10.7 ± 0.6 (Table 2.3). In addition c, D_{min} and $a_{W_{min}}$ showed some greater variation (Table 2.3). However the √M.S.E., the root mean square error, was smaller for both datasets with the pH_{max} term, 0.00521 compared to 0.00959 and 0.00502 compared with 0.00932 (Table 2.3). The inclusion of a pH_{max} term was found to improve the fit of the models according to the "extra sum of squares" principle (Appendix 5.4) (Draper and Smith, 1981).

A graphical representation of the fit of the data to the model is shown in Figure 2.1. Each part (a - f) of the figure shows the response of the data and the model to pH at that individual acid concentration. At high total acid concentrations the model fitted the data less well. Additional model predictions at other acid concentrations were superimposed to demonstrate the difference between the predicted inhibition at different total acid concentration and that shown by the data. It is important to note that the model is one overall equation covering all acid conditions in the range 0-500mM lactic acid and not an individual response fitted for each acid concentration i.e. each part of the figure.

Table 2.3 Estimated values and asymptotic standard errors of parameters for two different models fitted to two growth rate datasets showing lactic acid inhibition of *E. coli*. Parameters are defined in Eqn 2.2 & 2.3.

Equation Dataset √M.S.E.	c	pH_{min}	U_{min}	D_{min}	$a_{W_{min}}$	pH_{max}
Eqn 2.4 Dataset 1 0.00959	0.0289 ± 0.05	3.91 ± 0.03	10.7 ± 0.6	1036 ± 170	0.951 ± 0.01	N/A
Eqn 2.5 Dataset 2 0.00521	0.0248 ± 0.03	3.90 ± 0.02	10.7 ± 0.4	823 ± 237	0.934 ± 0.02	N/A
Eqn 2.6 Dataset 1 0.00932	0.0268 ± 0.05	3.91 ± 0.03	10.5 ± 0.6	1081 ± 189	0.942 ± 0.02	8.80 ± 0.2
Eqn 2.7 Dataset 2 0.00502	0.0229 ± 0.04	3.91 ± 0.02	10.6 ± 0.4	835 ± 235	0.922 ± 0.02	8.84 ± 0.2

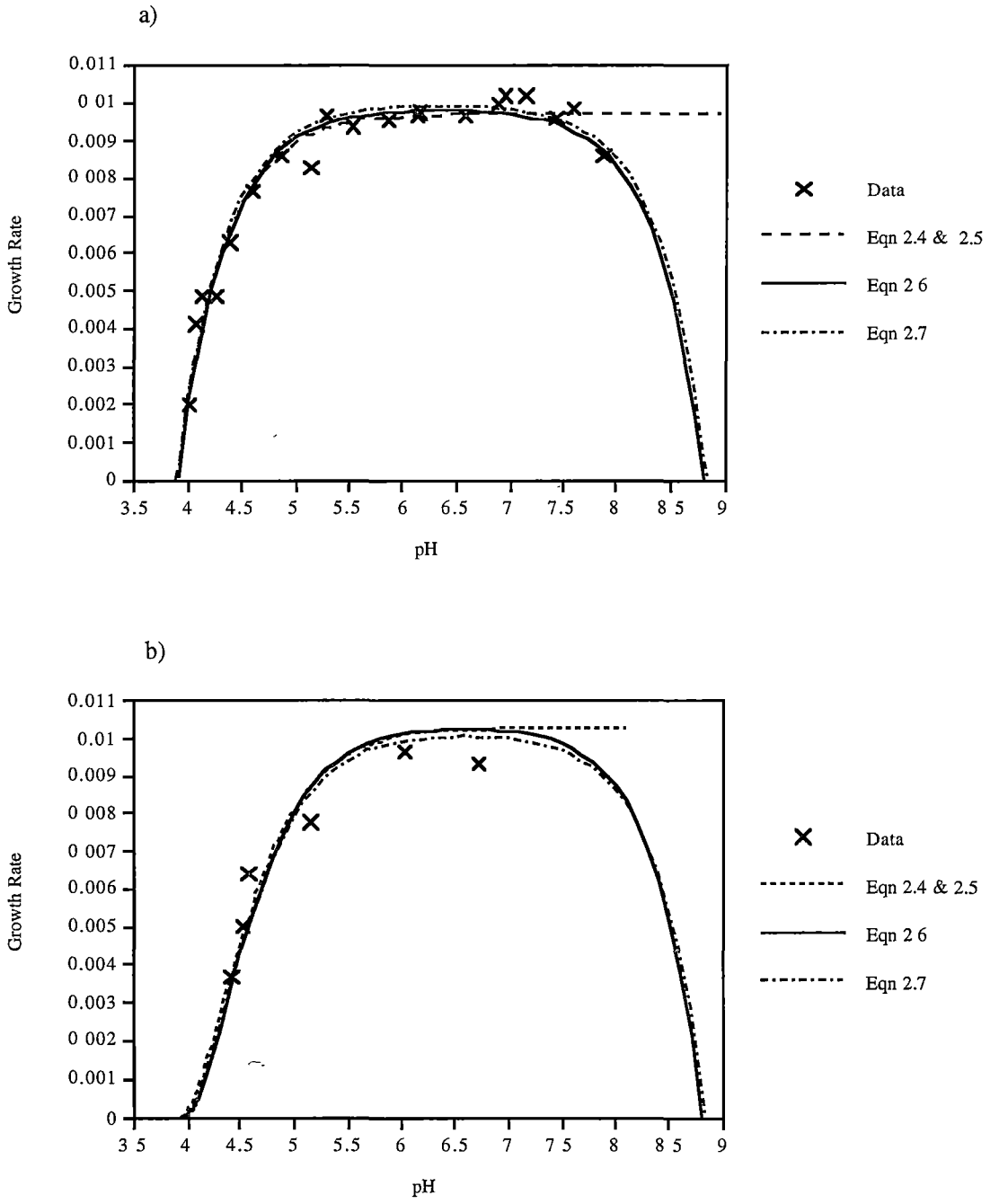


Figure 2.1 - Plot of pH versus growth rate (1/Generation time (min)) data and comparison with predictions of the fitted models for *E. coli* M23. a) 0mM and b) 25mM lactic acid

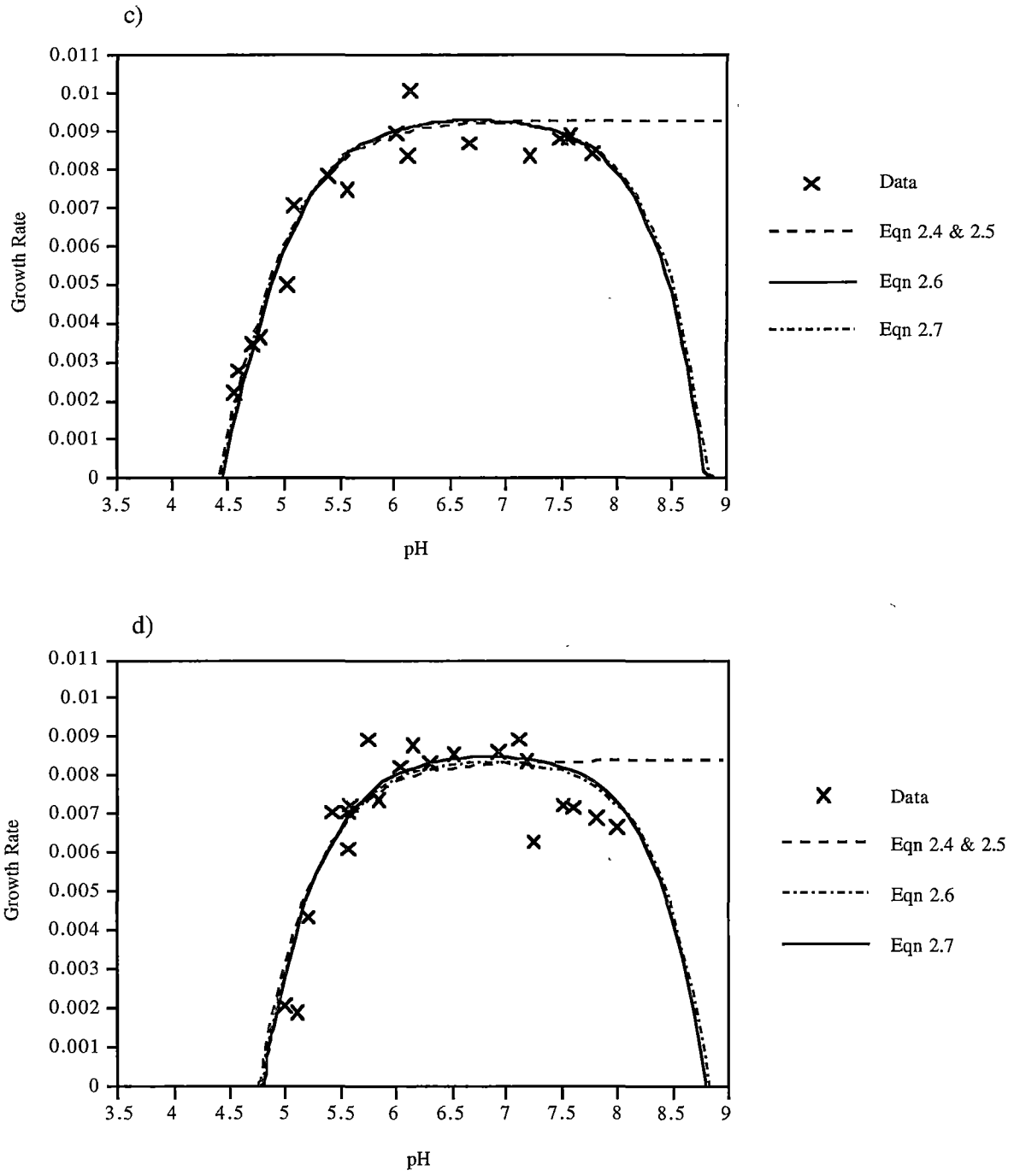


Figure 2.1 - Plot of pH versus growth rate (1/Generation time (min)) data and comparison with predictions of the fitted models for *E. coli* M23. c) 50mM and d) 100mM lactic acid

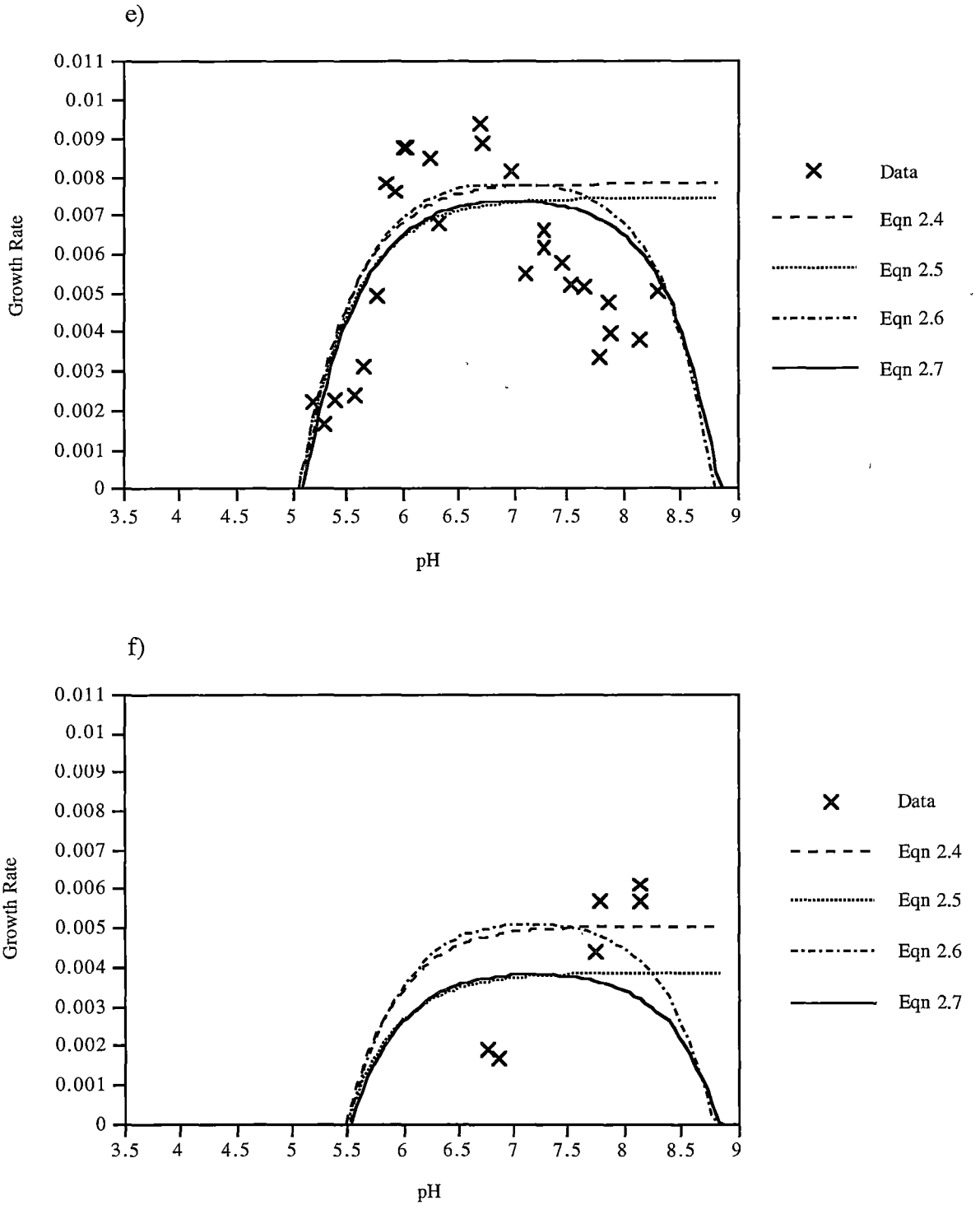


Figure 2.1 - Plot of pH versus growth rate (1/Generation time (min)) data and comparison with predictions of the fitted models for *E. coli* M23. e) 200mM and f) 500mM lactic acid. N.B. The data shown on this page was not included in the dataset used to create Eqn 2.5 and 2.7. These equations are shown here for comparision only.

2.4.2 Growth Rate of Different Strains at Suboptimal pH

Experiment 1 determined the inhibition of growth rate by low pH alone for 10 strains of *E. coli* (Appendix 1) at ~21°C (Data - Appendix 2.1). Water activity did not vary and no organic acid was used so the water activity (a_W) and organic acid (TAC) terms were not included in the model used to describe those results. The model used is as shown below.

$$\sqrt{k} = c(T - T_{\min})\sqrt{1 - 10^{pH_{\min} - pH}} \qquad (2.8)$$

where all terms are defined as for Eqn 2.2.

The following model, which contains a term for high pH, was used only when it was found to improve the fit of the model for the data of that strain.

$$\sqrt{k} = c(T - T_{\min})\sqrt{1 - 10^{pH_{\min} - pH}}\sqrt{1 - 10^{pH - pH_{\max}}} \qquad (2.9)$$

where all terms are defined as for Eqn 2.2.

A systematic error of overprediction and underprediction of growth rate as pH increased from $pH_{\min}$ to optimal pH was found with some strains. These included the pathogenic strains (R10, R31, R91 & R172) as well as non-pathogenic strains BR and MJR. Therefore a new model term for pH with a new coefficient, Q, was created and is shown below:

$$\sqrt{k} = c(T - T_{\min})\sqrt{1 - 10^{Q(pH_{\min} - pH)}} \qquad (2.10)$$

where the other terms are defined as for Eqn 2.2.

This new type of pH model with a factor (Q) added to the $pH_{\min}$ term allows the slope of the pH response to change. The Q factor itself is an indication of how different the data are from the older model. Eqns 2.8 and 2.9 are equivalent to having a fixed Q value of 1. For some strains the model with the added Q factor fitted better with the addition of a high pH term as shown below.

$$\sqrt{k} = c(T - T_{\min})\sqrt{1 - 10^{Q(pH_{\min} - pH)}}\sqrt{1 - 10^{pH - pH_{\max}}} \qquad (2.11)$$

where all terms are defined as for Eqn 2.2.

161 growth rate determinations were made. Six strains were nonpathogenic (M23, MJR, FT1, YY, SB & BR) and four were pathogenic (R10, R31, R91 & R172).

Table 2.4 - Number of datapoints used to fit the models for each strain

Strain	M23	MJR	FT1	YY	SB	BR	R10	R31	R91	R172
Data	31	21	18	12	12	12	13	13	13	13

For the pathogenic and non pathogenic groups, differences between the strains did not permit the creation of a good overall model for each group to enable comparison of the general characteristics of pathogenic and nonpathogenic strains. Therefore the data were modelled only for each of the individual strains despite some strains only having a small number of datapoints (Table 2.4). The fitted estimates of the parameters of Eqn 2.8 for each strain is shown in Tables 2.5 and 2.6.

For strain BR a large decrease in growth rate at the highest pH necessitated the use of Eqn 2.9 which contained a pH_{max} term. The fitted value of pH_{max} was 8.08 (±0.0564 A.S.E.). This addition improved the fit of the model significantly according to the "extra sum of squares" principle (Appendix 5.4) (Draper & Smith, 1981). The √M.S.E. dropped from 0.0118 (the highest value for Eqn 2.8) to 0.00586 for Eqn 2.9 (Table 2-5). For data of other strains, there were no data showing high pH growth rate inhibition, therefore the pH_{max} term did not to improve the model and values for pH_{max} less than 14 (maximum possible high pH value) were not estimated (data not shown).

Table 2.5 - Estimates of parameters, c and pH_{min} and their S.E.s and RMSE fitted using Eqn 2.8 (and 2.9 BR) for nonpathogenic *E. coli* strains.

Strain	c ± S.E.	pH_{min} ± S.E.	RMSE
M23	$0.00622 \pm 4.7\times10^{-5}$	3.91 ± 0.0124	4.24×10^{-3}
MJR	$0.00652 \pm 4.4\times10^{-5}$	3.90 ± 0.0129	2.93×10^{-3}
FT1	$0.00481 \pm 1.2\times10^{-4}$	3.85 ± 0.0787	7.28×10^{-3}
YY	$0.00476 \pm 1.7\times10^{-4}$	4.43 ± 0.0688	8.48×10^{-3}
SB	$0.00674 \pm 6.5\times10^{-5}$	3.90 ± 0.0157	3.09×10^{-3}
BR	$0.00540 \pm 2.4\times10^{-4}$	3.87 ± 0.0766	1.18×10^{-2}
BR (Eqn 2.9)	$0.00569 \pm 1.3\times10^{-4}$	3.90 ± 0.0307	5.87×10^{-3}

Table 2.6 - Estimates of parameters, c and pH_{min} and their S.E.s and RMSE fitted using Eqn 2.8 for the pathogenic *E. coli* strains.

Strain	c ± S.E.	pH_{min} ± S.E.	RMSE
R10	$0.00617 \pm 1.7\times10^{-4}$	3.74 ± 0.0313	9.48×10^{-3}
R31	$0.00654 \pm 1.2\times10^{-4}$	3.82 ± 0.0185	6.67×10^{-3}
R91	$0.00657 \pm 1.0\times10^{-4}$	3.82 ± 0.0227	5.81×10^{-3}
R172	$0.00633 \pm 1.1\times10^{-4}$	3.82 ± 0.0230	6.06×10^{-3}

The fitted estimates of the parameters of Eqn 2.10 for each strain is shown in Tables 2.7 and 2.8. The addition of the Q coefficient to the pH term resulted in a decrease in the √M.S.E. for all strains except M23 (Figure 2.2). The inclusion of the Q coefficient was found to improve the fit of the models for nonpathogenic strains MJR, FT1, YY, SB and BR and all the pathogenic strains according to the "extra sum of squares" principle (Appendix 5.4) (Draper & Smith, 1981). Fitting Eqn 2.10 changed the estimated values of pH_{min} (Figure 2.4) and c (Figure 2.3) for some strains compared to Eqn 2.8. The c estimate increased significantly ($P < 0.05$) for strain SB and the pathogenic strains and the pH_{min} estimate decreased significantly ($P < 0.05$) for strains SB, R91 and R172 (Z Test, Appendix 5.3).

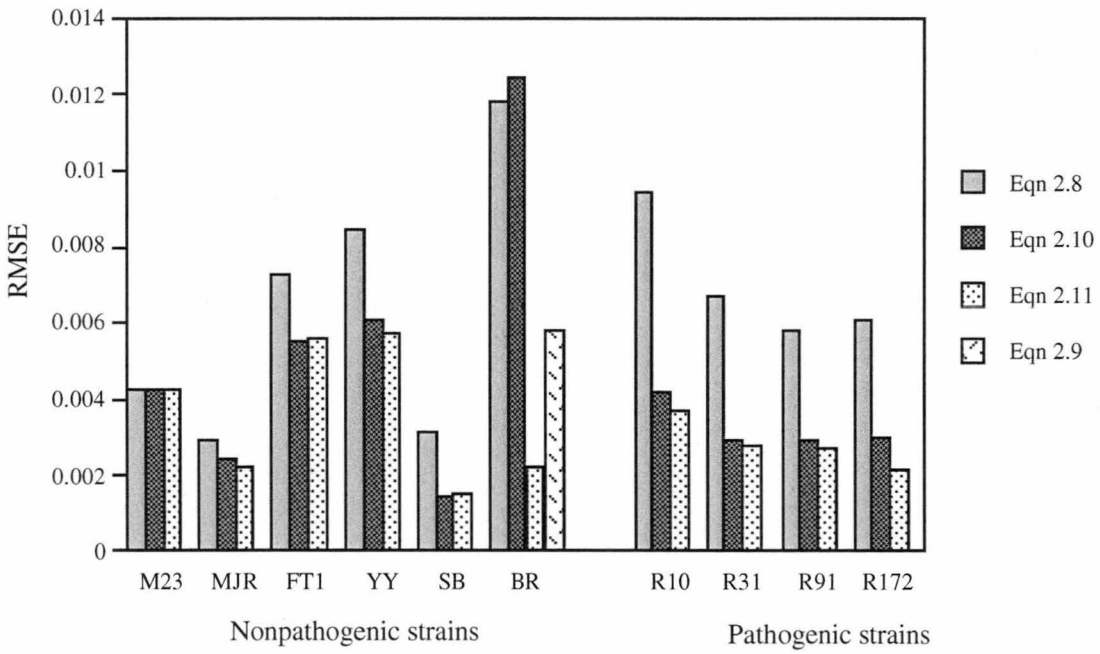


Figure 2.2 - Comparison of RMSE estimates of different strains.

For M23 fitting Eqn 2.10 showed that the addition of the Q term was not necessary to fit the model. The value of the Q factor was very close to 1, the estimated values of c (Figure 2.3) and pH_{min} (Figure 2.4) were very close to the previous model but most importantly the √M.S.E. was slightly higher with the new term (Figure 2.2).

Strains FT1 and YY again had high √M.S.E.s when fitted with Eqn 2.10 (Figure 2.2). This shows not all strains were like strain R10 where the √M.S.E. for this strain was more than halved when fitted with Eqn 2.10 compared with Eqn 2.8. Strain BR also did not show a good fit with only the Q term and no pH_{max} term (Eqn 2.10).

Table 2.7 - Nonpathogenic strains of *E. coli* - Estimates of parameters, c, pH_{min} and Q and their S.E.s and RMSE fitted for Eqn 2.10.

Strain	$c \pm$ S.E.	$pH_{min} \pm$ S.E.	$Q \pm$ S.E.	RMSE
M23	$0.00624 \pm 6.0 \times 10^{-5}$	3.90 ± 0.0254	0.924 ± 0.115	4.28×10^{-3}
MJR	$0.00663 \pm 5.5 \times 10^{-5}$	3.84 ± 0.0276	0.780 ± 0.0672	2.45×10^{-3}
FT1	$0.00527 \pm 2.7 \times 10^{-4}$	3.36 ± 0.366	0.331 ± 0.151	5.55×10^{-3}
YY	$0.00525 \pm 3.2 \times 10^{-4}$	4.19 ± 0.211	0.377 ± 0.164	6.06×10^{-3}
SB	$0.00692 \pm 4.6 \times 10^{-5}$	3.83 ± 0.0178	0.706 ± 0.0423	1.48×10^{-3}
BR	$0.00542 \pm 3.4 \times 10^{-4}$	3.86 ± 0.134	0.941 ± 0.563	1.24×10^{-2}

Table 2.8 - Pathogenic strains of *E. coli* - Estimates of parameters, c and pH_{min} and their S.E.s and RMSE fitted for Eqn 2.10.

Strain	$c \pm$ S.E.	$pH_{min} \pm$ S.E.	$Q \pm$ S.E.	RMSE
R10	$0.00680 \pm 1.6 \times 10^{-4}$	3.65 ± 0.0375	0.441 ± 0.0594	4.14×10^{-3}
R31	$0.00695 \pm 9.5 \times 10^{-5}$	3.77 ± 0.0173	0.585 ± 0.0490	2.96×10^{-3}
R91	$0.00689 \pm 8.9 \times 10^{-5}$	3.74 ± 0.0283	0.597 ± 0.0580	2.95×10^{-3}
R172	$0.00669 \pm 9.6 \times 10^{-5}$	3.74 ± 0.0270	0.573 ± 0.0569	2.96×10^{-3}

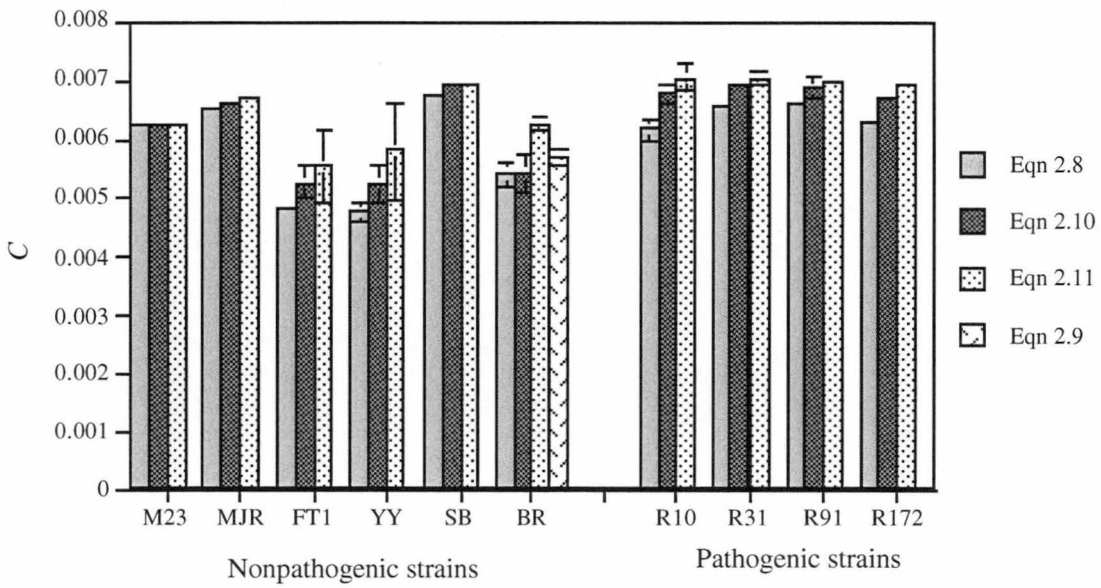


Figure 2.3 - Comparison of c estimates of different strains. Error bars are not visible where the error is too small to be presented.

The fitted estimates of the parameters of Eqn 2.11 for each strain are shown in Tables 2-9 and 2-10. For most strains the addition of the pH_{max} term resulted in a small decrease in √M.S.E. (Figure 2.2). Eqn 2.11 improved the fit compared to Eqn 2.10 for strains MJR, BR and the pathogenic strains. Fitting Eqn 2.11 changed the estimated values of pH_{min} and c for these strains compared to Eqn 2.10 according to the "extra sum of squares" principle (Appendix 5.4) (Draper & Smith, 1981). Similarly the c estimate increased (Figure 2.3) and the pH_{min} estimate decreased (Figure 2.4) to a varying degree depending on the strain but none changed significantly (Z Test, Appendix 5.3).

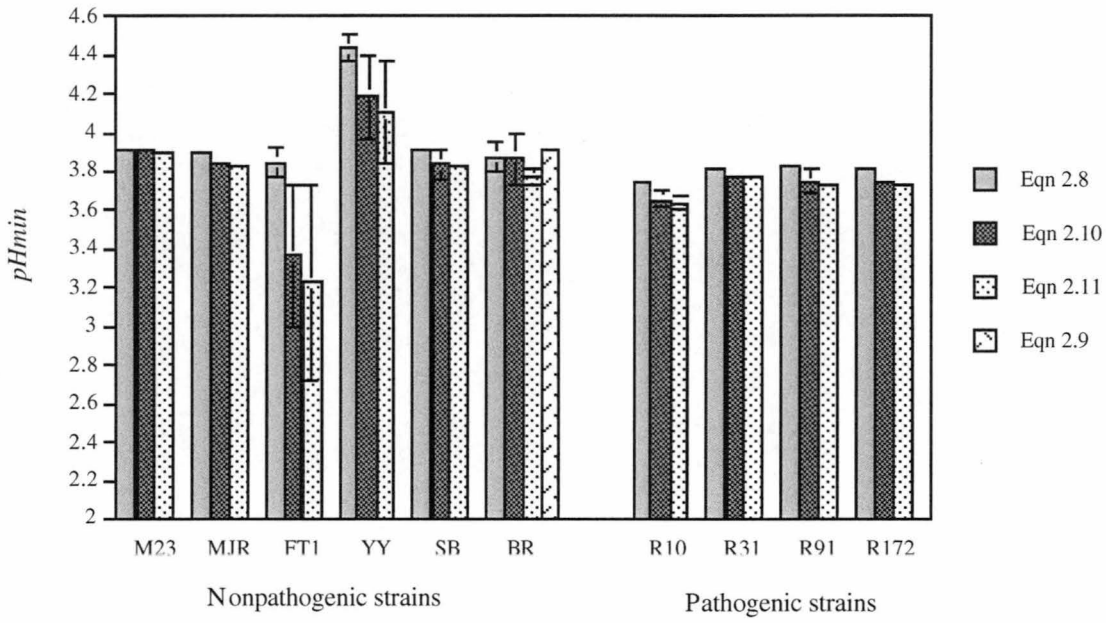


Figure 2.4 - Comparison of pH_{min} estimates of different strains. Error bars are not visible where the error is too small to be presented.

For M23 fitting Eqn 2.11 showed that the addition of neither the Q nor pH_{max} term was necessary to fit the data. The value of the Q factor was again very close to 1, the estimated values of c (Figure 2.3) and pH_{min} (Figure 2.4) were very close to the estimates obtained from both the previous models but most importantly the √M.S.E. was slightly higher again with the extra new term (Figure 2.2).

Strains FT1 and YY again had high √M.S.E.s when fitted with Eqn 2.11 (Figure 2.2). Strain BR with a pH_{max} term show an improved fit with the Q term added (Eqn 2.11 compared to Eqn 2.9) according to the "extra sum of squares" principle (Appendix 5.4) (Draper & Smith, 1981). The pH_{max} estimate for BR was slightly smaller for Eqn 2.11 (pH_{max} = 8.04) compared to Eqn 2.9 (pH_{max} = 8.08) but this change was not significant (Z Test, Appendix 5.3).

Table 2.9 - Nonpathogenic strains of *E. coli* - Estimates of parameters, c, pH_{min}, pH_{max} and Q and their S.E.s and RMSE fitted for Eqn 2.11.

Strain	$c \pm$ S.E.	$pH_{min} \pm$ S.E.	$pH_{max} \pm$ S.E.	$Q \pm$ S.E.	RMSE
M23	$0.00628 \pm 7.4 \times 10^{-5}$	3.89 ± 0.0268	9.29 ± 0.470	0.884 ± 0.117	4.29×10^{-3}
MJR	$0.00671 \pm 6.7 \times 10^{-5}$	3.82 ± 0.0276	9.15 ± 0.204	0.722 ± 0.0633	2.26×10^{-3}
FT1	$0.00554 \pm 6.0 \times 10^{-4}$	3.23 ± 0.502	8.92 ± 0.503	0.250 ± 0.172	5.60×10^{-3}
YY	$0.00580 \pm 8.3 \times 10^{-4}$	4.10 ± 0.267	8.67 ± 0.287	0.250 ± 0.167	5.71×10^{-3}
SB	$0.00695 \pm 6.1 \times 10^{-5}$	3.83 ± 0.0190	9.41 ± 0.478	0.687 ± 0.0467	1.49×10^{-3}
BR	$0.00626 \pm 1.2 \times 10^{-4}$	3.77 ± 0.0380	8.04 ± 0.017	0.447 ± 0.0530	2.25×10^{-3}

Table 2.10 - Pathogenic strains of *E. coli* - Estimates of parameters, c, pH_{min}, pH_{max} and Q and their S.E.s and RMSE fitted for Eqn 2.11.

Strain	$c \pm$ S.E.	$pH_{min} \pm$ S.E.	$pH_{max} \pm$ S.E.	$Q \pm$ S.E.	RMSE
R10	$0.00705 \pm 2.3 \times 10^{-4}$	3.64 ± 0.0377	8.51 ± 0.224	0.382 ± 0.0588	3.71×10^{-3}
R31	$0.00704 \pm 1.2 \times 10^{-4}$	3.77 ± 0.0177	8.89 ± 0.337	0.553 ± 0.0525	2.78×10^{-3}
R91	$0.00699 \pm 1.2 \times 10^{-4}$	3.73 ± 0.0288	8.83 ± 0.280	0.554 ± 0.0589	2.77×10^{-3}
R172	$0.00689 \pm 1.0 \times 10^{-4}$	3.73 ± 0.0217	8.52 ± 0.128	0.500 ± 0.0428	2.12×10^{-3}

Differences were found between the c and pH_{min} estimates for pathogenic and non pathogenic strains (Figure 2.3 & 2.4). The four lowest estimates of pH_{min} for Eqn 2.8 were for the four pathogenic strains (Table 2.5 & 2.6). The lowest pH_{min} estimates given by Eqn 2.10 and 2.11 were for pathogenic strains (Table 2.8 & 2.10) with the exception of FT1 for which the estimate was very imprecise (Table 2.7 & Table 2.8). Two of the nonpathogenic strains, FT1 and YY, had much slower growth rates which were reflected in significantly lower fitted c values for models 2.8 and 2.10 (Table 2.5, 2.7 & 2.9).

For most strains pH_{max} estimates had large standard errors (Figure 2.5). BR had the lowest and most precise estimate for pH_{max} which was significantly lower than for MJR (Z Test, Appendix 5.3). The four pathogenic strains, as well as YY and FT1, had low estimates of pH_{max} compared to the other nonpathogenic strains (Table 2.9 & 2.10) but these differences were not significant (Z Test, Appendix 5.3). Q estimates varied, with the pathogenic strains lower than M23, MJR and SB (Figure 2.6) however R10 was the only strain where Q was significantly lower (Z Test, Appendix 5.3). BR had a high Q estimate for Eqn 2.10 and a low estimate with Eqn 2.11. FT1 and YY had low estimates for Q, but those estimates are very imprecise (Figure 2.6).

The fit of each of the equations to the sets of data assessed using √MSEs is shown in Figure 2.2. It can also be judged from Figure 2.7. For example, this shows the good fit of the models to data for strain M23 (Figure 2.7a). It can also demonstrate the underprediction at low pH and an over prediction at high pH for nonpathogenic strains such as MJR (Figure 2.7b), SB (Figure 2.7e), BR (Figure 2.7f) and pathogenic strains (Figure 2.7g-j). For strains FT1 and YY the lack of fit of all the models can be seen as due to scatter and irregularity in the data, especially at low pH (Figure 2.7c & d).

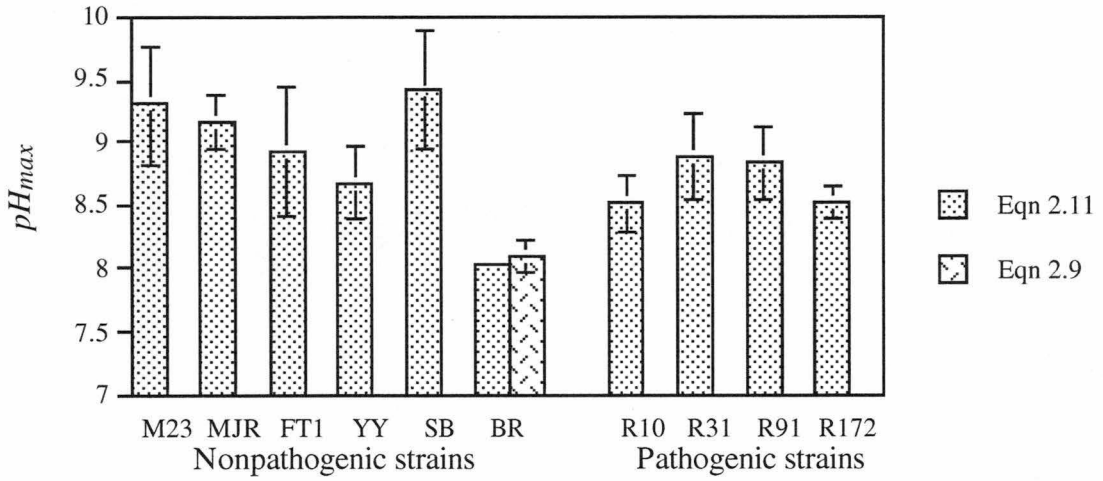


Figure 2.5 - Comparison of pH_{max} estimates of different strains. An error bar is not visible where the error is too small to be presented.

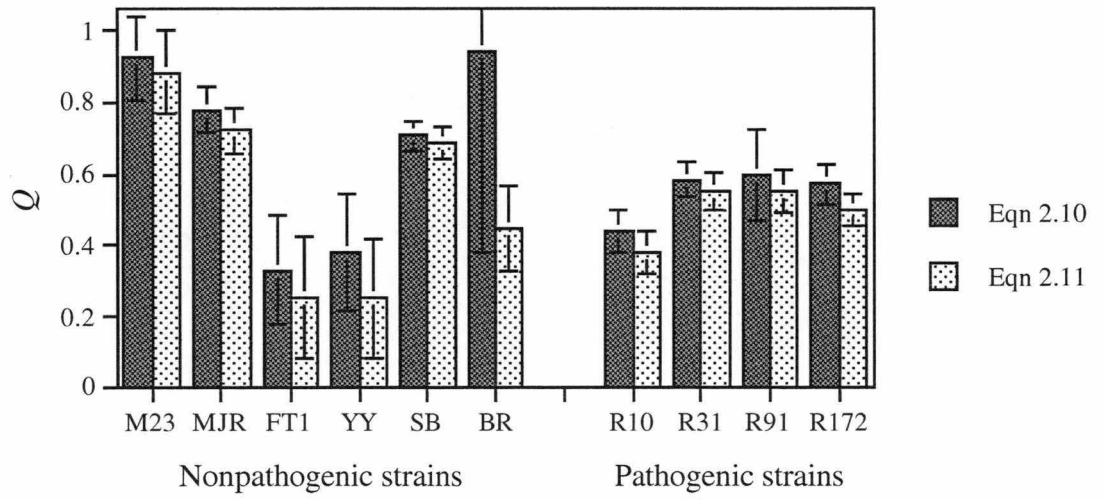


Figure 2.6 - Comparison of Q estimates of different strains.

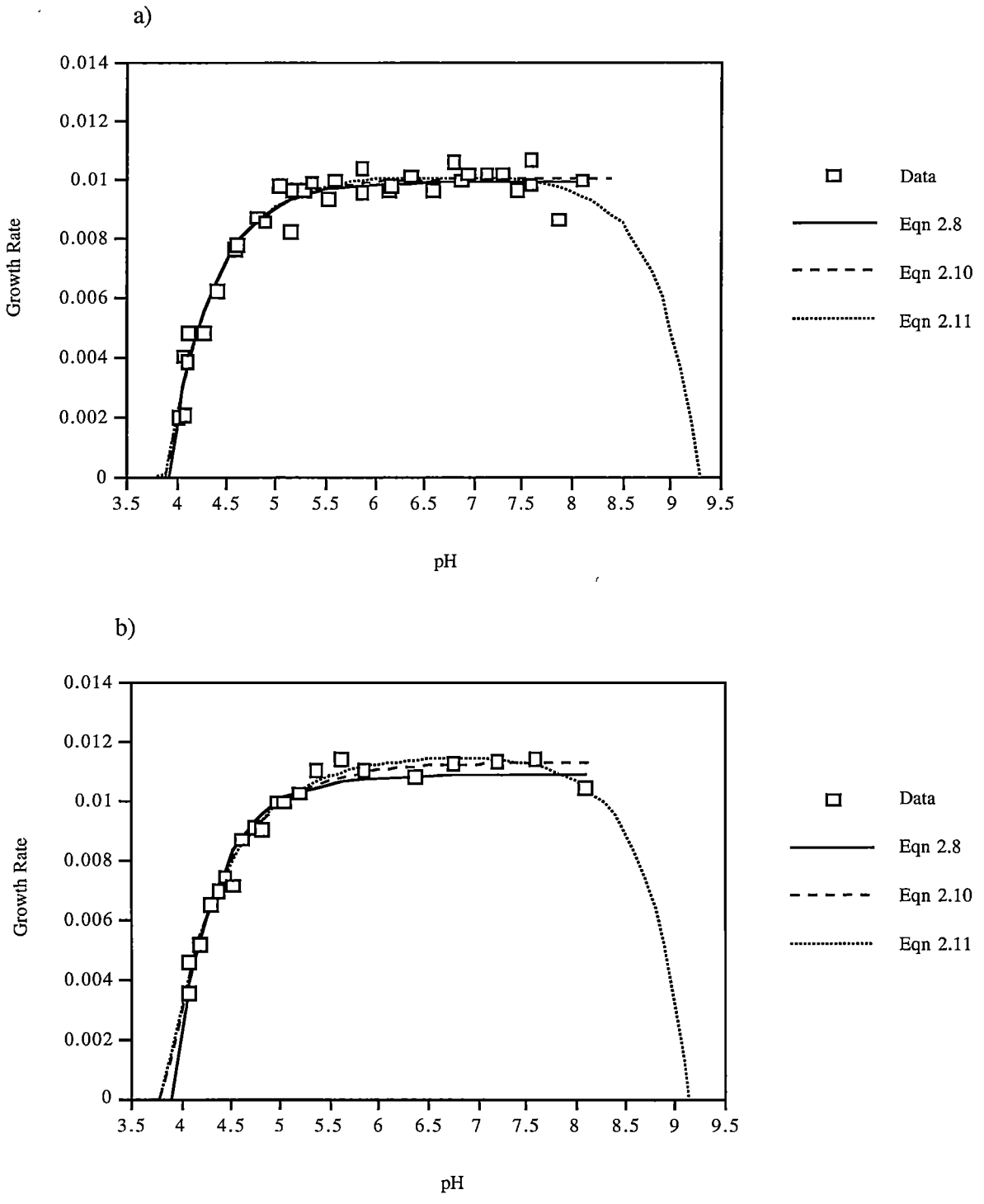
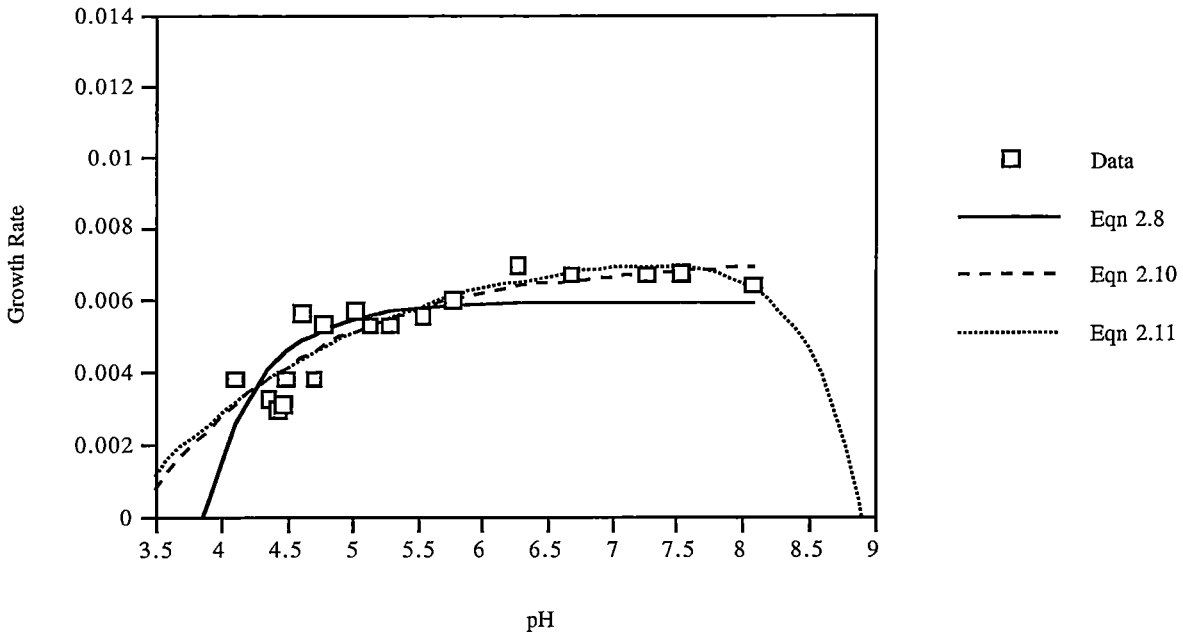


Figure 2.7 Plot of pH versus growth rate (1/Generation time (min)) data and fitted pH models for individual strains of *Escherichia coli* a) Strain M23 (nonpathogenic) b) Strain MJR (nonpathogenic).

c)



d)

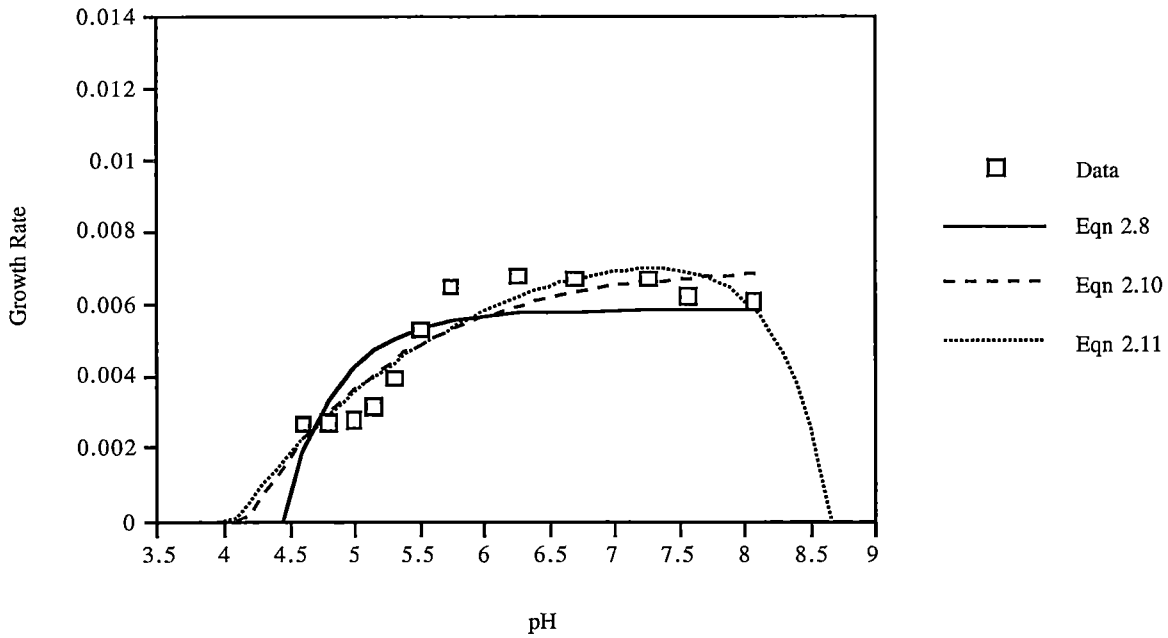
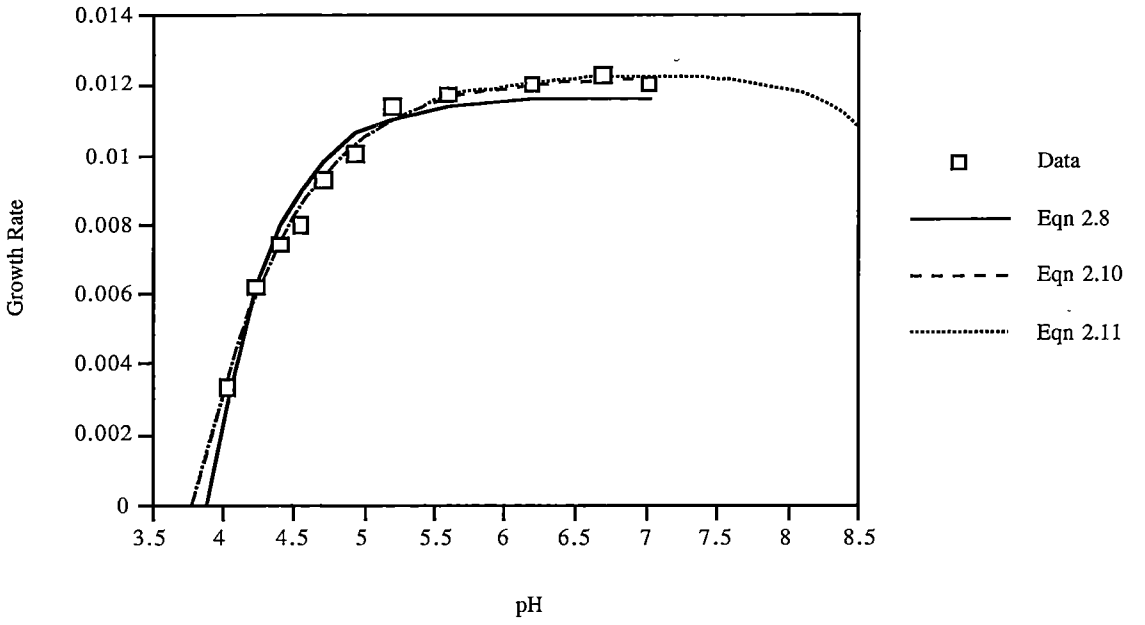


Figure 2.7 (continued) - Plot of pH versus growth rate (1/Generation time (min)) data and fitted pH models for individual strains of *Escherichia coli* c) Strain FT1 (nonpathogenic) d) Strain YY (nonpathogenic).

e)



f)

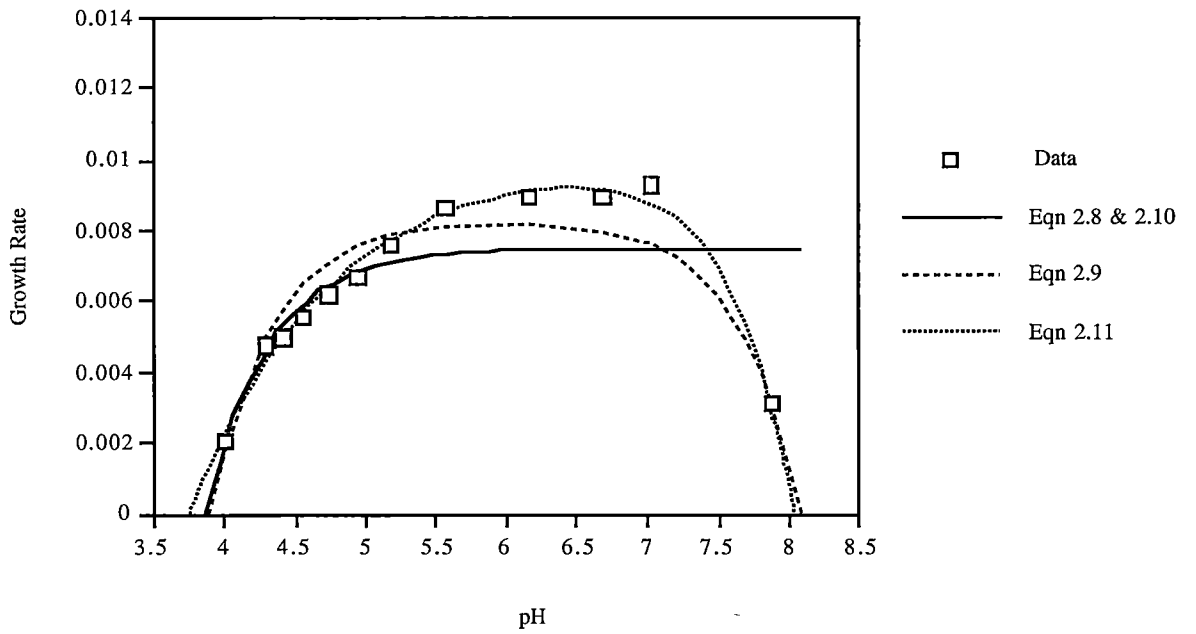
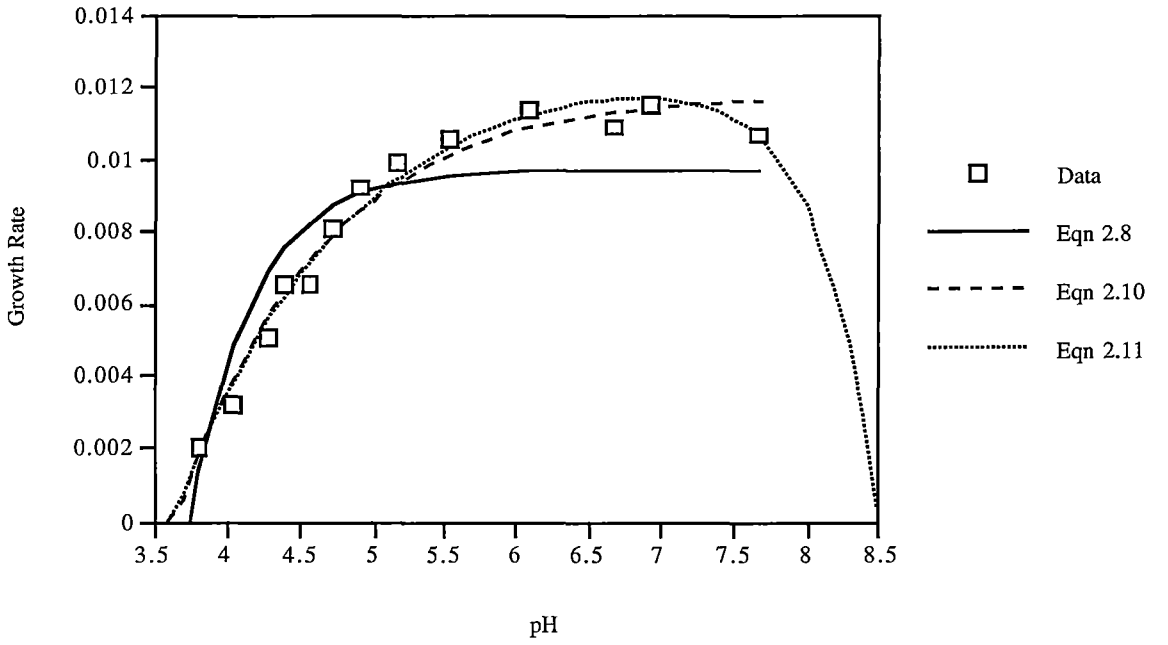


Figure 2.7 (continued) - Plot of pH versus growth rate (1/Generation time (min)) data and fitted pH models for individual strains of *Escherichia coli* e) Strain SB (nonpathogenic) f) Strain BR (nonpathogenic).

g)



h)

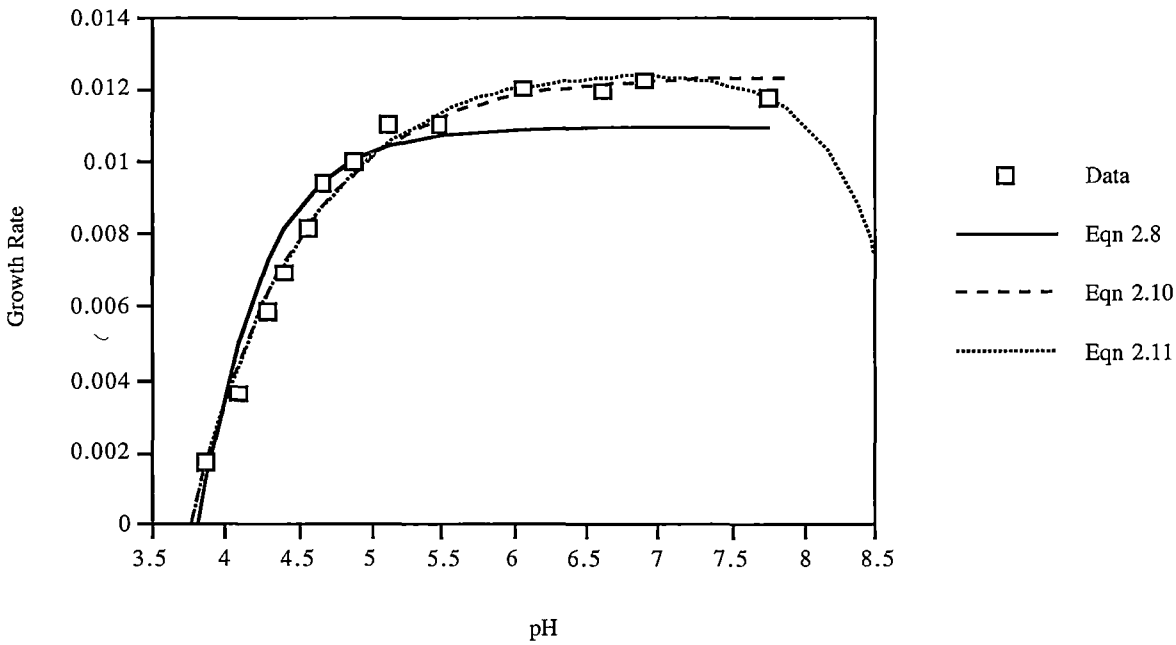
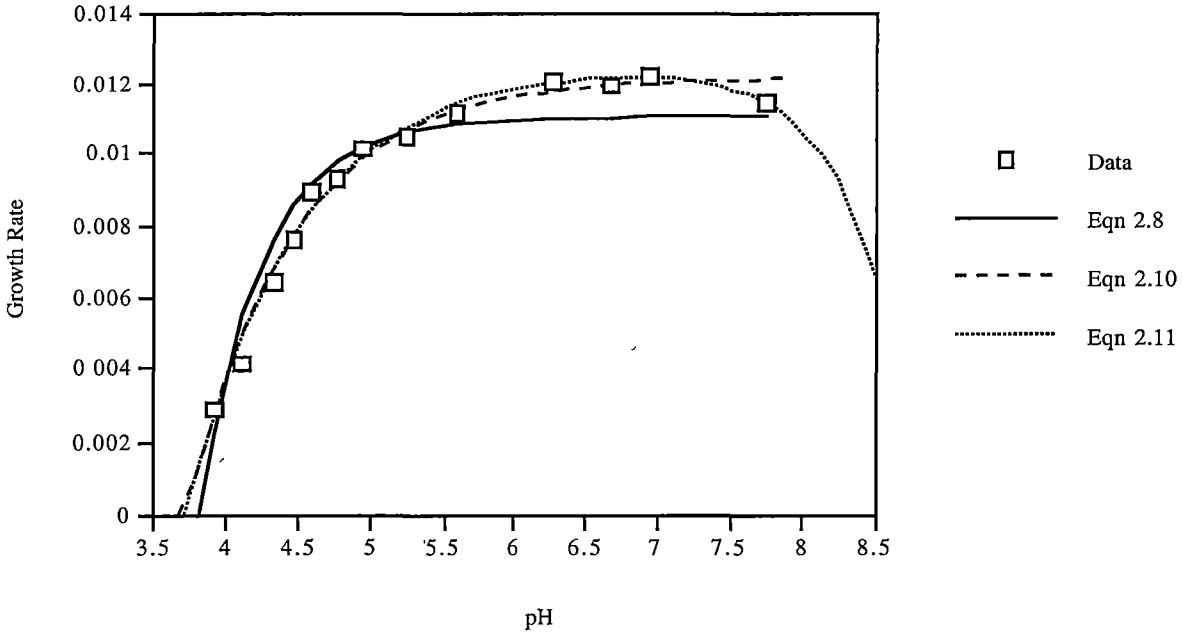


Figure 2.7 (continued) - Plot of pH versus growth rate (1/Generation time (min)) data and fitted pH models for individual strains of *Escherichia coli* g) Strain R10 (pathogenic) h) Strain R31 (pathogenic).

i)



j)

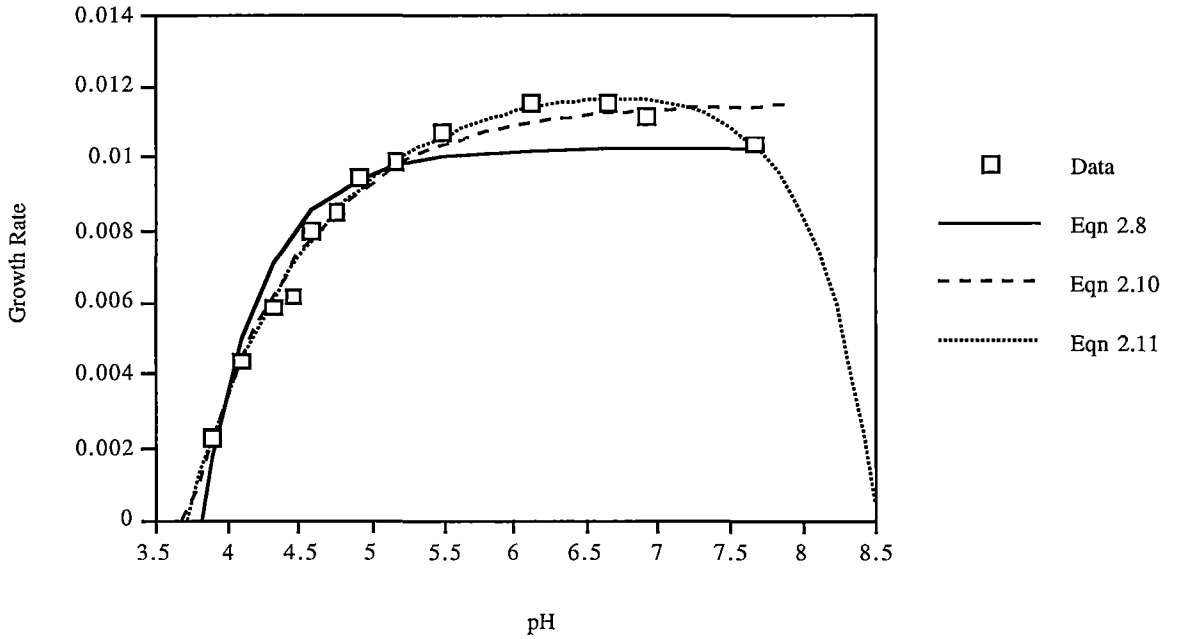


Figure 2.7 (continued) - Plot of pH versus growth rate (1/Generation time (min)) data and fitted pH models for individual strains of *Escherichia coli* i) Strain R91 (pathogenic) j) Strain R172 (pathogenic).

2.4.3 Growth Rate at Low pH and Low Water Activity

12 growth rates were determined at low pH and low water activity combinations with no organic acid present. These data were not modelled separately but were added to the data used to model acetic acid effects on *E. coli* growth rate.

2.4.4 Growth Rate Inhibition by Acetic Acid

The effect on growth rate of combinations of low pH (3-7) and acetic acid (0-800mM) on *E. coli* strain M23 was determined. Data were also collected describing the inhibitory effect of sodium acetate on growth rate.

Several growth rate models were developed for acetic acid to describe the 135 growth rate values obtained. To fit the model properly, datapoints which weighted the $U_{\min}$ to a higher value at the lowest pHs at 15, 20, 25 and 100mM were excluded for reasons discussed in Section 2.5.1.3. Also some rates were determined by manual measurement instead of fitting the modified-Gompertz function using the computer. The addition of a $pH_{\max}$ term to the model and the use of a new empirical term for inhibition by the dissociated acid molecule improved the fit of the model. Equation 2.12 shows the form of the new terms.

$$\sqrt{k} = c(T - T_{\min})\sqrt{a_w - a_{w\min}} \sqrt{1 - 10^{pH_{\min} - pH}}\sqrt{1 - 10^{pH - pH_{\max}}} \sqrt{1 - \frac{[AC]}{[U_{\min}]\left[1 + 10^{pH - pKa}\right]}}\sqrt{10^{\left(\frac{-D\,[AC]}{10^{pKa - pH} + 1}\right)}} + e \qquad (2.12)$$

where terms are as previously described in Eqn 2.2 and D is a new coefficient to be estimated within the new term for the effect of dissociated acid (for the derivation, see Appendix 5.1). The addition of the extra data for either higher concentrations of acetic acid or using sodium acetate resulted in similar estimates for model parameters. However, addition of data for higher concentrations of acid (200-800mM) increased the √M.S.E. due to greater scatter in the data. The √M.S.E. also increased with the addition of growth rates in the presence of sodium acetate. Shown in Table 2.7 are the fitted estimates of Eqn 2.12 for the lower concentration range of data (0-100mM) with the $pH_{\max}$ and D terms.

A graphical representation of the fit of the data to the model is shown in Figure 2.8. In the same way as for the lactic acid models, each part (a - k) of the figure shows the response of the data and the model to pH at that individual acid concentration (extra predicted model responses at lower concentrations are shown to demonstrate the "aberrant" data at the high acid conditions). It is important to note that the fitted model is one equation for all acetic acid conditions shown in Figure 2.8. The separate figures a)-k) showing the response to each acetic acid concentration (0-500mM) were not fitted to individual datasets (the data shown on each figure) as in Section 2.4.2, but the equation was fitted to the combined data including all acetic acid concentrations as in Section 2.4.1.

Table 2.7 - Estimates of parameters, $c, pH_{min}, U_{min}, D, a_{w_{min}}$ and pH_{max} and their A.S.E.s and √M.S.E. fitted for Eqn 2.12.

Model 7	c	pH_{min}	U_{min}	D	$a_{w_{min}}$	pH_{max}	√M.S.E.
Estimate	0.0335	3.93	3.18	0.00228	0.961	9.23	0.00784
A.S.E.	0.000608	0.0181	0.0786	0.000186	0.000947	0.0416	

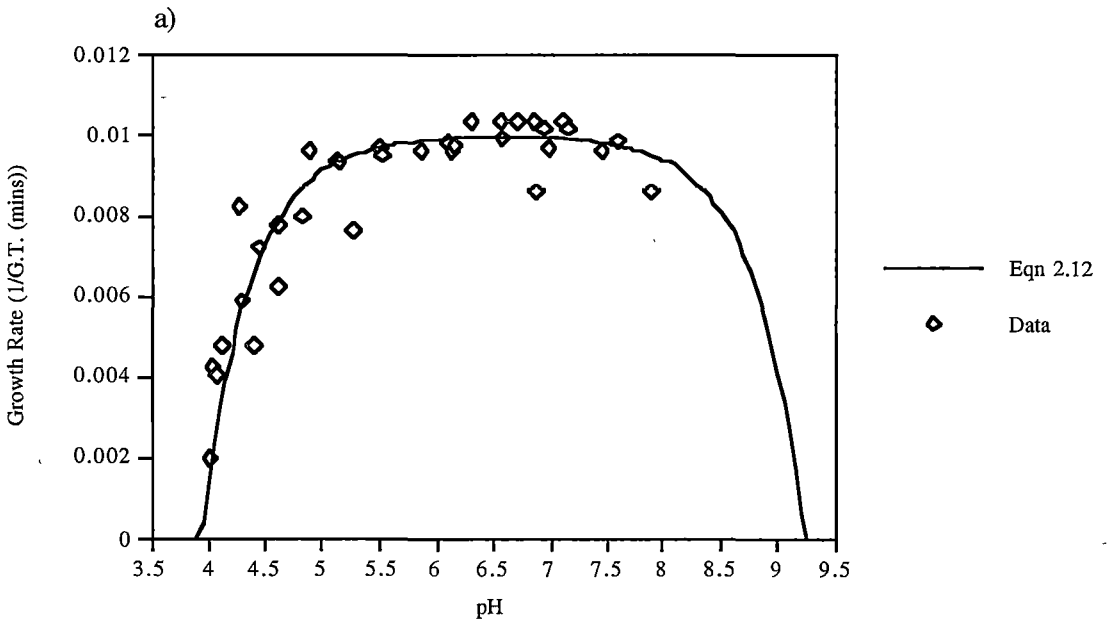


Figure 2.8 a) - Eqn 2.12 and data for *E. coli* M23 with no acetic acid

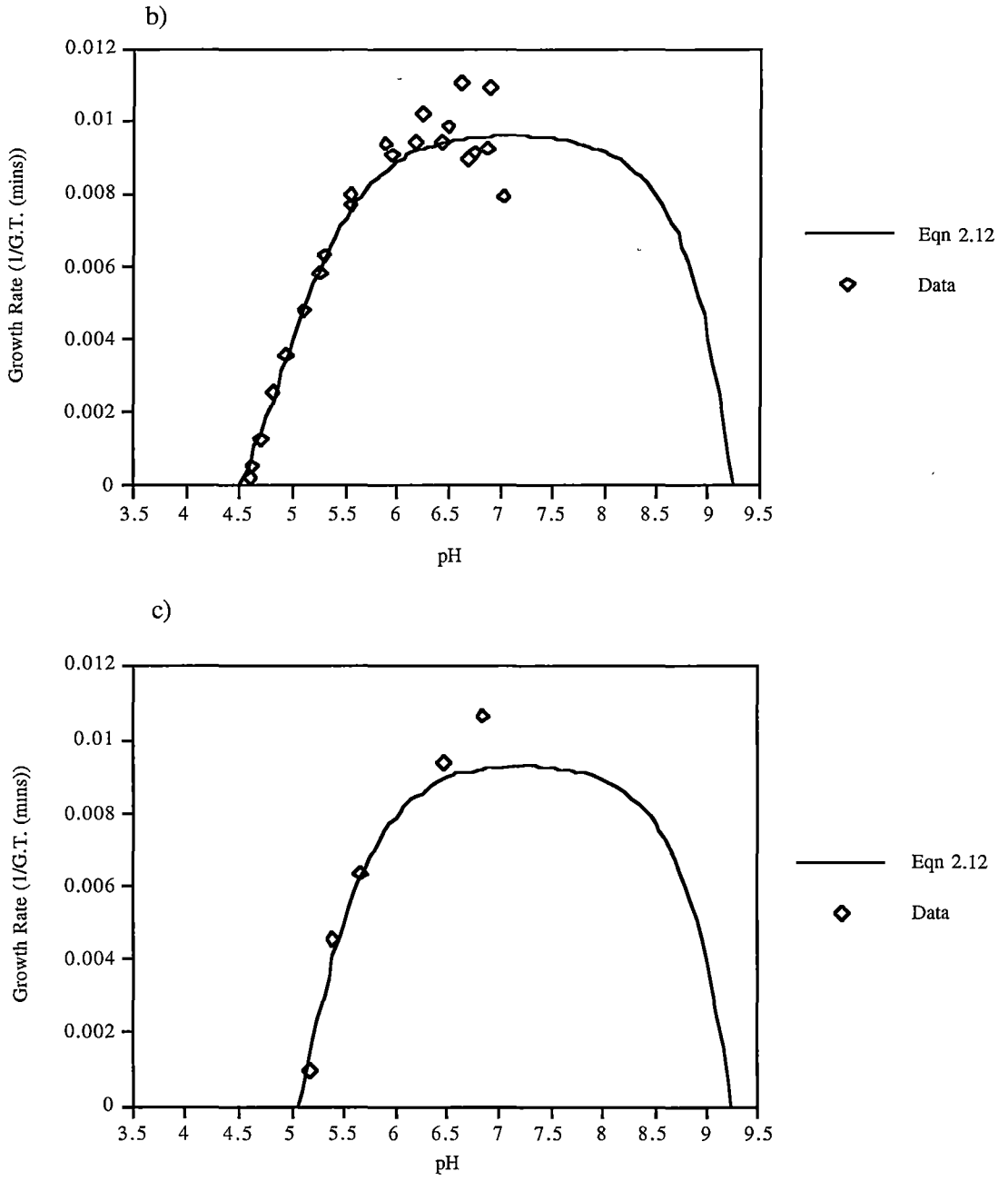


Figure 2.8 - (continued) - Eqn 2.12 and data for acetic acid for *E. coli* M23 b) 5mM acetic acid c) 10mM acetic acid

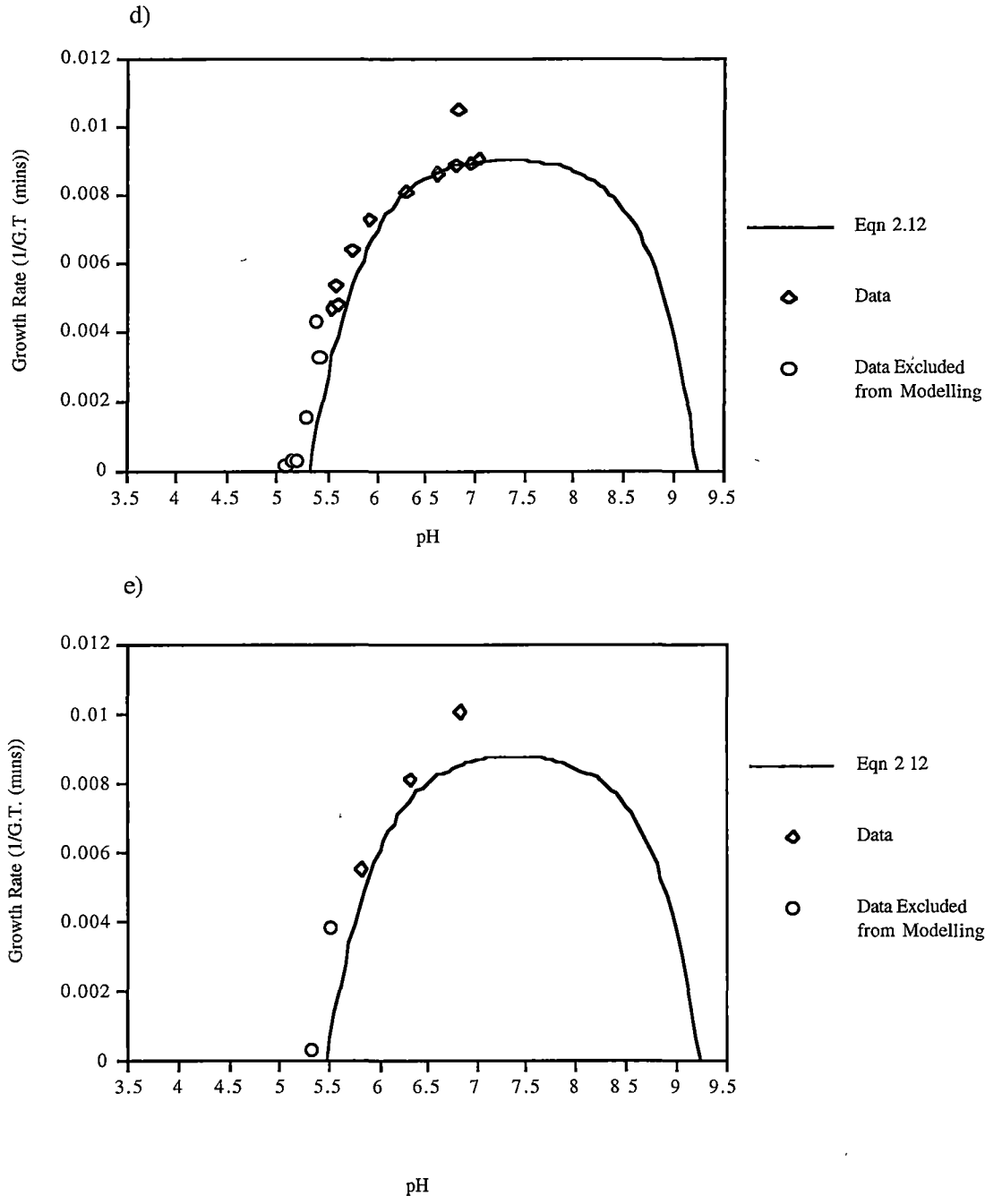


Figure 2.8 - (continued) - Eqn 2.12 and data for acetic acid for *E. coli* M23 d) 15mM acetic acid e) 20mM acetic acid. Data excluded from modelling is discussed in Section 2.5.1.3.

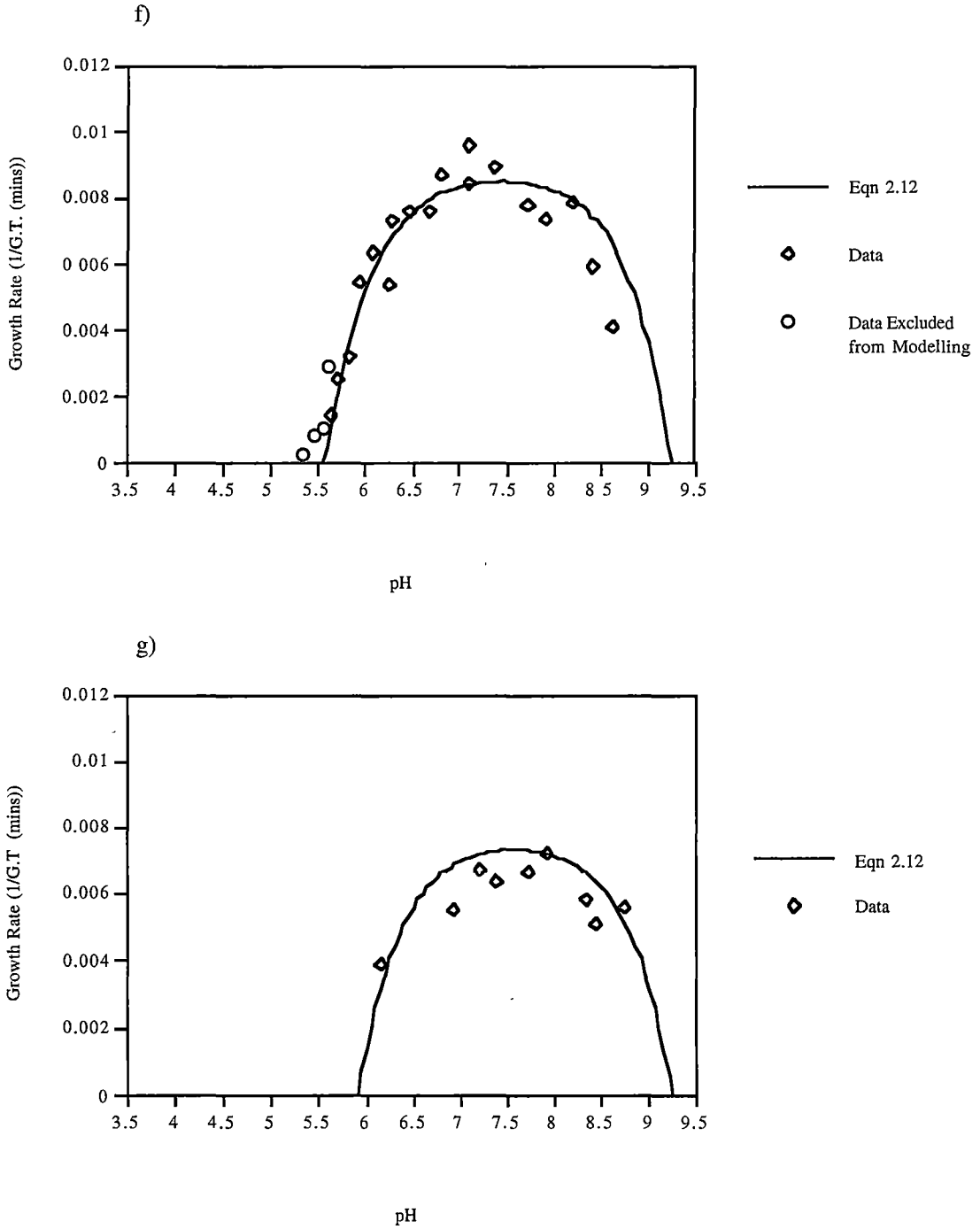


Figure 2.8 - (continued) - Eqn 2.12 and data for acetic acid for *E. coli* M23 f) 25mM acetic acid g) 50mM acetic acid. Data excluded from modelling is discussed in Section 2.5.1.3.

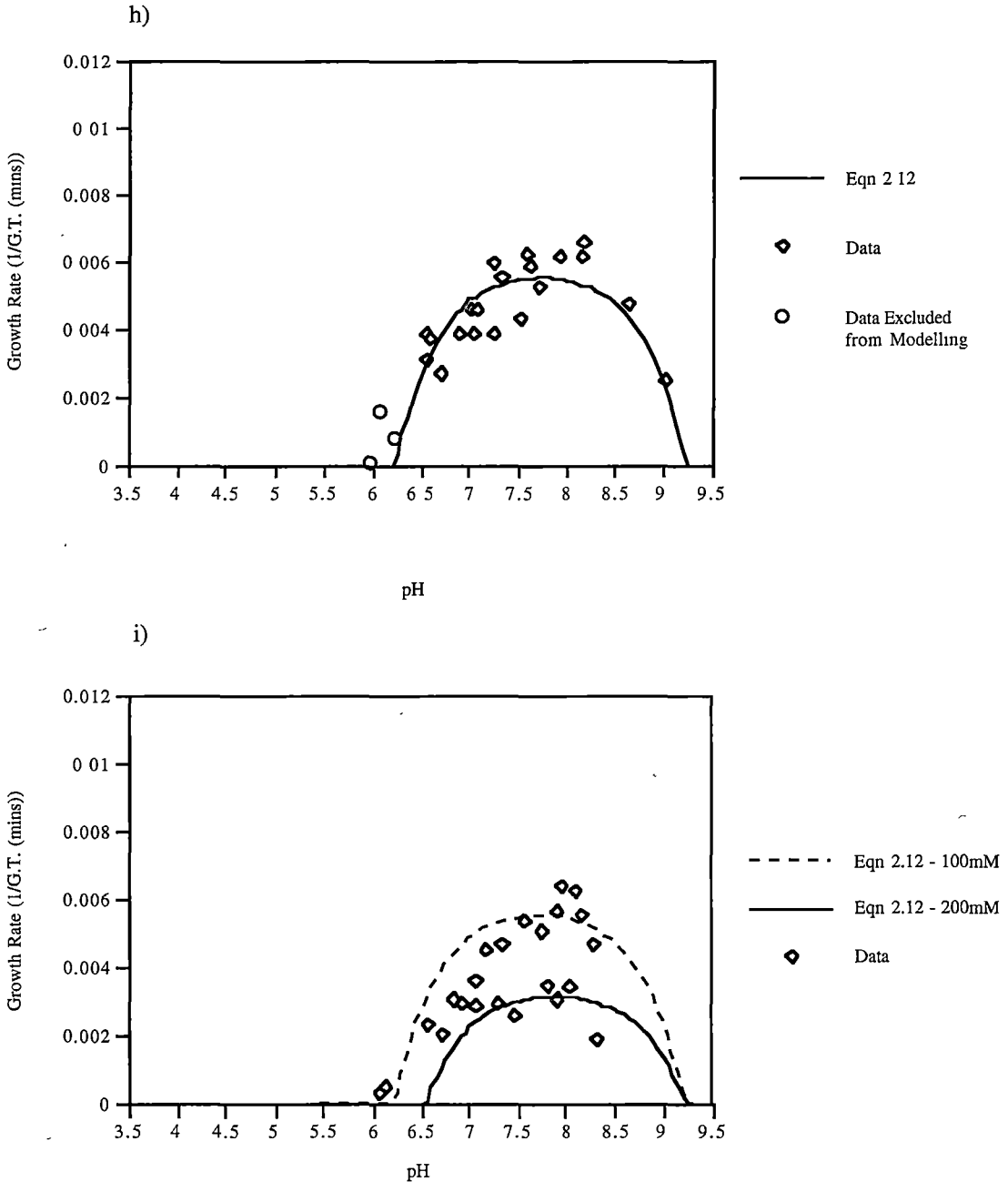


Figure 2.8 - (continued) - Eqn 2.12 and data for acetic acid for *E. coli* M23 h) 100mM acetic acid i) 200mM acetic acid. Data excluded from modelling is discussed in Section 2.5.1.3.

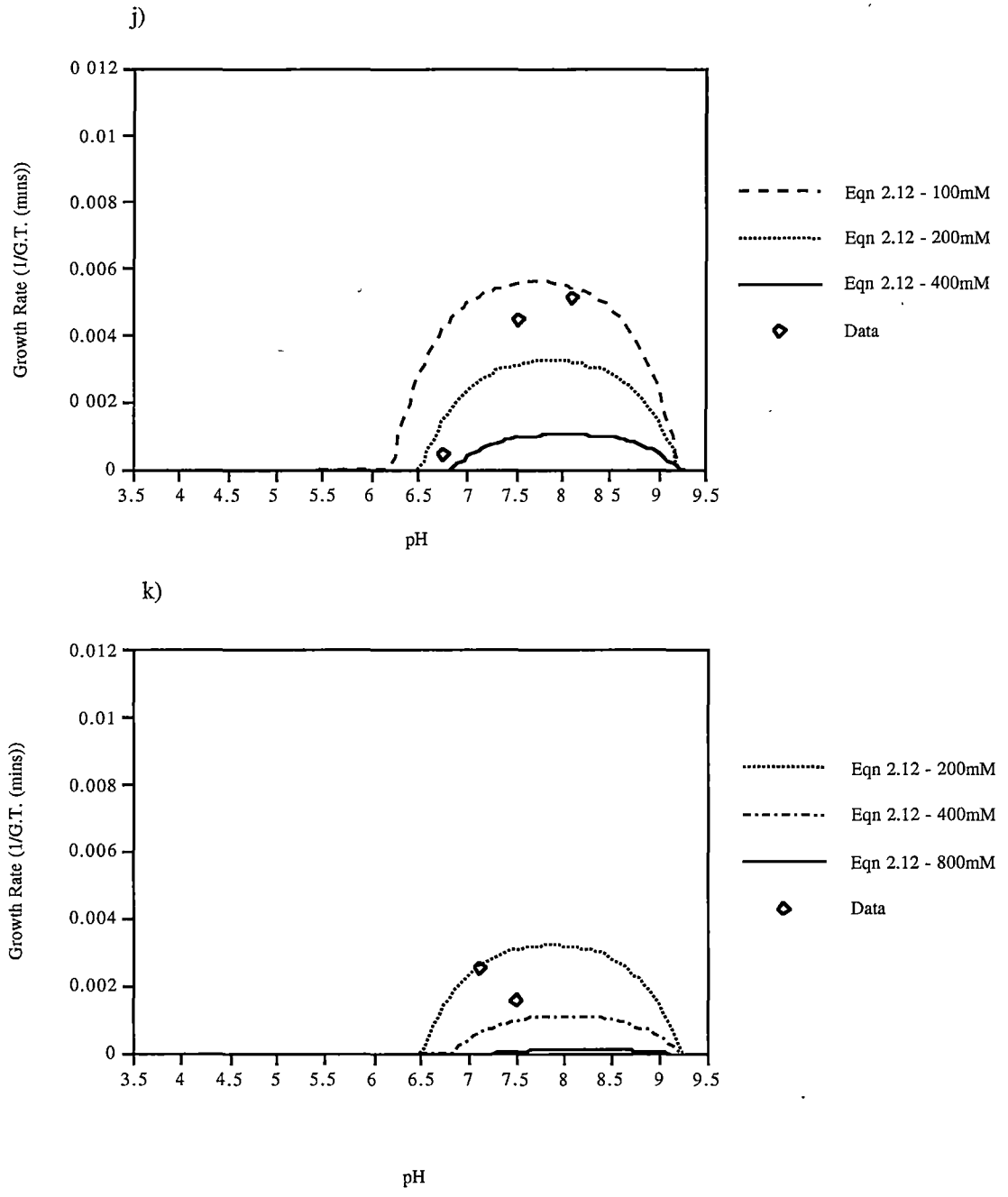


Figure 2.8 - (continued) - Eqn 2.12 and data for acetic acid for *E. coli* M23 j) 400mM acetic acid k) 800mM acetic acid.

2.4.5 Growth Rate Inhibition by High pH

Data were collected at 37°C to determine the response of strain M23 to high pH or inhibition by hydroxyl (OH^-) ions. Unfortunately at this high temperature there was little observed inhibition in growth rate until complete inhibition of growth, i.e. no growth, occurred at 9.5. However a model that described the data well was created as shown below.

$$\sqrt{k} = c\sqrt{1-10^{pH-pH_{max}}} \quad +e \qquad (2.13)$$

This model fitted to all 36 datapoints resulted in the following equation.

$$\sqrt{k} = 0.0289\sqrt{1-10^{pH-9.50}} \qquad (2.13a)$$

The model was also fitted with the two highest pH growth rate data and no growth data (shown as filled diamonds in Figure 2.9) excluded from the dataset. No growth data is often not included in growth rate modelling. The highest pH growth rates appeared to be inconsistent with the rest of the data. These exclusions resulted in the following fitted equation. The fitted equation 2.13b better fitted the data. The fit of these equations to the data is shown in Figure 2.9 below.

$$\sqrt{k} = 0.0283\sqrt{1-10^{pH-9.60}} \qquad (2.13b)$$

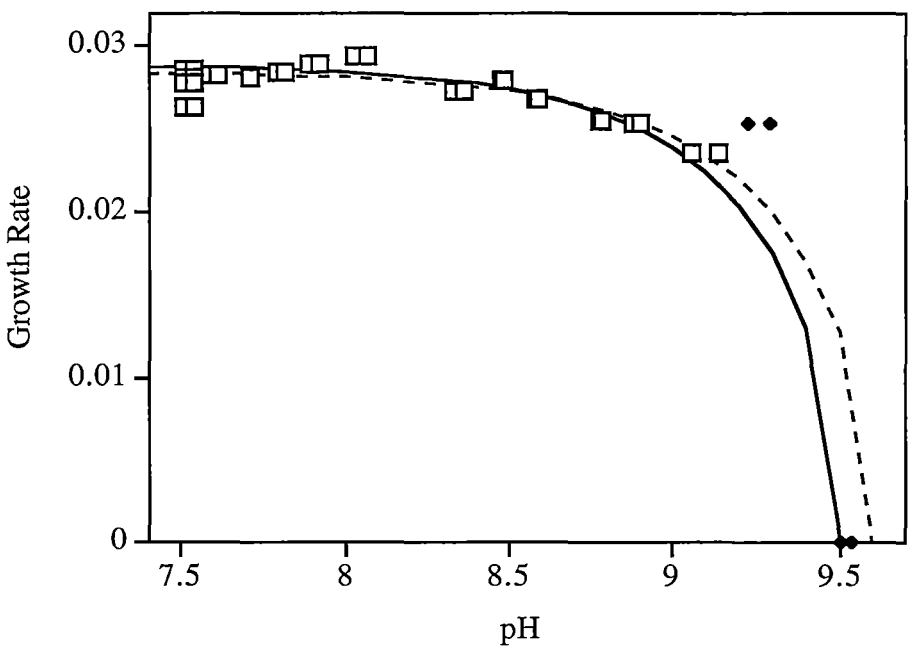


Figure 2.9 - High pH data (squares), data excluded from fitting equation 2.13b (filled diamonds) and predictions of fitted equations 2.13a (solid line) and 2.13b (dotted line) for growth of *E. coli* M23 at 37°C in nutrient broth alkalised with NaOH.

2.5 Discussion

2.5.1 Practical Problems and Possible Limitations to the Models

2.5.1.1 Lactic Acid Model

The lactic acid model fitted the data better when a high pH term was included. Although the data did not seem to indicate much high pH inhibition and the decrease in growth rate at high pH was usually small, there was a downward trend at the highest pHs for all acid concentrations. This implies that while high pH is not a common inhibitory factor in foods, the addition of a high pH term may help to better describe datasets that include pH values higher than optimal. The pH_{max} value given by the models is close to the pH maximum for *E. coli* growth of approximately pH 9 given by other studies (Gale and Epps, 1942; Glass *et al.*, 1992). Literature values given by general reviews are quite divergent for example from pH 9 (VanDemark and Batzing, 1986) to pH 10 (Desmarchelier and Grau, 1997). This study to model the high pH response showed a reduction in growth rate at pH ≥ 8.5, but that little growth rate inhibition occurred, i.e. growth rate was at least 80% of maximum rate at pH 9.1, before growth ceased abruptly at pH 9.5 (Figure 2.9). This may be partly because the measurement of high pH growth was determined at optimal temperature (37°C). At these temperatures there appears to be a more abrupt transition between where the organism can grow at a rapid rate and where growth ceases compared to lower temperatures at which growth can occur very slowly at the boundary.

It may be necessary to limit the predictions of these models to the lower concentrations of organic acid, i.e. not include predictions for >200mM lactic acid. These values are at the practical limit for experiments using the method of adding a known concentration of acid and then adjusting the pH to higher values. The results often show more scatter, possibly due to uncontrolled variables. These variable could include the difference in buffering capacity of the media in the presence of high acid concentrations, or minor variations in conditions such as temperature that would not measurably affect the growth rate under less stressful conditions.

It is not a significant limitation for applicability and validity of models (Eqns 2.4 - 2.7) in foods that the models are less reliable at high organic acid concentrations. The level of organic acids, such as lactic acid in most foods including meat, is well below these concentrations, e.g. 130-1055mg lactic acid per 100g in beef (Nassos *et al.*, 1985) or 65 to 130mM lactic acid in beef (Grau, 1981). Alternatively, foods with a high concentration of organic acid have low pH (< 4) below the pH range for growth of *E. coli* (Table 1.5, p.27). Therefore foods containing high organic acid concentrations would not support the growth of *E. coli* and the response could not be described by growth rate models. For these conditions different models are needed such as growth limit models or death rate models.

One situation in which there can be high concentrations of organic acid ions in food without low pH is the use of organic acid salts as food preservatives. Organic acid salts are the alkaline component of organic acid pH buffer systems which keep the pH at a constant level in an aqueous solution. For example sodium lactate completely dissolves in water (Eqn A) to give the anion or dissociated form of the acid ($Lactate^-_{aq}$) and sodium ions Na^+ (Eqn B). After dissolution the weak acid equilibrium comes into effect and the anions, acting as a weak base, will take up hydrogen ions from the water (Eqn C). This has the net effect of making the solution alkaline, containing an excess of hydroxyl ions, by formation of the undissociated form of the acid (Eqn D). However, (Eqn D) is an oversimplification. The dissociation reaction of lactic acid is an equilibrium governed by its pK_a so the different forms of the acid will exist in aqueous solutions at levels dependent on the total acid concentration and the pH of the solution.

$$H_2O \Rightarrow H^+_{aq} + OH^-_{aq} \quad (A)$$

$$Na^+ Lactate^-_s \Rightarrow Na^+_{aq} + (Lactate^-)_{aq} \quad (B)$$

$$(Lactate^-)_{aq} + H^+_{aq} \Leftrightarrow (H^+ Lactate^-)_{aq} \quad (C)$$

$$2(Na^+ Lactate^-)_s + 2H_2O \Rightarrow (H^+ Lactate^-)_{aq} + (Lactate^-)_{aq} + H^+_{aq} + 2OH^-_{aq} + 2Na^+_{aq} \quad (D)$$

Houtsma *et al.* (1994) studied the effect of sodium lactate on the growth of *Listeria innocua* and found that the minimum inhibitory concentration was strongly dependent on the pH. This supports the hypothesis presented here because at each concentration some inhibition will be due to each form of the acid. At pH 5.5 the minimum inhibitory concentration was 200mM sodium lactate. At pH 7, 1000mM was necessary for complete inhibition because a larger proportion was in the less inhibitory dissociated form. The results of Houtsma *et al.* (1994) describe the range of lactic acid concentration from 200 to 1000mM which is the region least well described in the results of this thesis although for a different organism. The minimum inhibitory concentration of lactic acid at pH 7, where almost all of the lactic acid will be in the dissociated form, is similar to the minimum concentration of dissociated lactic acid predicted to prevent *E. coli* growth using the growth rate models (Eqns 2.3-2.6). For their lowest pH of 5.5, the concentration of undissociated lactic acid found by Houtsma *et al.* (1994) to prevent *Listeria innocua* growth was approximately 4-5mM, which is similar to the minimum undissociated lactic acid concentration reported in this thesis to inhibit growth of *E. coli* completely. Similarly, Tienungoon (1998) found that the concentration of undissociated lactic acid required to prevent *Listeria monocytogenes* growth was in the range 3-6mM for two strains tested.

2.5.1.2 Pathogenic and Non pathogenic *E. coli* Strain Models

Due to logistic constraints, these experiments were primarily performed at a single temperature of 20°C, at which the maximum growth rate was slow enough to allow the measurement of up to 60 tubes growing simultaneously. Similar experiments at 37°C resulted in growth rates that were too fast to allow accurate measurement of rates for large numbers of cultures.

High pH (up to less than pH 8.5) was observed to be inhibitory to particular strains (e.g. BR), but was not generally a significant factor for the other strains. There was variation between the estimates of parameter values (c, pH_{min}, pH_{max}, Q) for each of the strains. However, for most strains there were similar trends. Pathogenic strains had generally higher maximum growth rates (as given by the c estimate) and lower Q and pH_{min} estimates than the nonpathogenic strains. These differences reflect a different shape of the pH response for pathogenic strains (Figure 2.7). The exceptions to this trend were the two slowest growing non pathogenic strains (FT1 and YY) which showed atypical responses to pH (Figure 2.7e & f) and where the data seemed to show a high degree of scatter.

It is possible that the restricted number of datapoints in some of the datasets caused problems in the fitting of the model. Other studies have used greater numbers of data, for example, 25 - 48 datapoints for each strain were used for creation of individual temperature models for different strains of *E. coli* (Salter, 1998). Generation of a larger number of data is useful to have confidence that the model predictions are not being displaced by influential outliers in the data. Also a lack of data in a specific region of the response will cause problems with fitting particular parameters. For example, the data for strain FT1 do not include any very slow growth rates at low pH and therefore, pH_{min} is not well estimated.

2.5.1.3 Acetic Acid Model

The growth rate of *E. coli* was more inhibited by acetic acid than lactic acid for the same total concentration of acid at neutral pH. 25mM lactic acid caused similar inhibition as 5mM acetic acid in terms of the pH at which growth ceased and maximum growth rate but at lower concentrations of undissociated acid. However, data for high concentrations of acetic acid were also collected. The shape of the response to acetic acid was similar to the response described for lactic acid but the same model (Presser *et al.*, 1997) did not fit the acetic acid data well.

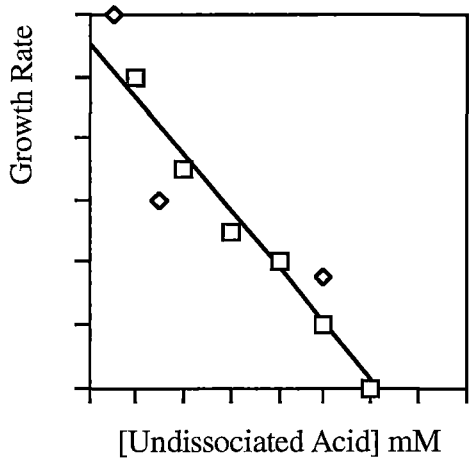
There was a systematic underestimation of the growth rate data at low pH and overestimation of the growth rate data at high pH. The fit overall of the model was not very good. Using a manual method of calculating the rate rather than fitting the modified-Gompertz function (Eqn 1.1), a slower estimate of rate at the very beginning of growth of the population was obtained. This implied that the conditions (pH and organic acid concentration) in those cultures changed as growth occurred and consequently the growth rate increased as the culture grew.

There are two possible reasons for an increasing growth rate, which causes the modified-Gompertz function to measure the fastest rate at conditions different to the initial conditions. Either the pH or the concentration of acetic acid in the broth was changing during the lag phase or early growth of the bacteria. This could be caused by metabolic action of the bacteria or by volatilisation of acid (Brockelhurst and Lund, 1990). While the pH is easily measurable it would not be so easy to measure the elimination of the acetic acid in the broth. In reality it is likely that both these processes may occur to varying degrees which makes the accurate measurement of both levels necessary. It is also possible that the production of other acidic or alkaline products by the bacteria would affect the pH and buffering capacity of the media and would be an important factor in the changing conditions.

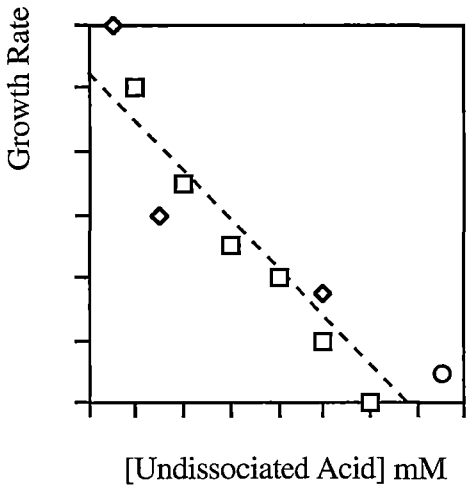
It is possible to investigate these hypotheses by determining what degree of change in the pH or acetic acid levels would be needed to change the growth rate to the level estimated by the modified-Gompertz function. Using 15 datapoints, at 15, 20, 25 and 100mM acetic acid concentrations, the degree of change in conditions needed to cause the faster measured growth rate was determined. These values were a fall in pH by 0.06 to 0.24 or a drop in undissociated acetic acid concentration of 0.42 to 2.38 mM. From this it seems reasonable that the proposed changes in either pH or acetic acid could occur, and if both occurred it would require even less change in either than calculated above. For example, the size of the change in pH is only twice the accuracy limit (0.1 unit) of the pH metering technology used throughout this thesis.

The method of estimating the value of the parameter U_{min} assumes a linear relationship of growth rate to undissociated acid concentration. Unfortunately data where rate has been overestimated, due to changes in pH or acid concentration, have a large influence on the U_{min} estimate. As shown in Figure 2.10a, the relationship between growth rate and undissociated acid is a negative linear correlation, as shown by the datapoints (squares) and the solid line (-). Even if some points are offset from the line (diamonds) the fit remains similar. Figure 2.10b shows the effect of the presence of an outlier datapoint at a high undissociated acid concentration on the right of the line (circle). The line of best fit moves to the right (dashed line) which slightly disrupts the fit with relation to all the other points.

a)



b)



c)

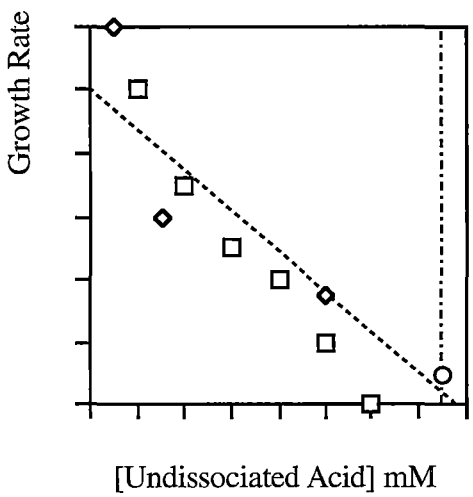


Figure 2.10 - Demonstration of the weighting effect of growth rate data, differentiated by plot symbols (see text for details) at high undissociated acid concentrations on the estimation of U_{min}. a) Normal fitting of a simple linear regression. b) The effect of an outlier (o) at high [UD] near the X axis. c) The effect of forcing the U_{min} value to be higher than the highest [UD] showing growth (i.e. the outlier point).

In addition, if the constraints that the SAS modelling program (SAS Institute Inc., 1989) uses are applied, as shown in Figure 2.10c, the undissociated acid concentration must be higher than all the points at which growth occurred. This causes the "line of best fit" to be shifted even further to the right (dotted line). The fitting routine requires that the intercept of the line must be greater than the maximum concentration of undissociated acid at which any growth was recorded (broken vertical line). This disrupts the fit of the line and results in a model that does not fit the total dataset very well. 15 datapoints were excluded for this reason, i.e. to avoid a biased weighting of the intercept for U_{min} to the right of the line of best fit for all the other points. The points excluded were at the lowest pHs for total concentrations greater than 15mM. Nonetheless in the final model all the excluded datapoints show a good fit to the model (see Figure 2.8).

The high pH term improved the fit of the model by allowing the deviations from the optimum rate at high pH to be fitted. The data at the highest acetic acid concentrations were inconsistent, scattered and showed a different rate of change of the slope to the other lower concentrations. For individual strains without organic acids (Section 2.4.2), this change in slope could be modelled by adding a Q coefficient to the pH term. However, as the models for organic acids are generated from the pH responses at all the concentrations taken together, adding a Q coefficient would not improve the model due to the changes in the pH response at different concentrations.

From a practical food microbiology standpoint there is an argument for the exclusion of the data at the highest organic acid concentrations as they are the least realistic. When high concentrations of acid are added the pH becomes very low. Consequently the pH has to be raised by 4 or 5 pH units in order to allow growth to occur. In real situations it is unlikely that these high concentrations will be present and that the bacteria will able to grow, for example, in foods. The pH range of foods and their acid contents given in Table 1.5 (p.27) shows that the normal range of conditions of food have been covered by the data gathered. Foods in which combinations of organic acids are present requires further study to determine the response to those combined stresses.

2.5.2 Mathematical Basis of the Models

Although these models may appear mathematically complex the conceptual basis of each term is simple. There are up to six terms, one for each factor: temperature, water activity, low pH, high pH, concentration of the dissociated form of the acid and the concentration of the undissociated form of the acid. This is in contrast to polynomial models which have a larger number of terms and combining the effect of several inhibitory factors in their terms. Square root type models frequently contain the temperature and water activity terms used in these models. These terms have successfully been used for a wide range of microorganisms (McMeekin *et al.*, 1987; Chandler and McMeekin, 1989; Ross, 1993; Miles *et al.*, 1997; Ross, 1999).

The T_{min} integer value of 4°C used in fitting these models was approximately the same as previous modelling estimates such as a square root temperature and water activity model for *E. coli* M23 (Ross, 1999) whose T_{min} was 4.14±0.63. The a_{Wmin}'s generated by fitting the growth rate models were 0.922, 0.934, 0.942, 0.951 and 0.961. Of these, the values for the lactic acid model with no pH_{max} fitted to dataset 1 (Eqn 2.4) was the same as that found when a_W was adjusted with NaCl 0.951 (Ross, 1999). For a pathogenic strain of *E. coli* (R31) a_{Wmin} was found to be 0.963 using a model with an a_{Wmax} term (Salter, 1998) which is very close to the a_{Wmin} value from the acetic acid model (Eqn 2.12) of 0.961. These favourable comparisons suggest that the humectants sodium chloride, acetic or lactic acid have similar inhibitory effect for water activity. The inhibitory effect of water activity ($a_{W_{min}}$) was independent of the humectant for some organisms (Ross, 1993) but not in all cases (Chandler & McMeekin, 1989). For example, glycerol which is not ionised produced an a_{Wmin} of 0.908 for *Staphylococcus xylosus* in comparison to an a_{Wmin} of 0.838 with NaCl.

Novel terms for the pH and organic acid factors (Derivations - Appendix 5.1 & 5.2) were developed based on the following hypotheses. First, that growth rate inhibition by pH is proportional to hydrogen ion concentration. Second, that growth rate inhibition by organic acid is proportional to undissociated acid concentration and dissociated acid concentration. The inhibition due to each novel factor is given as a value by which the maximum growth rate is inhibited from 0 (completely inhibitory conditions) to 1 (optimal conditions, no inhibition). Other studies have used the pH at the midpoint of growth, the point where rate is measured, as the variable against which growth rate is modelled (Ross, 1993). While this is theoretically more valid in these broth systems where the broth pH changes as growth occurs, it does not represent the possible practical outcome of the use of the model in the food industry.

Originally the pH term of the model was based on a mathematical function that describes a linear rise, in terms of hydrogen ion concentration, from a threshold value to an optimum value (Presser *et al.*, 1997). A linear response of growth rate to hydrogen ion concentration has been reported (Cole *et al.*, 1990) and was observed in this study under some conditions. The form of 10^{-pH} is used to translate pH into hydrogen ion concentration as pH is the negative of the $\log_{10}$ of hydrogen ion concentration. The pKa is equivalent to the pH of a weak acid solution at which half the acid is in each of the undissociated and dissociated forms. The proportions of the two forms of acid can be calculated for any acid of known pKa, at any pH, using the Henderson-Hasselbalch equation. The total concentration of acid and the pH are used to calculate the concentration of undissociated acid and dissociated acid respectively in each term of the models described in this thesis. Therefore the pH must appear in each of these three terms of the model.

The addition of the Q factor in the pH model for the individual strains allows a greater flexibility of the model to fit the slope of the curve for the data. The size of the Q coefficient gives the degree to which the slope of the curve is flattened. The dissociated acid term was altered to a new term which included the D coefficient because of the shape of the growth rate response to high concentrations of dissociated acetic acid. The response was best described by a mathematical relationship of the form $10^{D[\text{Dissociated acid}]}$. This relationship was found to describe the growth rate response to dissociated acid concentration better and it was still based on the concentration of the dissociated form of the acid. The addition of the high pH term allows high pH inhibition by hydroxyl ions to be modelled as a mirror image of low pH inhibition by hydrogen ions.

2.5.3 Comparison of pH Response of *E. coli* to Other Studies

The growth rate response of *E. coli* to pH, acetic and lactic acid rises steeply from pH_{min} to a plateau region of similar optimal growth rates for a range of near neutral pHs. Similar pH responses to that described in this thesis have been reported for *Listeria monocytogenes* in response to HCl and lactic acid (Ross, 1993; Tienungoon, 1998) and for *Vibrio parahaemolyticus* in response to HCl and lactic acid (Miles, 1994). Other workers have described pH as causing only a small decrease in growth rate except at pHs close to the growth limit where a significant decrease in the growth rate occurs abruptly (Gibson *et al.*, 1988; Cole *et al.*, 1990). Many studies which report the effect of pH are limited by the amount and number of variations in pH that they have measured. Gibson and Roberts (1986) tested growth rates only at three pH values (5.6, 6.2 & 6.8), all of which are well within the growth range of *E. coli*. This problem has widely occurred when other variables that are considered more important to growth, such as temperature, have been determined in detail. The variables considered less important, such as pH, are tested at only a few points. Considering that pH inhibition is only significant at near limiting pH values (e.g. within 1 pH unit of the minimum pH) it is not surprising that such studies found little pH effect on the growth response except where other factors where limiting.

These models have been expanded to include a high pH term which allows the effect of high pH or hydroxyl ions (OH^-) to be taken into account. Ironically it was the inhibiting presence of organic acid at high pH which first led to a noticeable decline in growth rate at higher than neutral pH. This emphasizes the fact that the inhibition of organic acids is independent of their pH lowering effect. The data collected did not generally extend above the optimal pH range and foods rarely have an alkaline pH (Table 1.5). Inhibition occurred at near to neutral pHs when other conditions were limiting such as the presence of organic acids. Considerable variation was observed in the pH_{max} values of the various strains of *E. coli*. Non pathogenic strain BR in particular seemed to have a much lower pH_{max} values compared to all other strains.

2.5.4 Comparison with Other pH Models

These models (Eqn 2.4 - 2.12) for the growth rate of *E. coli* in response to pH, acetic and lactic acid provides a good description of the datasets. The models differ from to other published pH models because they describe a pH response that rises steeply from pH_{min} to a plateau region of similar optimal growth rates for a range of near neutral pHs.

Square root derived models, unlike the polynomial models that include the effect of pH (Buchanan *et al.*, 1993; McClure *et al.*, 1993; Bhaduri *et al.*, 1995), enable the comparison of the fitted model values for pH minima and pH maxima between strains and species. Although all models for pH described are empirical, square root derived models separate the response to each factor and may be used to further clarify which are the major causes of inhibition. This approach has been termed the 'gamma concept' by Zwietering *et al.* (1996) and is used in the cardinal parameter models of Rosso *et al.* (1995). While polynomial models can also separate the effects their complexity makes interpretation of the response to each factor more difficult. A comparison of square root models and polynomial models reported that square root models were more accurate in their predictions than polynomial types (Heitzer *et al.*, 1991; Delignette-Muller *et al.*, 1995). However, polynomial models in general describe more variables and therefore can fit more closely to an individual dataset but often predict less well to other or more general datasets.

Further, square root type mathematical models described in this study are based on underlying hypotheses which were used to develop the form of equation. If these models are found to fit the data well, this gives support to the physiological causes of inhibition as embodied in the hypotheses. Therefore the models described in this thesis demonstrated that inhibition of organic acids is due to separate effects of undissociated acid, dissociated acid and pH and that hydrogen ions and undissociated acid are the main inhibitory factors, each one dominating depending on the conditions.

Square root models describing the effects of temperature and water activity are well established in the literature (McMeekin *et al.*, 1987; Chandler & McMeekin, 1989; Heitzer *et al.*, 1991; Ross and McMeekin, 1991; Miles, 1994). However, square root models that incorporate a term for pH, in comparison to other square root models are infrequently reported in the literature and inconsistent in their approaches.

The use of a water activity term from a square root model as a prototype for a pH term was moderately successful (Adams *et al.*, 1991) but unsuccessful for modelling survival (Little *et al.*, 1992b). Likewise the substitution of the entire temperature range term to describe the whole range of pH, with both pH_{min} and pH_{max} terms, was attempted (Wijtzes *et al.*, 1993) (Section 1.3.1.5, Eqn 1.9). However, this model was subsequently replaced with a purely empirically derived parabolic pH term as well as a model that used the form of the square root superoptimal temperature term to allow for asymmetry in the data (Wijtzes *et al.*, 1995) (Section 1.3.1.6, Eqn 1.10). These models also described the suboptimal and superoptimal regions of pH inhibition.

Figure 2.11 below shows the fit of these models to growth rate data showing pH inhibition (Miles, 1994) for *Vibrio parahaemolyticus* strain 38.349 . These data were collected at ~ 20.75 ± 0.3 with the individual temperature readings for each growth rate determined. Therefore to graph the data and the models together the rates were adjusted using the relative rate concept (McMeekin *et al.*, 1993) to a temperature of 20°C with a T_{min} of 5.86 as reported by Miles (1994).

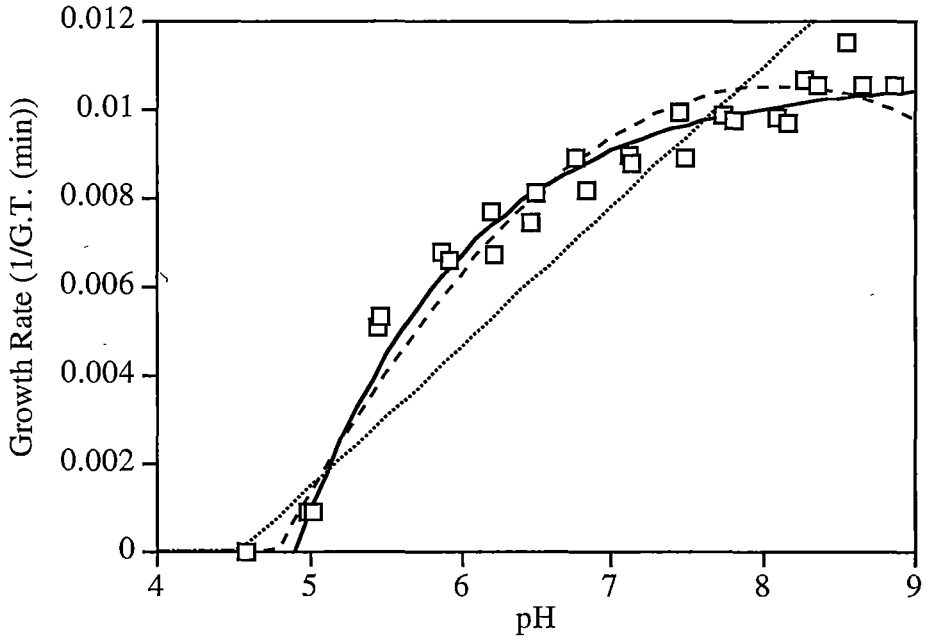


Figure 2.11 - Fit of pH models from this study (Eqn 2.10) (solid line), (Wijtzes *et al.*, 1995) (dashed line) and (Adams *et al.*, 1991) (broken line) to growth rate data of *Vibrio parahaemolyticus* strain 38.349 at 20°C in tryptone soy broth + 3% salt (Miles, 1994). Model fitted values and goodness of fit using Chi² are shown in Appendix 2.6 .

The shape of each model's response to temperature can be seen from Figure 2.11. The pH model of Adams (1991) was developed with data that were mainly influenced by temperature but had a pH inhibitory component. It follows a straight line from a minimum pH value and was not found to fit these data (Figure 2.11) or any of the other datasets well (data not shown). The various "Wijtzes" models (Wijtzes *et al.*, 1993; Wijtzes *et al.*, 1995) showed a similar shape when fitted to the data (Figure 2.11). These models achieve a good fit of the data but are constrained by the form of the model to describe a decreasing growth rate to a maximum pH even though the data themselves show no high pH inhibition. This causes a deviation from the data with an underprediction at lower pH and an overprediction at optimal pH which is larger for Wijtzes *et al.* (1993) (not shown) than for Wijtzes *et al.* (1995). The model developed in this study, containing a Q coefficient showed the best fit to the model, and did not need to include a pH_{max} term.

Figure 2.12 shows the fit of these models to growth rate data showing pH inhibition (Ross, 1993) for *Listeria monocytogenes* Scott A. These data were collected at 19.5 ± 0.5°C.

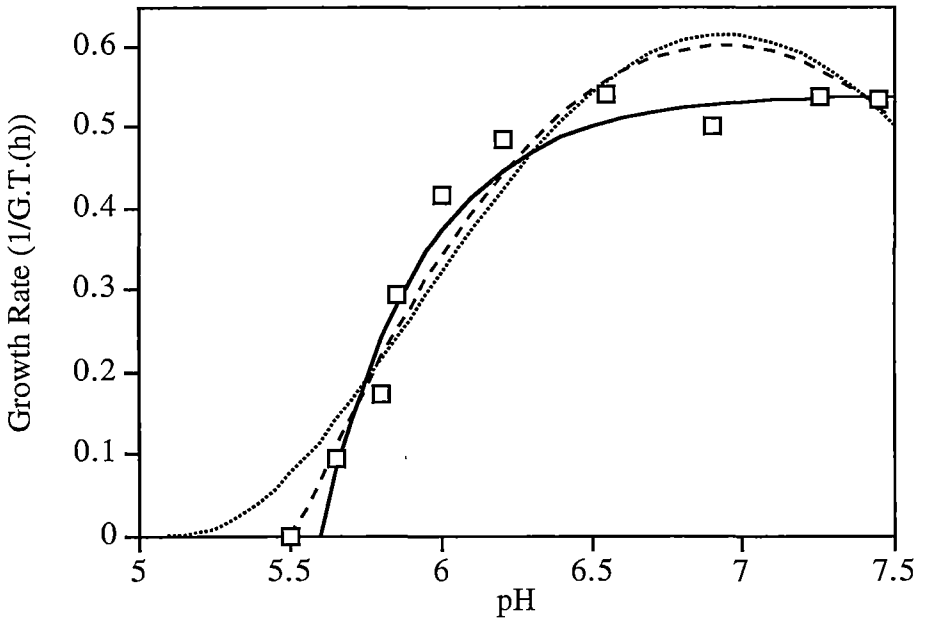


Figure 2.12 - Fit of pH models from this study (Eqn 2.10) (solid line), (Wijtzes *et al.*, 1995) (dashed line) and (Wijtzes *et al.*, 1993) (dotted line) to growth rate data of *Listeria monocytogenes* Scott A at 19.5 ± 0.5°C in tryptone soy broth + 0.2M lactate (Ross, 1993). Model fitted values and goodness of fit using Chi^2 are shown in Appendix 2.6.

The various models of Wijtzes *et al.* (Wijtzes *et al.*, 1993; Wijtzes *et al.*, 1995) again showed a similar shape when fitted to the data (Figure 2.12) and the same deviation. The model developed in this study containing a Q coefficient showed the best fit to the model, and did not need to include a pH_{max} term as the data were over a restricted range of pH all less than 7.5.

Petran and Zottola (1989) published another study on the pH inhibition of *Listeria monocytogenes* Scott A (Figure 2.13). There were a smaller number of points but these points were over the whole biokinetic range of pH and showed high pH inhibition. However, there are still relatively large regions where data are lacking. In the region where growth occurs, the measurements of growth rate are 1 pH unit apart, so that the measured growth rate increases by three and a half times from one observation at pH 5 to the next at pH 6, and falls again by almost the same amount from pH 8 to pH 9. There are insufficient data points in the optimal growth area to evaluate the shape of the pH response critically.

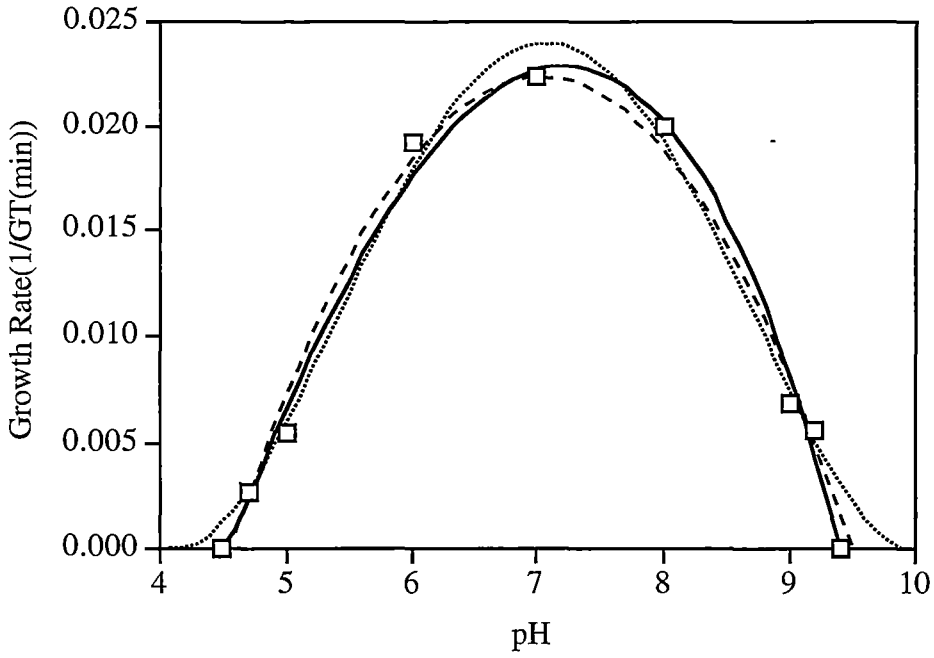


Figure 2.13 - Fit of pH models from this study (Eqn 2.14) (solid line), (Wijtzes *et al.*, 1995) (dashed line) and (Wijtzes *et al.*, 1993) (dotted line) to growth rate data of *Listeria monocytogenes* Scott A at 30°C in tryptic soy broth (Petran & Zottola, 1989). Model fitted values and goodness of fit using Chi^2 are shown in Appendix 2.6.

The models of Wijtzes *et al.* (1995) showed their characteristic parabola shape when fitted to the data of Petran and Zottola (1989) (Figure 2.13). Unlike the previous two datasets described, the use of a new asymmetrical model with both a suboptimal pH term containing Q and a superoptimal pH term containing an analogous R (Eqn 2.14) resulted in an improved fit of the model.

$$\sqrt{k} = c(T - T_{\min})\sqrt{1 - 10^{Q(pH_{\min} - pH)}}\sqrt{1 - 10^{R(pH - pH_{\max})}} \qquad (2.14)$$

The model of Wijtzes *et al.* (1993) gave a better fit to the data than that of Wijtzes *et al.* (1995) and the model developed in this study, despite the fact it contained both a Q coefficient and an equivalent R coefficient for the pH_{max} term. However, with more datapoints in the region between pH 5 and 9 a better fit by the model developed in this study would be possible. Comprehensive studies of the pH and organic acid response of *Listeria monocytogenes* strains have found that models of the type developed in this study can be fitted for both pH and lactic acid inhibition (Tienungoon, 1998).

There are few literature datasets with a large number of datapoints for *E. coli* showing pH inhibition. Datasets such as Buchanan *et al.*(1993) contain data at different pH but it is only a minor variable and data are given at only a few (4-6) datapoints over the pH range at any given temperature. This kind of data can be modelled using the pH models in this thesis (not shown) but the restricted data range makes it difficult to draw conclusions about the relative fit of each of the models to the data.

The disadvantage of few datapoints can also be seen for the data of Glass *et al.* (1992) (Figure 2.14). There is a large degree of uncertainty in the "shape" the model should take. Data for the inhibition of *E. coli* by lactic acid were also collected in this study but were unable to be modelled because the method used involved using lactic acid as the acidulant to adjust the pH, so the amount of acid at each pH was unknown.

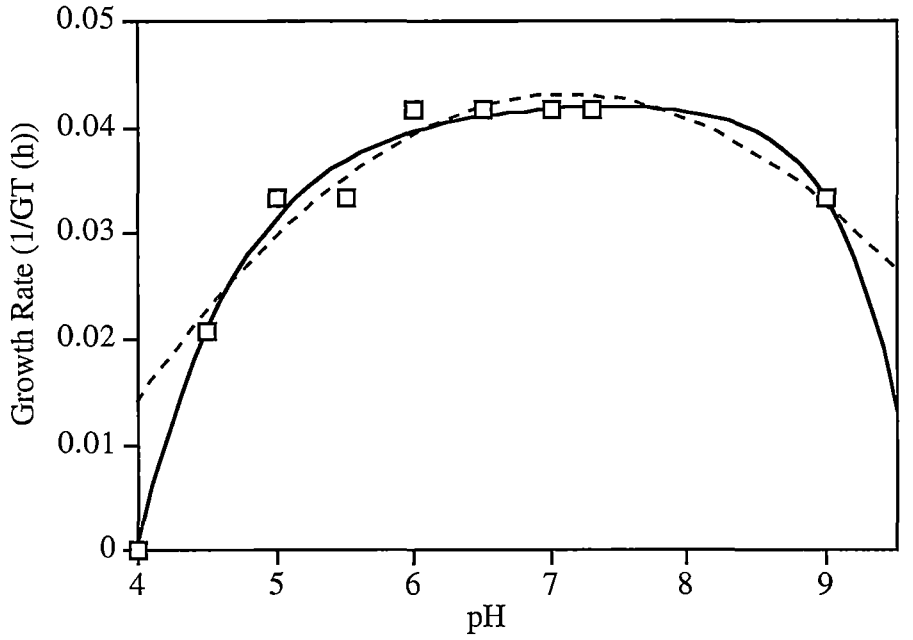


Figure 2.14 - Fit of pH models from this study (Eqn 2.11) (solid line) and (Wijtzes *et al.*, 1995) (dashed line) to growth rate data of *E. coli* O157:H7 at 37°C in trypticase soy broth (Glass *et al.*, 1992). Model fitted values and goodness of fit using Chi^2 are shown in Appendix 2.6.

Fitted Wijtzes models (Wijtzes *et al.*, 1995) and (Wijtzes *et al.*, 1993) overlapped (not shown) for the data of Glass *et al.* (1992) . These Wijtzes models showed a similar goodness of fit to the data (Figure 2.14) as the other model developed in this study (Eqn 2.11), containing a Q coefficient and a pH_{max} term. However, if more data were available this would determine whether the shape of the response is fitted better by the models developed in this study. For instance the predictions of the pH_{min} and pH_{max} values given by the Wijtzes models are very wide, given that no growth was measured at pH 4.

Another *E. coli* dataset is found in the work of Gale and Epps (1942). It has similar limitations to that of Glass *et al.* (1992), with few datapoints to cover the whole range of pH. However, these data were collected at two temperatures which allows for more data to be included in the model fitting, provided the temperature can also be modelled. The data were modelled using the T_{min} of 4°C previously used for *E. coli*.

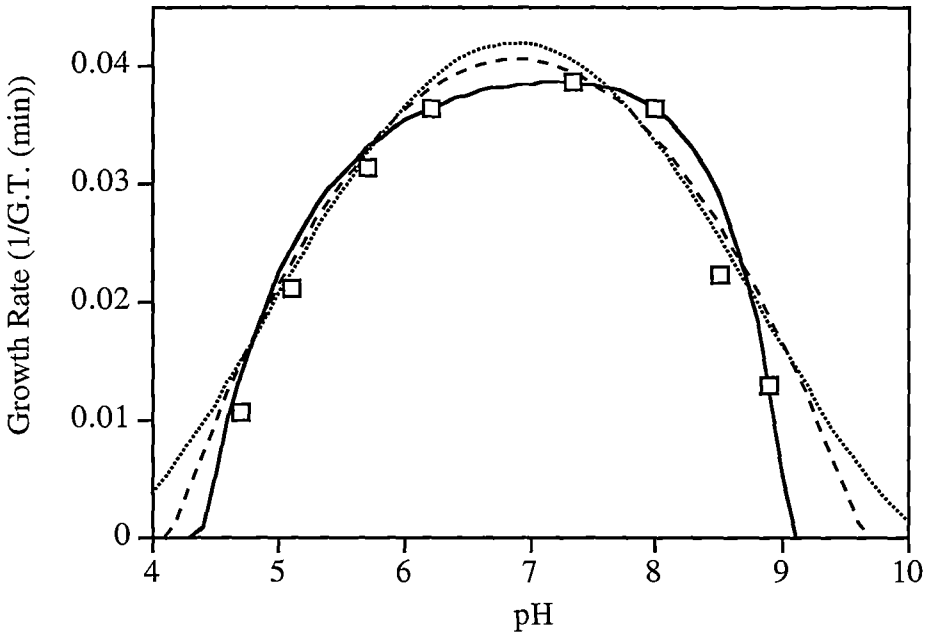


Figure 2.15 - Fit of pH models from this study (Eqn 2.11) (solid line), (Wijtzes *et al.*, 1995) (dashed line) and (Wijtzes *et al.*, 1993) (dotted line) to growth rate data of *E. coli* at 37°C in broth (Gale & Epps, 1942). Model fitted values and goodness of fit using Chi² are shown in Appendix 2.6.

The Wijtzes models (Wijtzes *et al.*, 1993; Wijtzes *et al.*, 1995) showed a better goodness of fit to the data (Figures 2.15 & 2.16) than the model developed in this study, containing a Q coefficient and a pH_{max} term (Eqn 2.11). However, this is a problem with the temperature modelling which caused the predicted values at 27°C to be underestimated for all models (Figure 2.16). Therefore the Wijtzes models, which overestimate in the optimal growth region, have a better fit for these data. All of the models fitted the data better when T_{min} was lowered suggesting that the T_{min} value used in the modelling (4°C) might not be correct for the strain used by Gale and Epps (1942). Similarly to Glass *et al.* (1992) there is a lack of data in the region of slow growth rates, data which are important to determine the overall shape and estimates of the pH_{min} and pH_{max} values. Again the predictions of the pH_{min} and pH_{max} values given by the Wijtzes models are very wide but observations of no growth were not described by Gale and Epps (1942).

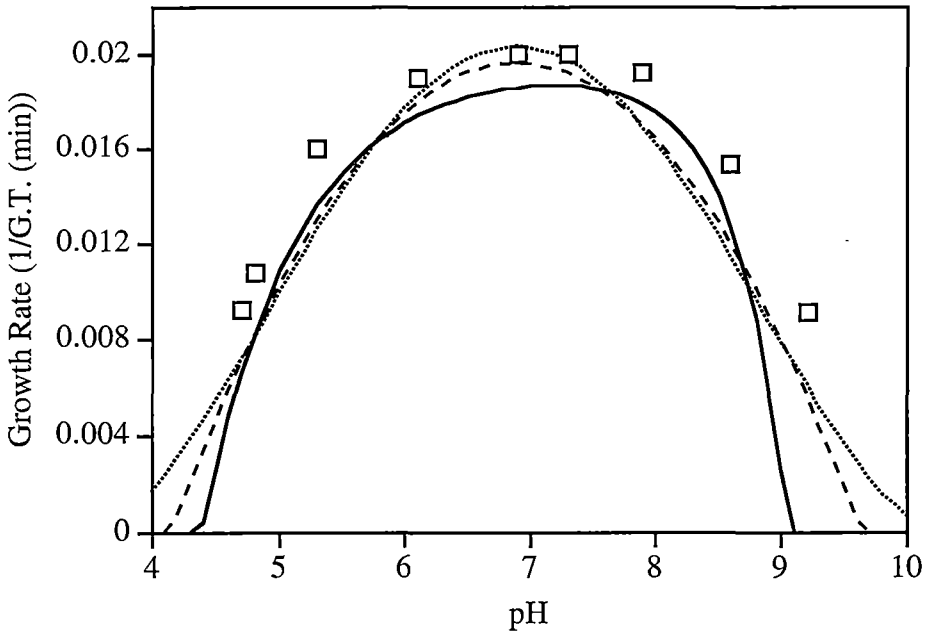


Figure 2.16 - Fit of pH models from this study (Eqn 2.11) (solid line), (Wijtzes *et al.*, 1995) (dashed line) and (Wijtzes *et al.*, 1993) (dotted line) to growth rate data of *E. coli* at 27°C in broth (Gale & Epps, 1942). Model fitted values and goodness of fit using Chi^2 are shown in Appendix 2.6.

Another dataset that reveals pH inhibition at several temperatures is that used by Wijtzes *et al.* (1995) to create their model for *Lactobacillus curvatus*. It has a large number of datapoints at each temperature which cover most of the biokinetic range of pH. Wijtzes *et al.* (1995) modelled the data at each temperature separately and there was a wide variation in estimates of pH_{min} and pH_{max} at each temperature (Wijtzes *et al.*, 1995). In this study we modelled the combined data from Wijtzes *et al.* (1995) for three temperatures (6°C, 15°C and 29°C). The temperature can also be modelled using an approximate T_{min} of -3°C obtained by Wijtzes *et al.* (1995).

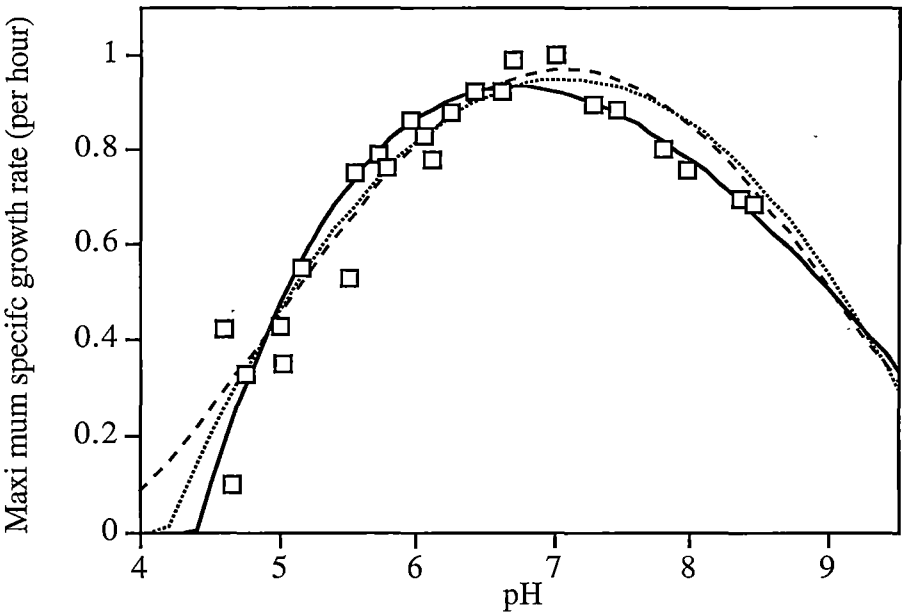


Figure 2.17 - Fit of pH models from this study (solid line), (Wijtzes *et al.*, 1995) (dashed line) and (Wijtzes *et al.*, 1993) (dotted line) to growth rate data of *Lactobacillus curvatus* at 29°C in broth (Wijtzes *et al.*, 1995). Model fitted values and goodness of fit using Chi^2 are shown in Appendix 2.6.

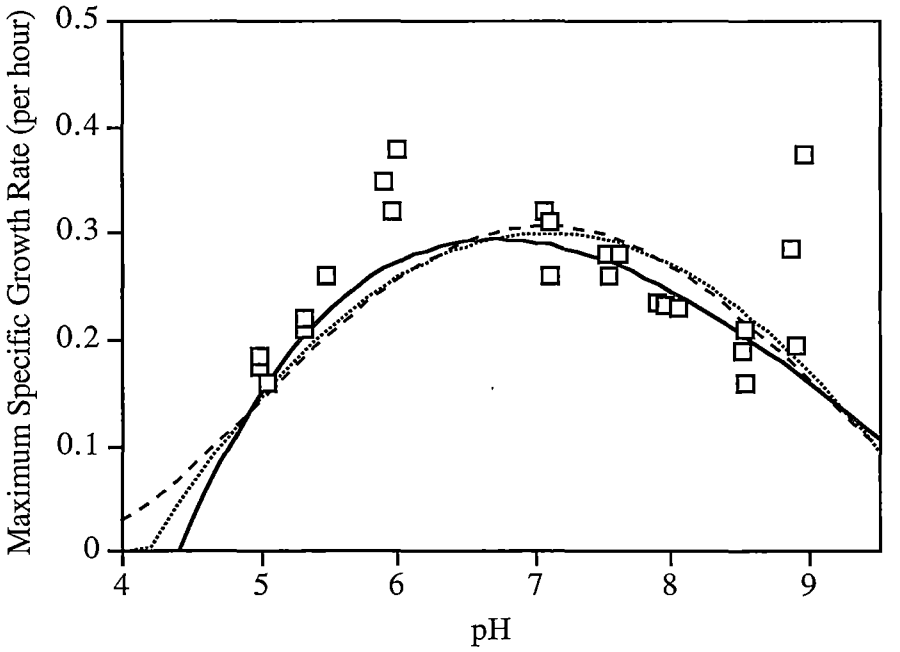


Figure 2.18 - Fit of pH models from this study (solid line), (Wijtzes *et al.*, 1995) (dashed line) and (Wijtzes *et al.*, 1993) (dotted line) to growth rate data of *Lactobacillus curvatus* at 15°C in broth (Wijtzes *et al.*, 1995). Model fitted values and goodness of fit using Chi^2 are shown in Appendix 2.6.

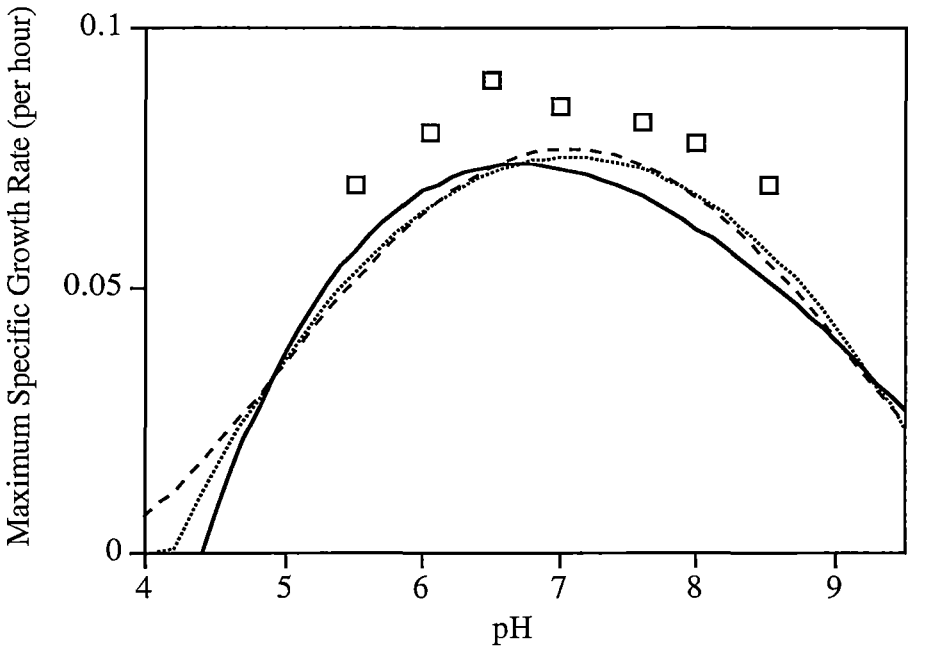


Figure 2.19 - Fit of pH models from this study (solid line), (Wijtzes *et al.*, 1995) (dashed line) and (Wijtzes *et al.*, 1993) (dotted line) to growth rate data of *Lactobacillus curvatus* at 6°C in broth (Wijtzes *et al.*, 1995). Model fitted values and goodness of fit using Chi^2 are shown in Appendix 2.6.

The fit of the two Wijtzes models is similar to that of the model developed by this study (Eqn 2.14). This estimation of pH_{min} and pH_{max} was difficult due to the lack of very slow growth rate data at high and low pH extremes. There is also a large amount of scatter in the data at 15°C at pH ~5.7 and pH ~9. This scatter, especially at the high pH extreme, caused problems in obtaining a pH_{max} estimate (not shown). There was also a difficulty with the temperature modelling, possibly an inappropriate estimate of T_{min}, that causes all the models to underestimate the growth rate at the lowest temperature.

In conclusion, the pH response reported in this thesis, and in other rigorous studies of the pH response of various organisms, can be accurately and thoroughly described by the new models developed in this study. Other models can also describe the data well, but lack the flexibility to describe all of the datasets well. Comparison of the fit of different models to data is often hampered by a lack of large datasets for pH inhibition.

Another type of model that is used to describe the response of microorganisms to pH uses a minimum, maximum and an optimal value. First developed for temperature modelling (Rosso *et al.*, 1993), these models have also been used for pH (Rosso *et al.*, 1995). The cardinal pH model developed by Rosso *et al.* (1995) was compared favourably by them to both the model of Wijtzes *et al.* (1993) and Zwietering *et al.* (1992). Rosso *et al.* (1995) also showed a good fit of their model to several datasets of rumen bacterial growth rates with pH, which were characterised by low pH optima for growth and a sharp decline in growth rate above optimal pH.While showing a good fit to these datasets this model includes an extra term to give a biological meaning to the model by defining the maximum possible growth rate and the values of the optimal conditions.

The use of these mathematically convenient terms has been shown to not necessarily provide the most accurate description of the shape of the pH response. It is an oversimplification from the available data to assume that a pH term could be exactly the same as terms for either water activity or temperature and that these terms could simply be substituted to model pH. The inadequacy of these models to describe some pH data sets (Ross, 1993; Miles, 1994) points to the need for new comprehensive pH models based on the unique properties of pH inhibition. Further, no previous square root models have successfully modelled the inhibitory effects of organic acids using only a pH term. This also supports the strategy described here to model organic acids as new terms distinct from the inhibition given by pH alone.

The presence of organic acid changes the observed response of microbial growth rate to the concentration of hydrogen ion concentration and supports the use of extra terms for organic acid in the model. The additional inhibitory effect of the organic acid can be seen in the changes in the observed pH response as organic acid concentration increases. As the concentration of total acid increases not only does the observed minimum pH for growth (X intercept) change but the slope of the line of growth rate versus hydrogen ion concentration also becomes steeper and the maximum growth rate decreases. The inhibition due to organic acid and pH is a complex and interdependent system, as each factor affects the level of inhibition by the other.

2.5.5 Physiological Significance of Modelling Results

Rosso *et al.* (1997) observed a correlation between the pK_a of thirteen organic acids and the resulting minimum pH at which *Salmonella* initiated growth, using the data of Chung and Goepfert (1970). Each acid was used individually to acidify the medium (tryptone-yeast extract-glucose broth).

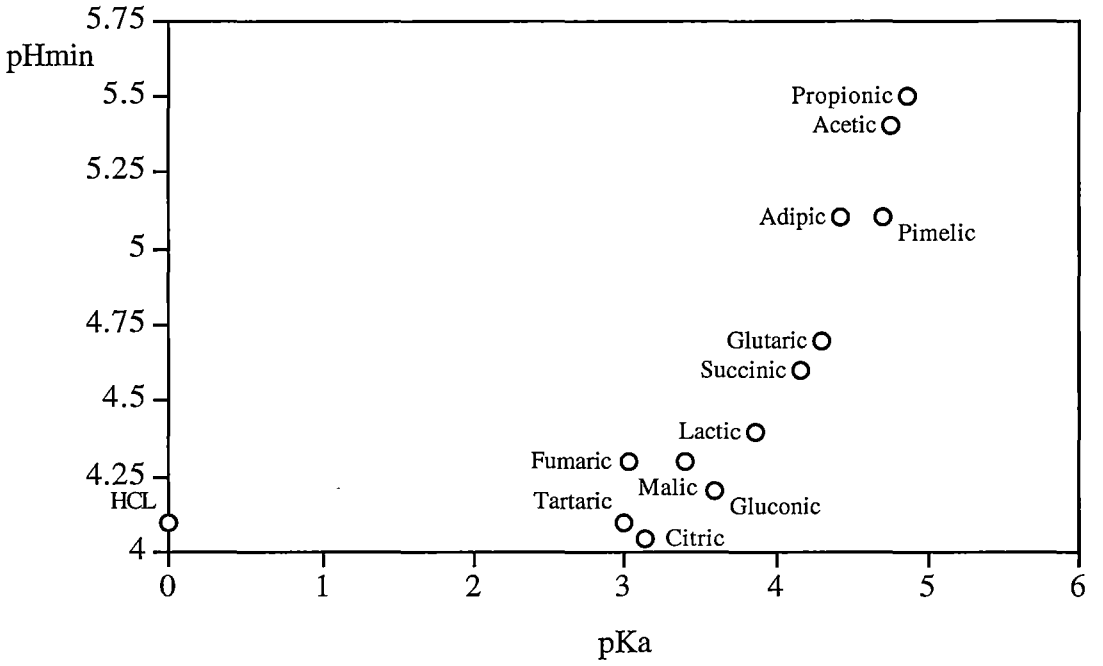


Figure 2.20 - Correlation between pH_{min} and pK_a for Salmonellae. Data from Chung and Goepfert (1970).

Table 2.8 - The pK_a constants of organic acids used by Rosso *et al.* (1997) that differ or are not included in Table 1.6 (p.31)

Acid	pK_a (value from Table 1.6)
Tartaric	3.00 (3.04)
Gluconic	3.60
Glutaric	4.30
Adipic	4.43 (4.40)
Pimelic	4.71
Acetic	4.75 (4.76)

Rosso *et al.* (1997) defined the minimum pH for growth as the interval between the measured pH_{min} and the next value for which growth was observed (precision ±0.025). They described the correlation between pH_{min} and pK_a values is in two parts:
i) below a threshold pK_a value, pK_a° , pH_{min} seems to be constant at pH_{min}°
ii) from pK_a° to the maximum pK_a studied (propionic acid = 4.87) pH_{min} seems to describe a second degree polynomial relationship.

Rosso *et al.* (1997) developed the following equations to describe these trends in the data.

$$pK_a < pK_a^\circ \text{ , } pH_{min} = pH_{min}^\circ$$

$$pK_a \geq pK_a^\circ \text{ , } pH_{min} = k(pK_a - pK_a^\circ)^2 + pH_{min}^\circ \quad (1)$$

And $$pH_{min}^\circ = pK_a^\circ + 1 \quad (2)$$

Therefore adding (2) into (1) gives

$$pK_a \geq pK_a^\circ \text{ , } pH_{min} = k(pK_a - pH_{min}^\circ + 1)^2 + pH_{min}^\circ \quad (3)$$

Rosso *et al.* (1997) fitted equation (3) to the *Salmonella* data of Chung and Goepfert (1970) using the ordinary least square criterion. The values obtained were pH_{min}° = 4.10 (3.95 - 4.25) and k =0.453 (0.398 - 0.515). One acid, fumaric acid was not included in the model; it was taken as an outlier because this organic acid is hardly soluble in water. The following figure shows the data with Rosso's model overlaid.

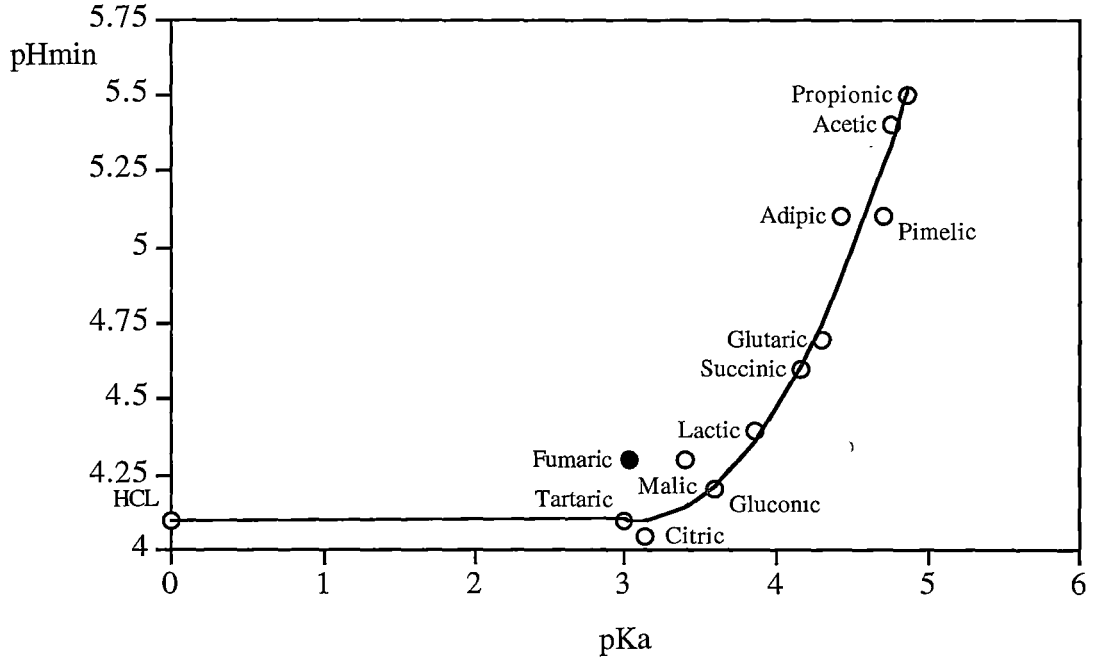


Figure 2.21 - Correlation between pH_{min} and pK_a for Salmonellae. Data from Chung and Goepfert (1970) and Model (Equation 3) from Rosso *et al.* (1997).

A similar relationship was observed between the pK_a of the acid added to lower the pH and the minimum pH for growth of *E. coli* in Mueller Hinton broth using eight of the same acids (Rosso *et al.*, 1997). Rosso et al. (1997) fitted equation (3) to the *E. coli* data also using the ordinary least squares criterion. The values obtained were $pH_{min}^{\circ} = 4.20$ (4.12 - 4.29) and k =0.355 (0.320 - 0.390).

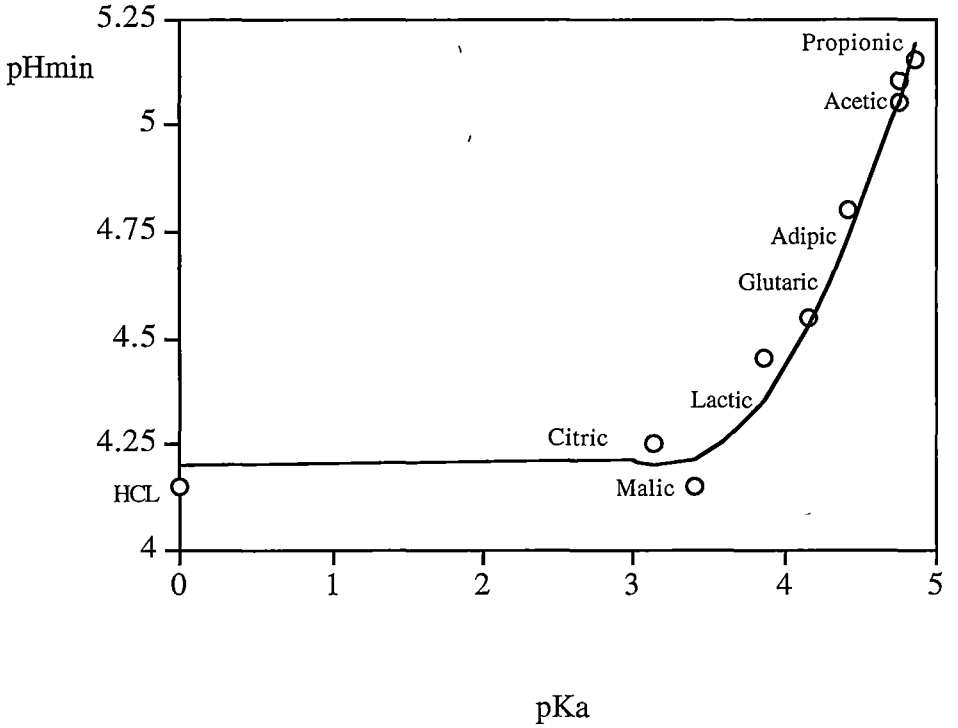


Figure 2.22 - Correlation between pH_{min} and pK_a for *E. coli*. Data and Model (Equation 3) from Rosso *et al.* (1997).

Rosso et al. (1997) used this relationship to predict, given the minimum pH for growth for a strong acid (hydrochloric) and an organic acid (acetic), the minimum pH for growth for *Listeria* for three other organic acids (malic, citric and lactic) using the data of Sorrells *et al.* (1989). These predictions were relatively accurate although many of the observations for malic acid and about half for citric acid were qualitative (<4.40) where the predictions were 4.40 or 4.30. About 20% of the predictions were accurately predicted, all at pH 4.40. For citric acid about 30% of the predictions were less than observed by 0.1-0.2 pH units. For malic acid more than half were not predicted accurately with variation of -0.25 to +0.05 pH units. Importantly the predictions all showed a trend with pH decreasing with decreasing temperature. This is in contrast to the observations for malic acid where, for 3 out of the 4 strains, the observed minimum pHs were 4.60 at 35°C, 4.40 at 25°C and then their values increased again to 4.60 at the lowest temperature of 10°C.

Rosso *et al.* (1997) formulated two hypotheses to explain the two parts of the correlation described by their model. First, that the pH_{min} seen in strong acids is the absolute minimum pH and corresponds to the maximum proton concentration pH_{min}° . If organic acids have a low pK_a, well below the minimum pH for growth, the pH_{min} is the same as for a strong acid. But if they have a higher pK_a, a second effect of organic acids occurs which depends on their pK_a and appears to be an effect of the acid.

While Rosso *et al.* (1997) have clearly effectively modelled the correlation that they have observed there are several limitations to their approach. First, in a practical sense their observation is limited in application to broth systems where a single organic acid is the sole acidulant used to alter the pH. In contrast to this many foods have a complex mixture of organic acids present, and while some may be present at sub-inhibitory levels, they could still significantly affect the starting pH and buffering capacity of the system. Currently using this approach a new model would have to be created for each broth system in which the buffering capacity was significantly altered. Also the information given by the model is only single limiting value (pH_{min}) for each bacteria and acid in comparison to the models presented in this study which can predict the response of bacterial strains to a wide range of combinations of pH and concentrations of organic acid. Other practical considerations include the fact that growth for *E. coli* was only monitored for two days at 35°C which allows for the possibility that some slower growth was not detected.

More significantly there is a basic theoretical explanation for the relationship observed by Rosso *et al.* (1997). Models developed in this study can better and more comprehensively describe the response of bacteria to pH and organic acids because they are based on an understanding of the chemistry of organic acids unlike the approach of Rosso *et al.* (1997).

The relationship between the pK_a of an acid and the proportion of the acid in each of its dissociated (D) and undissociated (UD) forms given by the Henderson-Hasselbalch equation (1.16) is shown in Figure 2.23 for three organic acids. As the pH is lowered the proportion of the acid in the dissociated form decreases and the undissociated form increases. The pK_a is the point at which half the acid is in each form (where the two lines cross) and corresponds to 3.13 for citric acid, 3.86 for lactic acid and 4.76 for acetic acid. It should be noted that citric acid has three acid groups and therefore can contribute 3 protons to the solution. However, it is usual to use the lowest pKa of the three acid groups when describing the behaviour of citric acid.

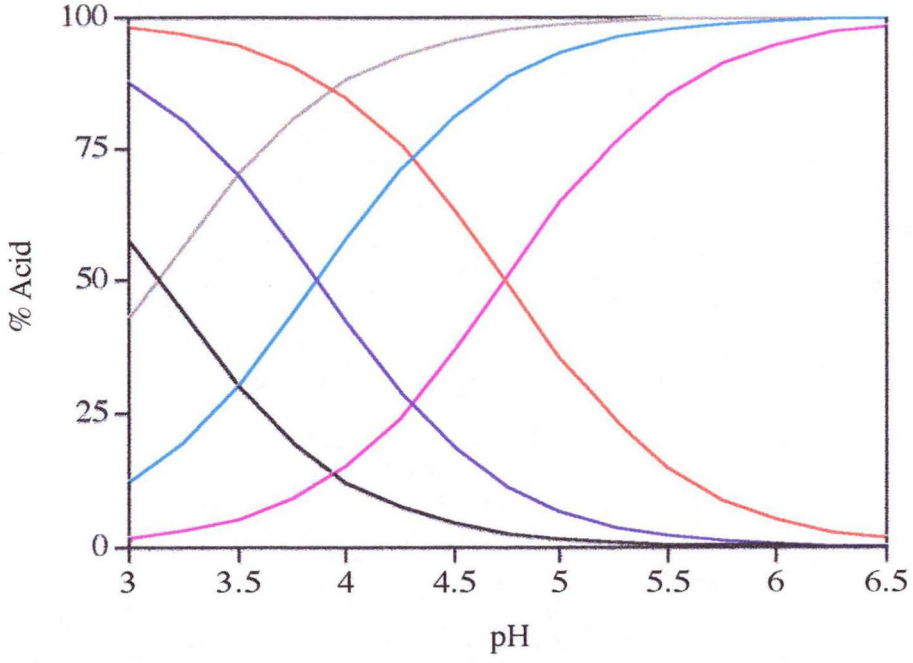


Figure 2.23 - Relationship between pH, the proportion of acid in each form, undissociated (UD) and dissociated (D), and the pK_a of organic acids: citric 3.13 (UD-black, D-grey), lactic 3.86 (UD-blue, D-cyan) and acetic 4.76 (UD-red, D-pink).

The pK_a also governs the buffering effect of the acid. It is very hard to lower the pH below the pK_a of the acid because of the buffering effect in the region $pK_a \pm 1$. Another way of describing this is that an acid will lower the pH of an aqueous solution according to its "strength" or ability to donate hydrogen ions. For organic acids "strength" can be measured by the pK_a; the lower the pK_a, the stronger the acid. Therefore citric acid is the strongest, then lactic and then acetic of the three acids pictured above (Figure 2.23). When added to an aqueous solution in equal molar concentrations more citric acid (than lactic or than acetic in turn) will dissociate increasing the proportion of dissociated ions and lowering the pH.

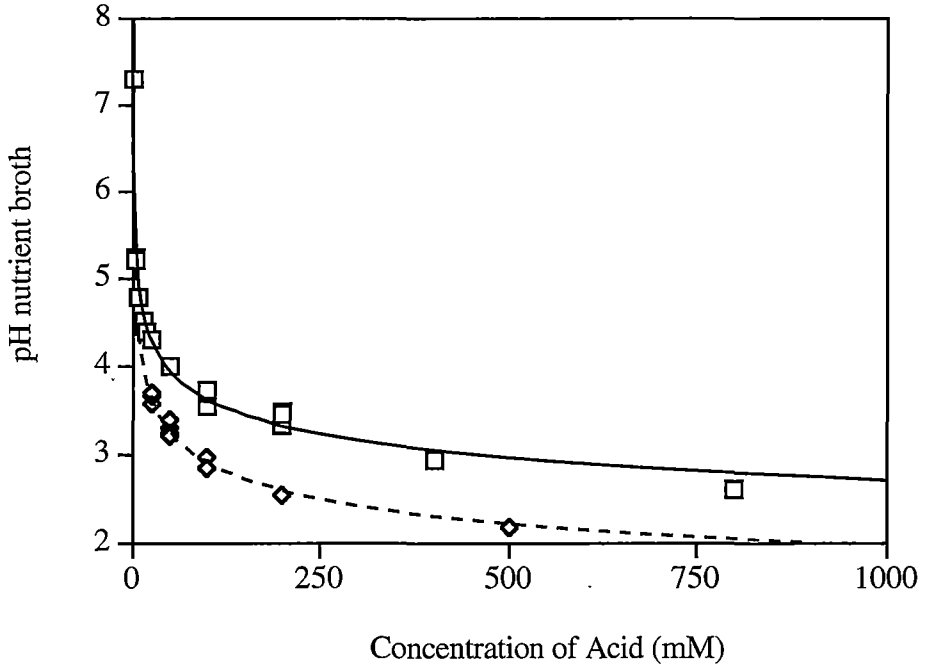


Figure 2.24 - Data for the relationship between the broth pH and the total concentration of organic acid, lactic acid (diamonds) or acetic acid (squares) added. The fitted lines for acetic acid is $y = 6.433\ x^{(-0.125)}$ and for lactic acid is $y = 6.356\ x^{(-0.170)}$.

This difference between acids can be shown by data recorded in this study but not previously used (Figure 2.24). The following relationship was apparent between the concentration of the two acids, lactic and acetic, and the resulting pH of the broth when these concentrations of acid were added. There were significant differences in the exponent term for the different acids (-0.125 compared to -0.170) and it was found by trial and error that this determined the slope of the curve and the lowest pH to which it tended.

In terms of bacterial growth inhibition there are two inhibitory factors, low pH (high concentration of hydrogen ions) and an inhibitory concentration of undissociated acid. Therefore, given the pH_{min} value and approximating the inhibitory undissociated acid concentration present, a method of predicting the relationship found by Rosso *et al.* (1997), between the pH that is inhibitory for each organic acid and the pK_a, is shown in Figure 2.25. It can be seen from this graph that the complete inhibition of *E. coli* growth occurs when either the low pH boundary or the undissociated acid boundary is reached. For stronger acids with low pK_a values the addition of even low concentrations of acid results in a large drop in the pH. Therefore the pH_{min} value is reached before the concentration of undissociated (more inhibitory form) organic acid is present in sufficient concentration to inhibit growth. In contrast the addition of weaker organic acids does not reduce the pH below the pH_{min} and inhibition of growth is due to the presence of inhibitory concentrations of the undissociated acid.

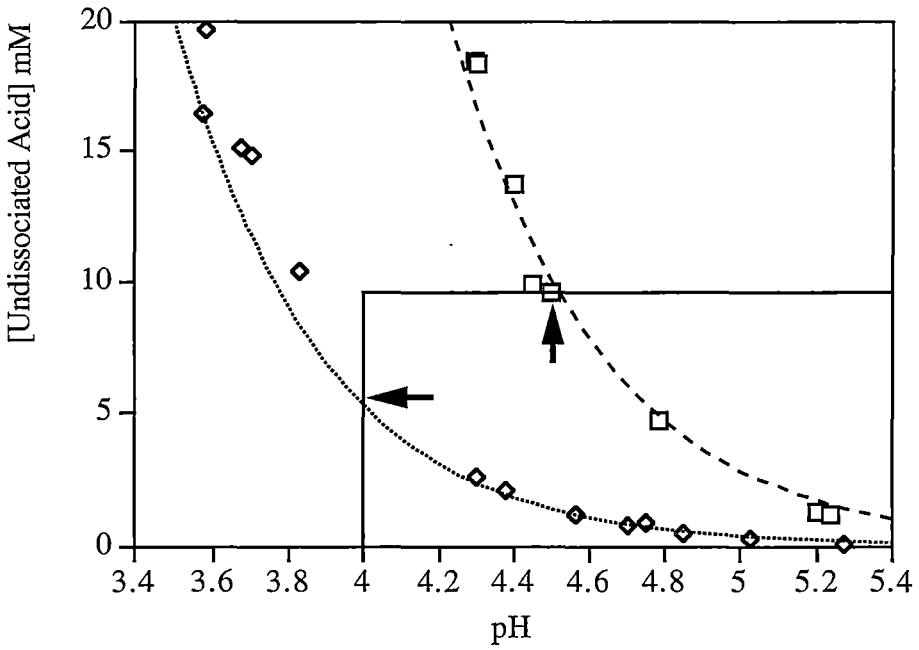


Figure 2.25 - The relationship between the addition of lactic acid (diamonds) and acetic acid (squares) and the resulting pH, in terms of the undissociated acid concentration. The curve fit for each acid shows which factor inhibits *E. coli* growth for each acid, for lactic acid (dotted line) the pHmin is reached (solid vertical line), for acetic acid (dashed line) the inhibitory concentration of undissociated acid is reached (solid horizontal line).

If it is assumed that the relationship between the acid concentration and the resulting pH is primarily dependent on the pK_a of the acids the response of the other acids described by Rosso *et al.* (1997) can be "interpolated" between the curves for acetic and lactic acid. For those acids with a pK_a in the region just above pH_{min} it is probable that there is inhibition in part by both pH and undissociated acid. It is also possible that partial inhibition by each factor can cause complete cessation of growth, so that the shape of the lines of complete inhibition is not a sharp corner where the two limiting conditions meet, but a more restrictive concave curve. In contrast the growth rate models developed in this study only predict a zero growth rate where one condition is completely limiting. Under all other conditions these models predict a very small but positive growth rate. In reality conditions where two factors are very close to their limits may also produce no growth as it has been hypothesised (Ross, *pers. comm.*) that there is a lower limit, as well as an upper one, to the rate at which bacteria can grow at any given temperature.

The minimum growth pH values for acetic and lactic acid obtained by Figure 2.25 do not match the numerical values obtained by Rosso *et al.* (1997) for *E. coli*. This is either due to the difference in the strains used and/or the buffering capacity of Brain Heart Infusion Broth (Rosso *et al.*, 1997) compared to Nutrient Broth (this study). The pH resulting from addition of organic acids to broth was very different to that when the same concentration of organic acid was added to water to make an aqueous solution. This implies that there is a significant buffering effect of the broth components, which include proteins, peptides and free amino acids such as histidine.

It is perhaps significant that all of the acids used by Rosso *et al.* (1997) have a similar inhibitory undissociated acid concentrations. All of the acids are found to be similarly inhibitory and the more inhibitory and chemically more complex acids such as benzoic and sorbic were not included. Some inhibitory undissociated acid concentration values could be calculated from the data of Rosso *et al.* (1997) where the total concentration of acid was given for some of the pH_{min}s. These varied from 5 - 11mM; with 8mM for lactic, 6mM for glutaric, 11mM for adipic, 6mM for acetic and 5mM for propionic acid.

The ability to describe a single critical value that describes the complete inhibition of growth of a particular organism by a particular acid is an important step in being able to describe the pH and acid responses of microorganisms. For example, the models developed in this study are based on the hypothesis that the inhibition of the growth rate was equivalent for the same concentration of undissociated organic acid regardless of the total organic acid or dissociated organic acid concentrations. Also that the cessation of growth occurred consistently at a single undissociated organic acid concentration regardless of other non-limiting environmental conditions. One of the few examples of studies where the effects of organic acids and the pH has been separated is that of Brockelhurst and Lund (1990) for *Yersinia enterocolitica*. Their results like those in this study showed an increase in the pH at which complete inhibition of growth occurred as the total concentration of lactic acid increased. The minimum inhibitory concentrations of undissociated lactic acid was found to be 5, 10 and 5mM for lactic acid concentrations of 22, 44 & 111mM respectively at both 10 and 20°C. While the study of Brockelhurst and Lund (1990) is limited it is encouraging that these values support the hypothesis that one minimum inhibitory concentration of undissociated lactic acid can be used to describe the inhibition due to this organic acid under many other environmental conditions.

The fitted models for organic acid developed here suggest that inhibition by organic acids is only completely described by an effect of hydrogen ion concentration, an effect of the undissociated acid molecule and an effect of the dissociated acid molecule with each acting in proportion to their concentration. Other studies have separated the effect of dissociated and undissociated acid (Eklund, 1983; Salmond *et al.*, 1984). However, some investigators (Ahamad and Marth, 1989; El-Shenawy and Marth, 1989) state that the dissociated form of the acid does not have a significant inhibitory effect. Dissociated acid is much less inhibitory on growth rate than undissociated acid, approximately 100 times less for lactic acid and 70-300 times less for acetic acid inhibition. However, this does not mean that it can be disregarded, as when the total organic acid concentration and pH are high, the inhibitory effect of the dissociated form may be the most significant (Eklund, 1989; Houtsma *et al.*, 1994). Such conditions were not extensively tested in this work, but even here the inclusion of the inhibitory effect of the dissociated form of the acid in the model was necessary to describe the data well.

The possible mechanism of inhibition of the dissociated ion is difficult to postulate. Unlike the undissociated molecule the dissociated anion is negatively charged and so presumably cannot freely diffuse into the cell. It is possible that the molecule acts on the exterior of the cell, or that it is taken up by the bacterial cell.

Dissociated acid will also be present inside the cell after the undissociated form diffuses across the cell membrane and dissociates inside the cell. As shown in Figure 2.26 Russell (1992) found that the effect of undissociated acid on *E. coli* was in fact due to the accumulation of the dissociated anion. This occurred because the cells maintained a high intracellular pH (by removing the excess hydrogen ions) in the presence of high external organic acid concentration. For one pathogenic strain of *E. coli*, the internal pH was allowed to fall in the presence of high concentrations of organic acids. This successful strategy for overcoming organic acid inhibition suggests that adaptation of cell physiology to a lower internal pH was possible whereas acclimatization to anion accumulation was not possible or desirable. This also suggests that the removal of anions accumulated within the cell during organic acid stress is a rate limiting step in the process in comparison with the mechanism of removal of excess hydrogen ions in pH stress.

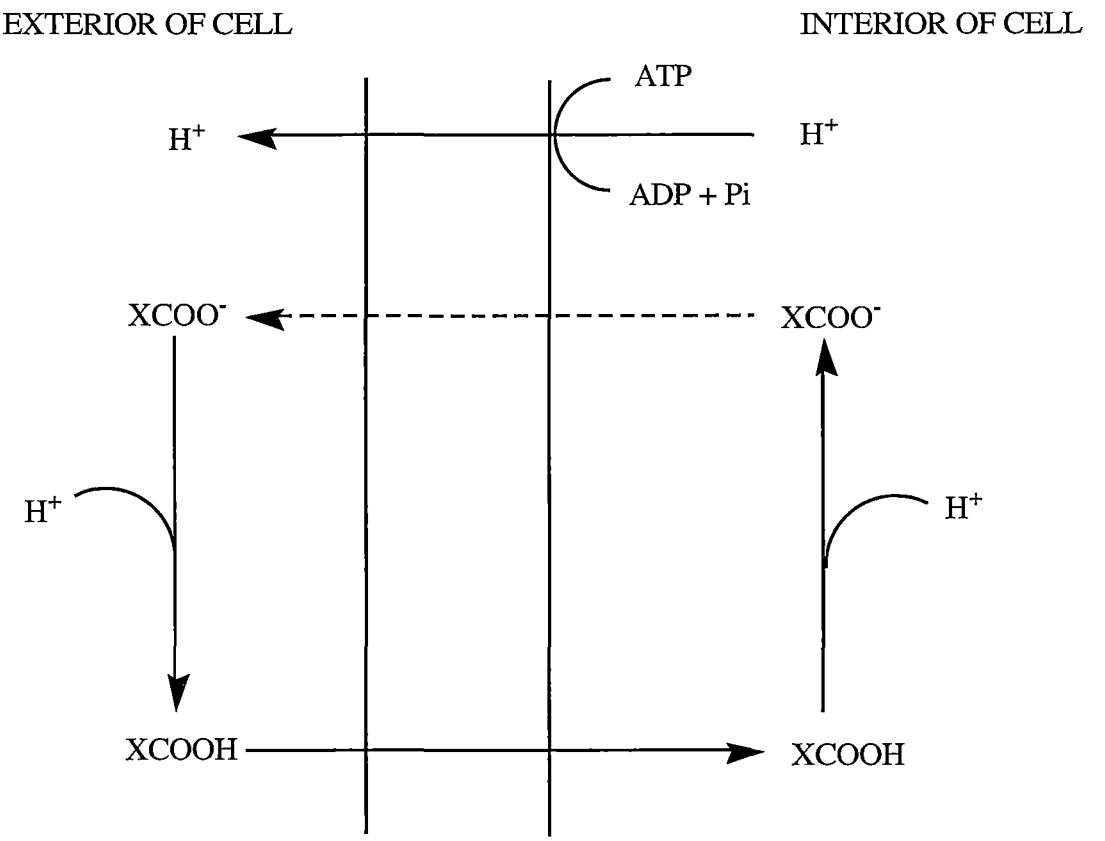


Figure 2.26 - Mechanism of anion accumulation showing the permeability of the undissociated molecule, XCOOH (solid arrow), the impermeability of the dissociated molecule, $XCOO^-$ (dotted arrow) and the ATPase pump that pumps out excess protons. Adapted from Russell (1992).

The mechanisms of action of organic acids and pH on microorganisms are not well understood. Modelling studies that accurately describe microbial growth rate responses to inhibition may help to understand the underlying causes of the inhibition. These studies also provide useful practical information to the food industry. However in some cases the aspect of the microbial response to be modelled is not the growth rate but the boundary between those conditions which permit growth and those which prevent it.

3 GROWTH/NO GROWTH EXPERIMENTS AND MODELLING

3. Growth/No Growth Experiments and Modelling

3.1 Summary

The form of the growth rate model previously described in this study was used to create probability models for definition of the growth/no growth interface of *E. coli* . Existing data (Presser, 1995) were used for ranges of pH (2.8 - 6.9), lactic acid concentration (0 - 500mM) and temperature (10 - 37°C). These data were specifically generated to elucidate whether growth could occur or not at these conditions. Similarly generated new data were used to clarify the response to water activity in this study over a range of temperature (8 - 37°C), pH (3-7) and water activities (0.945 - 0.996) with NaCl as the humectant.

The methods used to generate these probability models have evolved. Previously the parameter estimates for modelling had to be fixed at values determined by growth rate modelling (Presser, 1995; Presser *et al.*, 1998). Subsequent advances in modelling have allowed the potential estimation of all parameters within the growth/no growth modelling process. New models have been developed using different combinations of terms for temperature, water activity, including both suboptimal and superoptimal terms, to describe the inhibition observed. A novel term for pH described earlier (2.4.2) has been introduced to growth/no growth modelling. Also a SAS (SAS Institute, 1989) procedure that selects terms to be modelled was employed to determine the usefulness of the addition of squared and cross product terms to the models. Much progress has been made on the methods for the mathematical modelling of growth/no growth data.

During these mathematical modelling studies new insights have been developed regarding the way antimicrobial factors affect the position of the growth interface. Differences have been observed in the way these same factors affect the growth rate of the same bacteria. The depth of knowledge gained has led to clearer objectives for future experimentation and an understanding of some possible restrictions on this technique.

3.2 Introduction

There are many foods in which low pH and the presence of organic acids are significant constraints on microbial growth. In some of these foods, growth of neutrophilic organisms is very inhibited, for example, foods with pH < 4.5 and those with a high organic acid content. Therefore it has been conventionally assumed that food poisoning could not occur from consumption of low pH foods.

Outbreaks of foodborne illness linked to consumption of low pH and high acid foods have challenged this assumption. Outbreaks of HUS have been linked to consumption of low pH and high organic acid foods such as apple cider and mayonnaise where the pH is close to the pH limit or even too low to support growth of *Escherichia coli.* However, for some organisms, large numbers of organisms are not required to cause disease. There is epidemiological evidence that the infectious dose is very low for *E. coli* (Anon., 1996a; Anon., 1996b; Easton, 1997), perhaps less than fifty organisms (Tilden *et al.*, 1996).

In these cases of *E. coli* food poisoning it appears that sufficient numbers of bacteria survived in the food despite the low pH and organic acid conditions and were able to cause disease. Therefore, for this example, where a pathogenic organism with a low infectious dose is present in food, a reduction of the growth rate of this organism will be insufficient to prevent illness if the organism is already present in sufficient numbers to cause disease. It is often not possible to change the formulation of foods, so that they are lethal to bacteria without adversely affecting the palatability and other consumer acceptance criteria. Growth/no growth modelling can be used to assess whether food formulation modifications, for example, to remove preservative, will change conditions enough to allow bacterial growth.

New methods to model the response of organisms to these kinds of limiting conditions have been explored. Methods that model the rate of growth or death are less reliable in this growth/no growth interface region where growth may or may not occur and growth rates or death rates may be very slow. At several sets of environmental conditions within the growth/no growth interface region, replicate observations yielded opposite results (Bolton and Frank, 1999). For example, at a single condition the three replicates included one observation of growth, one of death and one of a less than 0.5 log change in numbers of *Listeria monocytogenes.* All possible combinations of these three observations were observed in the three replicates, such as two observations of growth and one of death. There was no obvious pattern to the environmental conditions that caused these type of results which supports the need for a probabilistic approach to modelling the boundary. Near the limits for growth bacterial responses are erratic and the variability in conditions makes the prediction of a discrete rate inadequate to describe the bacterial response.

3.3 Materials and Methods

3.3.1 Generation of New Data - Experiment 1

This experiment was designed to determine the growth/no growth interface of *E. coli* M23 as dictated by temperature, pH and water activity with no lactic acid.

3.3.1.1 Preparation of pH broths for Experiment 1

Overstrength Nutrient Broths (Appendix 1) were prepared using addition of NaCl, a common humectant in the food industry, to lower the water activity. Broths were prepared at the following final concentrations of NaCl: 7%w/v which gave $a_w = 0.955$, 5.25%w/v which gave $a_w = 0.965$, 3.5%w/v which gave $a_w = 0.975$ and 2%w/v which gave $a_w = 0.985$ in nutrient broth. The broth at each water activity was then divided into six aliquots and dispensed to separate flasks. Each flask was kept refrigerated until its pH was individually adjusted (to give the final six values for each water activity between 3 and 5.8 shown in Table 3.1) using HCl and NaOH solutions. The broths were made up to their final volumes with water. The pH was retested to check no further pH change had occurred and the broth filter sterilised (Activon filter, 0.45 μm) into sterile bottles.

Table 3.1 pHs of nutrient broths for each water activity in Experiment 1

Broth#	pH for 0.955	pH for 0.965	pH for 0.975	pH for 0.985
1	4.5	4.5	4	3
2	5	4.7	4.5	4
3	5.5	5	4.7	4.3
4	5.6	5.1	4.8	4.4
5	5.7	5.2	4.9	4.5
6	5.8	5.3	5	4.6

These pH values were selected because they fell on either side of the anticipated interface estimated from the growth rate model. Broths were kept for a week at room temperature, by which time any contamination present was presumed to have become visible as turbidity. Contaminated broths were discarded.

3.3.1.2 Standardisation of Inocula for Experiment 1

The inoculum of *E. coli* M23 was prepared by pipetting 5ml of an overnight static culture (37°C) into 45ml of Nutrient Broth in a side-arm flask. This was incubated at 37°C with shaking for approximately 2 hours until the absorbance was 0.8. An absorbance of 0.8 was found from previous experiments to give cells in the late exponential phase of growth. Due to time constraints the inoculum was occasionally kept at 15°C until the bottles were inoculated. The maximum delay was 20 minutes and this was calculated not to affect the cell density significantly. (The generation time of *E. coli* at 15°C is approximately 120 minutes, so that within 20 minutes a maximum of 1/6 of one generation's growth could have occurred).

3.3.1.3 Growth/No Growth Determinations

0.5 ml of the *E. coli* M23 inoculum described above (3.3.1.2) was pipetted into the bottles containing 30 ml of each pH-adjusted broth. The inoculated broths were thereafter kept in a waterbath (4 - 10°C) to ensure that growth was minimised until the inoculated broths were dispensed and placed at the selected temperatures. This inoculum level usually gave just visible turbidity but due to the small volume of broth in the well plates, this level of turbidity was not visible during the experiment. For each water activity, 2ml of each pH broth was pipetted into each of the 4 wells of a 24 well plate (Appendix 1). Thus, each 24 well plate contained 4 replicates of the six different pH broths for one water activity. One 24 well plate per water activity (4 in total) was incubated at each of 5 temperatures using 25°C, 30°C and 37°C incubators and constant temperature rooms at 10°C and 20°C. There was a total of twenty 24 well plates in the experiment.

3.3.1.4 Methods of Evaluating Growth or No Growth

Turbidity

If visible turbidity of the broth developed within the well during incubation, growth was presumed to have occurred. Turbidity was recorded daily for each well for approximately the first 21 days and thereafter less frequently until the end of the experiment (up to 50 days for the lowest temperature 10°C).

Ecometric Method

An ecometric technique (Mossel *et al.*, 1981; Mossel *et al.*, 1983) was developed and used to determine an approximate level of bacterial numbers as described below. Loopfuls (~1/50 ml) of each cultures were streaked onto plates. The streak pattern, shown in Appendix 2, was followed by using guidelines marked on a template placed under the plate and then traced as accurately as possible with the loop. The loop was not flamed between streaking the four sections of the plate. The extent of growth along the streak lines after 48 hour incubation at 20°C or 24 hours at 37°C was recorded. A standard curve for the ecometric technique showed a correlation between the last streak on which the number of colonies exceeded five and the cell density (log cfu/ml) (Presser, 1995).

This indicated that the cell numbers had decreased from inoculum levels if there were fewer than five colonies on the second streak line. For wells where there was no visible turbidity but a deposit of cells in the base of the well, or no visible change at all, the wells were ecometrically streaked. Each replicate well was streaked onto a separate plate and the extent of growth determined as described above.

Final pH

The final pH of the broth was measured and compared to the initial pH. An increase of approximately 2 or more pH units was considered indicative of growth (Presser, 1995).

3.3.1.5 Verification of *E. coli* Growth or No Growth

E. coli growth was presumed to have occurred in wells that demonstrated increased turbidity and an increase in pH. Wells in which growth was observed were then streaked on Nutrient Agar (NA) to verify that they were a pure cultures and subsequently streaked onto Eosin Methylene Blue Agar (EMB) to verify typical colonies of *E. coli.* In non turbid wells the absence of growth was confirmed by the ecometric streaking method as well as in some instances a spread plate of 0.1ml of culture on a nutrient agar plate on which growth was determined after 48 hours at 37°C. Finally a lack of significant increase in the pH (less than 1 pH unit) was also presumed to indicate no growth had occurred.

3.3.2 Generation of New Data - Experiment 2

This experiment was designed to gather more data on the growth/no growth interface of *E. coli* M23 as dictated by temperature, pH and water activity. These data were gathered to increase the amount of data on and near the position of the interface for combinations of low a_w, T and pH. The methods for Experiment 1 were employed with the following exceptions.

3.3.2.1 Preparation of pH Broths

Broths were prepared at the following final concentrations of NaCl: 8.5%w/v which gave a_w = 0.945, 7%w/v which gave a_w = 0.955, 5.25%w/v which gave a_w = 0.965, 3.5%w/v which gave a_w = 0.975 and 2%w/v which gave a_w = 0.985 in nutrient broth. The broth at each water activity was then divided into 5 flasks for a_w = 0.955 and 0.945, 8 flasks for a_w = 0.985 and 9 flasks for a_w = 0.965 and 0.975. Each flask was kept refrigerated until its pH was individually adjusted (to give final pH values between 4 and 7) using HCl and NaOH solutions. The pH values for water activities of 0.965, 0.975, and 0.985 at the different temperatures are shown in Table 3.2. pHs for water activities of 0.945 and 0.955 were 5.0, 5.5, 6.0, 6.5 and 7.0 for all temperatures.

Table 3.2 - pH values of Broths at various Water Activity & Temperature Combinations

Temperature (°C)	Water Activity	pH				
8	0.965	5.00	5.50	6.00	6.50	7.00
	0.975	5.00	5.50	6.00	6.50	7.00
	0.985	4.00	5.00	5.50	6.00	7.00
12	0.965	5.00	5.50	6.00	6.50	7.00
	0.975	5.00	5.50	6.00	6.50	7.00
	0.985	4.00	4.25	4.50	4.75	5.00
15	0.965	5.00	5.50	6.00	6.50	7.00
	0.975	5.00	5.50	6.00	6.50	7.00
	0.985	4.00	4.25	4.50	4.75	5.00
19	0.965	5.00	5.50	6.00	6.50	7.00
	0.975	4.00	5.00	5.50	6.00	7.00
	0.985	4.00	4.25	4.50	4.75	5.00
22	0.965	4.00	5.00	5.50	6.00	7.00
	0.975	4.00	4.25	4.50	4.75	5.00
	0.985	4.00	4.25	4.50	4.75	5.00
26	0.965	4.00	4.25	4.50	4.75	5.00
	0.975	4.00	4.25	4.50	4.75	5.00
	0.985	4.00	4.25	4.50	4.75	5.00
30	0.965	4.00	4.25	4.50	4.75	5.00
	0.975	4.00	4.25	4.50	4.75	5.00
	0.985	4.00	4.25	4.50	4.75	5.00
33	0.965	4.00	4.25	4.50	4.75	5.00
	0.975	4.00	4.25	4.50	4.75	5.00
	0.985	4.00	4.25	4.50	4.75	5.00
37	0.965	4.00	4.25	4.50	4.75	5.00
	0.975	4.00	4.25	4.50	4.75	5.00
	0.985	4.00	4.25	4.50	4.75	5.00
40	0.965	4.00	4.25	4.50	4.75	5.00
	0.975	4.00	4.25	4.50	4.75	5.00
	0.985	4.00	4.25	4.50	4.75	5.00

3.3.2.2 Growth/No Growth Determinations

0.5ml of an overnight static culture (37°C) was added to 25ml of each broth at each water activity and pH combination. 2ml of each broth was pipetted into 5 wells of the 25 well plates (Appendix 1), under sterile conditions in a laminar flow hood according to the scheme shown in Table 3.3.

Table 3.3 Layout of Different Broths in the 25 Well Plates

a_w	pH	pH	pH	pH	pH
0.945	5.00	5.50	6.00	6.50	7.00
0.955	5.00	5.50	6.00	6.50	7.00
0.965	1st broth	2nd broth	3rd broth	4th broth	5th broth
0.975	1st broth	2nd broth	3rd broth	4th broth	5th broth
0.985	1st broth	2nd broth	3rd broth	4th broth	5th broth

"1st broth" indicates the lowest pH broth of that water activity tested at that temperature, "2nd broth" is the next lowest, and so on to the 5th and highest pH broth of that water activity for that temperature. For details of the water activity and pH combinations tested at each temperature see Table 3.2. One well plate was incubated at each of 10 temperatures (8-40°C) on each shelf of a vertical temperature gradient incubator (Appendix 1).

3.3.2.3 Evaluation of Growth/No Growth

Turbidity

If visible turbidity of the broth developed within the well during incubation, growth was presumed to have occurred. Apparent turbidity was recorded daily for approximately one week and then weekly for each well for one month.

3.3.2.4 Verification of *E. coli*

E. coli growth was presumed to have occurred in wells that demonstrated an increase in turbidity. Wells that were obviously contaminated by fungal or pigmented bacterial growth that was clearly not *E. coli* were excluded from the results.

3.3.3 Other data

Previously published data (Presser, 1995) were used to fit the following growth/no growth models described in Section 3.3.4. These data showed a small variation in water activity due to the presence of lactic acid but there were no data with NaCl added to determine a water activity response. Most data used were growth/no growth data. Growth rate data were also used because observations of no growth in no growth experiments were only made after at least two weeks incubation. This long time period was to ensure that all growth, however slow, was observed as growth. Growth rate experiments gave only single observations of growth or no growth. In addition 38 growth rate data at high pH (7.7-10.6) and optimal temperature (34-35°C) were used in fitting some of the models as shown in the Table below.

Experiment	Type of Data	Datapoints	Replicates	Observation
Presser (1995)	Growth/No Growth	330	4	1-330
Presser (1995)	Growth Rate	177	1	331-507
Experiment 1	Growth/No Growth	120	4	508-627
Experiment 2	Growth/No Growth	206	1	628-833
High pH	Growth Rate	38	1	834-871

3.3.4 Growth/No Growth Modelling

3.3.4.1 Creation of Growth/No Growth Models

A square root growth rate model based on Ratkowsky *et al.*(1982) and fitted for *E. coli* (Presser *et al.*, 1997) was used to create the first growth/no growth model for *E. coli* M23 inhibited by lactic acid and pH at suboptimal temperatures and water activities (Presser *et al.*, 1998). Both these models contained terms for inhibition due to low temperature, low water activity, low pH and the presence of the two components of lactic acid. Subsequently more complex models that contain additional terms for inhibition by superoptimal conditions have been proposed (Ross, 1999). An example of a model that contains terms for inhibition due to low temperature, high temperature, low water activity, low pH, high pH, undissociated lactic acid and dissociated lactic acid respectively is shown in Eqn. 3.1a.

$$k = c(T - T_{\min})^2(1 - \exp(d(T - T_{\max})^2(a_w - a_{w_{\min}}) \times (1 - 10^{pH_{\min} - pH})(1 - 10^{pH - pH_{\max}}) \times \left[1 - \frac{LAC}{U_{\min}(1 + 10^{pH - pK_a})}\right]\left[1 - \frac{LAC}{D_{\min}(1 + 10^{pK_a - pH})}\right] + e \qquad (3.1a)$$

The terms are as previously defined (2.3.5). This model contains the same pH_{max} term as that introduced in Eqn 2.9. The T_{max} term was taken from other kinetic modelling (Ratkowsky *et al.*, 1983) where T_{max} is a notional upper value of temperature where growth rate is predicted to be zero and d is a novel constant of proportionality. Also in Chapter 2 a new type of term for pH inhibition was introduced (2.4.2 : Eqn 2-10). This new type of pH term can be used instead of the pH term described in Eqn 3.1a and is given below.

$$(1-10^{Q(pH_{\min}-pH)}) \qquad (3.1b)$$

Square root growth rate models were used to derive four new growth/no growth models (Eqn 3.2-3.5) discussed in the following sections. The growth/no growth model is created by mathematical transformation of the growth rate model. The natural logarithm (ln) of both sides of the equation is taken and then the left hand side is replaced with a logit function : logit (P) = ln{P/(1-P)} where P is probability of growth (0-1). The log transformed terms for the same parameters are now added together rather than multiplied. Each term has a constant (b_1-b_n) as well as the constant at the beginning of the equation (b_0). These constants are subsequently estimated in the modelling process. Eqn 3.2 is the growth limits model constructed using this approach from the growth rate model described above (Eqn 3.1a).

$$\begin{aligned} Logit(P) = {} & b_0 + b_1 \ln(T - T_{\min}) + b_2 \ln(1 - \exp(d(T - T_{\max}))) + b_3 \ln(a_w - a_{w_{\min}}) \\ & + b_4 \ln(1 - 10^{pH_{\min} - pH}) + b_5 \ln(1 - 10^{pH - pH_{\max}}) \\ & + b_6 \ln\left[1 - \frac{LAC}{U_{\min}(1 + 10^{pH - pK_a})}\right] + b_7 \ln\left[1 - \frac{LAC}{D_{\min}(1 + 10^{pK_a - pH})}\right] \end{aligned} \qquad (3.2)$$

Four main types of models have been used to model the growth/no growth interface and an example for each is given in detail in the sections below. The first type used fixed estimates of parameters given by kinetic modelling studies of the same organism under the same conditions. The next type of model included T_{max} (Salter *et al.*, 2000; Tienungoon *et al.*, 2000) and pH_{max} terms (Tienungoon, unpublished, 1999) which improved the fit of the data when included in other growth/no growth interface models. In the third type of model these superoptimal terms were only included selectively and the suboptimal pH term was modified to include an extra scaling coefficient, equivalent to the Q factor in kinetic modelling, which greatly improved the fit of the model to the data. In the final model type, squared and cross-product terms were added to a model containing suboptimal terms.

3.3.4.2 Model with Fixed Parameter Estimates

A growth rate model describing the effects of temperature (10-37°C), water activity (0.955-0.999), pH (3-7) and lactic acid (0-500mM) on the growth rate of a nonpathogenic strain of *E. coli* was published by Presser *et al.* (1997). That model was used to create a growth limits model (Presser *et al.*, 1998). The growth limits model describes the effects of temperature (10-37°C), water activity (0.955-0.999), pH (3-7) and lactic acid (0-500mM) on the growth rate of a nonpathogenic *E. coli* M23 strain. This model was created using 413 datapoints from Presser (1995) and Experiment 1. This data set was created by the elimination of all data with a pH less than 3.9 and a concentration of undissociated acid greater than 10.7mM, that fell outside the pH limit or undissociated acid limit for growth according to the growth rate parameters to be used in this modelling. This growth limits model was created by the method of Ratkowsky and Ross, (1995). The "cardinal" theoretical limits T_{min} , $a_{W_{min}}$, pH_{min}, U_{min} and D_{min} were taken to be fixed constants from Presser *et al.* (1997) and the pK_a of lactic acid from Budavari (1989). These values were not estimated by the growth/no growth modelling process but the "*b*" coefficients preceding the model terms were estimated (Presser *et al.*, 1998). The superoptimal terms for temperature or pH were not included in this model (Eqn 3.3).

$$\begin{aligned} Logit(P) = {} & b_0 + b_1 \ln(T - T_{\min}) + b_3 \ln(a_w - a_{w_{\min}}) \\ & + b_4 \ln(1 - 10^{pH_{\min} - pH}) \\ & + b_6 \ln\left[1 - \frac{LAC}{U_{\min}(1 + 10^{pH - pK_a})}\right] + b_7 \ln\left[1 - \frac{LAC}{D_{\min}(1 + 10^{pK_a - pH})}\right] \end{aligned} \qquad (3.3)$$

3.3.4.3 Models with Superoptimal pH and Temperature Terms

The next type of growth limits model was created using Eqn 3.2. Different datasets were used, the first model was fitted to the same 413 datapoints as Eqn 3.3. The "cardinal" theoretical limits T_{min}, T_{max},$a_{W_{min}}$, pH_{min}, U_{min} and D_{min} were estimated by the growth/no growth modelling process. The dataset did not contain data over the superoptimal temperature range from which T_{max} values could be estimated reliably. The estimated values for the T_{max} term coefficient, "*d*" (0.317) and T_{max} (49.13°C) from growth rate temperature modelling for this strain (Salter, 1998) were used.

Approximate values for bounds on the pH_{max} estimate were trialled. However the model tended to fit a large negative coefficient for the pH_{max} term, which described the reverse of inhibition, i.e. stimulation by high pH (Eqn 3.7 - Section 3.4.1.2). In order to counteract this, in fitting Eqn 3.8 (Section 3.4.1.2), high pH data were included in the dataset along with the Experiment 2 data. These were added to the data from Presser (1995) and Experiment 1. This data set contained the full 871 total datapoints with no data eliminated. Also, when fitting Eqn 3.8 the coefficients were constrained to be greater than zero.

3.3.4.4 Model with Q factor

The third type of growth limits model was created using a total dataset of 641 points. This dataset was created by the elimination of all data with a pH greater than 7.5 and a concentration of undissociated acid greater than 10.88mM. These were data greater than the estimated pH optimum or the undissociated acid limit for growth (10.88mM was the highest concentration of undissociated acid at which growth was observed). These data were removed to facilitate the fitting process. The "cardinal" theoretical limits T_{min}, $a_{W_{min}}$, pH_{min}, U_{min} and D_{min} were estimated by the growth/no growth modelling process. The substitution of the Q type pH term (Eqn 3.1b) into Eqn 3.2 and removal of the T_{max} and pH_{max} terms gives the following equation:

$$\begin{aligned} Logit(P) = {} & b_0 + b_1 \ln(T - T_{\min}) + b_3 \ln(a_w - a_{w_{\min}}) \\ & + b_4 \ln(1 - 10^{Q(pH_{\min} - pH)}) \\ & + b_6 \ln\left[1 - \frac{LAC}{U_{\min}(1 + 10^{pH - pK_a})}\right] + b_7 \ln\left[1 - \frac{LAC}{D_{\min}(1 + 10^{3.86 - pK_a})}\right] \end{aligned} \quad (3.4)$$

3.3.4.5 Suboptimal Temperature and pH Model with Squared and Cross Product Terms

The final type of growth limits model described here was created using the same dataset as the model in the Section 3.3.4.4. The "cardinal" theoretical limits T_{min}, $a_{W_{min}}$, pH_{min}, U_{min} and D_{min} were estimated by the growth/no growth modelling process. The addition of a squared suboptimal temperature term and a cross-product suboptimal temperature and water activity term into Eqn 3.4 gives the following equation:

$$\begin{aligned} Logit(P) = {} & b_0 + b_1 \ln(T - T_{\min}) + b_3 \ln(a_w - a_{w_{\min}}) \\ & + b_4 \ln(1 - 10^{Q(pH_{\min} - pH)}) \\ & + b_6 \ln\left[1 - \frac{LAC}{U_{\min}(1 + 10^{pH - pK_a})}\right] + b_7 \ln\left[1 - \frac{LAC}{D_{\min}(1 + 10^{pK_a - pH})}\right] \\ & + b_8 \ln(T - T_{\min})^2 + b_9 \ln(T - T_{\min}) \ln(a_w - a_{w_{\min}}) \end{aligned} \quad (3.5)$$

3.3.4.6 Fitting Growth/No Growth Models

Model fitting was undertaken in consultation with D.A. Ratkowsky, who developed the various codes for model fitting. The first method of fitting a nonlinear logistic regression model containing nonlinear terms (Eqn 3.2) was using "linear" logistic regression. Previously this was the only method generally available for fitting parameters when the response variable is a "presence/absence" variable such as growth/no growth (Ratkowsky, 1993). Using "linear" logistic regression the nonlinear parameters (T_{min}, pH_{min}, $a_{W_{min}}$, U_{min} and D_{min}) needed to be set to fixed values to model the data and estimate values for the coefficients. Therefore, it was necessary to use values estimated from growth rate modelling studies for the nonlinear parameters. The original growth/no growth interface model described (Eqn 3.3) was fitted to the initial subset of the data containing 413 points using SAS PROC LOGISTIC (SAS Institute Inc., 1989), a procedure for linear logistic regression modelling (Presser *et al.*, 1998) .

Modelling advances have allowed estimation of all coefficients and parameters, including parameters such as T_{min} and $a_{W_{min}}$, in the growth/no growth modelling process using nonlinear regression modelling with SAS PROC NLIN (SAS Institute Inc., 1989) (Tienungoon *et al.*, 2000; Salter, *et al.*, 2000; Presser *et al.*, 1999). This method is still new and relatively little used. Subsequently another method was used to determine if single linear terms for each inhibitory factor were the only terms needed in the model or if other terms such as squared or even cubed terms for each parameter were needed to fit the data better. To achieve this, stepwise logistic regression was performed in SAS PROC LOGISTIC (SAS Institute Inc., 1989) for suboptimal temperature and pH, superoptimal temperature and pH, undissociated acid, dissociated acid and water activity terms for various powers as well as cross products of these terms. Also using SAS PROC NLIN (SAS Institute Inc., 1989) the estimates for the parameters and their coefficients could be constrained between specified bounds.

3.3.4.7 Assessment of Growth/No Growth Models

Several measures have been used to assess the goodness of fit of growth/no growth models. Those available in SAS PROC LOGISTIC (SAS Institute Inc., 1989) include the Receiver Operating Characteristic (ROC) curve (Lemeshow and Le Gall, 1994), the Hosmer-Lemeshow goodness-of-fit statistic (Hosmer and Lemeshow, 1989) and the maximum rescaled R^2 statistic (Nagelkerke, 1991).

Sensitivity is the proportion of observed events, in this case growth, that were correctly predicted and specificity is the proportion of non events, in this case no growth that were correctly predicted. A plot of sensitivity against the complement of specificity gives the area under the ROC curve, *c*, which gives a measure of the discrimination where at optimal discrimination *c* has a value of 1. In a practical example such as epidemiological studies $c > 0.7$ is considered acceptable discrimination, $c > 0.8$ is considered excellent discrimination and $c > 0.9$ is considered outstanding discrimination (Lemeshow & Le Gall, 1994). However in the study of predictive microbiology a higher degree of discrimination should be possible because the factors which influence the growth response and the range of their values are better known and are more easily controlled experimentally than the factors influencing epidemiological outcomes.

The Hosmer-Lemeshow goodness-of-fit statistic was developed for estimating the goodness-of-fit where there is little or no replication in any of the subpopulations of a dataset. To calculate the statistic, subjects are grouped into a contingency table and a Pearson chi-square statistic is calculated. A small Hosmer-Lemeshow goodness-of-fit statistic for a given number of degrees of freedom gives a large probability (e.g. $P > 0.05$) which indicates a good fit of the model to the data. The coefficient of determination R^2 is commonly used in regression applications to determine goodness of fit where the error is normally distributed. The maximum rescaled R^2 was developed by Nagelkerke (1991) as a generalisation of this coefficient. The closer R^2 is to 1 the better is the prediction of the response variable from the explanatory variables.

For all models the accuracy of prediction of the model was also finally tested using the fitted values of the model's parameters and coefficients to predict probabilities of growth. The probabilities of growth were calculated for the range of conditions over which the data were collected and were then compared with the observed incidences of growth/no growth in the data. This method allowed any systematic errors or trends present in the predictions to be observed and described for each model.

Initially, the predictions of the models were classified as overpredictions if the predicted value was > 0.5 and ≥ 50% of the replicates yielded no growth. Similarly, the predictions of the models were classified as underpredictions if predicted probability of growth was < 0.5 and ≥ 50% of replicates grew. Another, less stringent, set of criteria were also used. Most commonly four replicates were used, which gives a resolution of 25%, i.e. the possible observed results were 0.0, 0.25, 0.5, 0.75 and 1.0. These alternative criteria considered overprediction to occur if the value of the prediction was > 0.75 when the no growth was observed in ≥ 50% of replicates, and underprediction if the value of the prediction was < 0.25 when growth was observed in ≥ 50% of replicates.

3.4 Growth/No Growth Results

3.4.1 Models

The results of the modelling of the four main types of models are given in detail in the sections below. At each stage of refinement of the models there was improvement in the fit of the model to the data. This section describes the goodness of fit of the models, their differences and difficulties in fitting to data. In Section 3.4.2, the fit of the model to the data is examined in detail.

3.4.1.1 Model with Suboptimal Terms and Fixed Parameter Estimates

The first type of model (based on Eqn 3.3) created used fixed estimates of parameters that were given by kinetic modelling studies of the same organism under the same conditions. The "cardinal" theoretical limits T_{min} , $a_{W_{min}}$, pH_{min}, U_{min} and D_{min} (fixed values from Presser *et al.* (1997)), the pK_a of lactic acid (Budavari (1989)) and the b coefficients (Presser *et al.*, 1998) (estimated by the growth/no growth modelling process) as well as the standard errors for each parameter estimate are given in Table 3.4 for the data set of 413 points. The ROC (c) was 0.973, the maximum rescaled R^2 was 0.7981 and the Hosmer Lemeshow goodness of fit characteristic was 8.728 with 8 degrees of freedom which corresponds to an acceptable probability of 0.3658. The fitted model is given below:

$$\begin{aligned} Logit(P) = {} & 28.0 + 8.90\ln(T - 4.00) \\ & +2.01\ln(a_w - 0.934) \\ & +4.59\ln(1 - 10^{3.90-pH}) \\ & +6.96\ln\left[1 - \frac{LAC}{10.7(1+10^{pH-3.86})}\right] \\ & +3.06\ln\left[1 - \frac{LAC}{823(1+10^{3.86-pH})}\right] \end{aligned} \qquad (3.6)$$

Table 3.4 - Parameter and Coefficient Estimates for Growth/No Growth Interface Modelling for the Different Types of Model

	Eqn 3.6 Estimate ± S.E.	Eqn 3.7 Estimate ± S.E.	Eqn 3.8 Estimate ± S.E.	Eqn 3.9 Estimate ± S.E.	Eqn 3.10 Estimate ± S.E.
b_0	27.98 ± 2.45	29.63 ± 13.29	16.97 ± 3.78	36.60 ± 7.05	67.56 ± 14.69
b_1 (T_{min})	2.013 ± 0.250	2.243 ± 1.426	1.706 ± 0.457	1.413 ± 0.152	0.376 ± 7.651
b_2 (T_{max})	-	43.14 ± 27.80	53.60 ± 17.22	-	-
b_3 (a_{Wmin})	8.897 ± 0.742	9.365 ± 4.928	5.544 ± 1.431	9.76 ± 3.10	26.42 ± 7.95
b_4 (pH_{min})	4.587 ± 0.469	5.186 ± 2.474	15.18 ± 10.55	23.7 ± 40.6	23.65 ± 38.98
b_5 (pH_{max})	-	-150.9 ± 189110	3.0129 ± 5.447	-	-
b_6 (U_{min})	6.964 ± 0.573	18.37 ± 16.48	8.658 ± 2.751	29.5 ± 28.2	66.00 ± 129.8
b_7 (D_{min})	3.065 ± 0.488	1.030 ± 1.943	0.4928 ± 0.7576	1.199 ±1.137	1.474 ± 1.347
b_8 (T_{min})^2	-	-	-	-	-2.652 ± 1.637
b_9 (T_{min})(a_{Wmin})	-	-	-	-	-5.564 ± 1.841
T_{min} (°C)	**4**	4.268 ± 6.632	6.000 ± 1.758	7.892 ± 0.080	2.583 ± 3.234
a_{Wmin}	**0.934**	0.9352 ± 0.0150	0.9344 ± 0.0075	0.933 ± 0.011	0.938 ± 0.007
pH_{min}	**3.90**	3.885 ± 0.102	3.268 ± 0.241	2.400 ± 1.359	2.400 ± 1.386
pH_{max}	**-**	9.045 ± 542.274	9.470 ± 0.119	**-**	**-**
U_{min}	**10.7**	19.45 ± 11.70	17.60 ± 3.40	36.46 ± 29.32	69.43 ± 126.0
D_{min}	**823.4**	539.4 ± 210.4	510.4 ± 83.9	507.2 ± 42.7	507.3 ± 40.87
T_{max} (°C)	-	**49.13**	**49.13**	-	-
d	-	**0.317**	**0.317**	-	-
Q	-	-	-	0.274 ± 0.171	0.219 ± 0.190
Data (#)	413	413	871	641	641

Numbers in bold were fixed in the modelling process, numbers underlined were estimates that hit bounds and thus the value given is not an estimate but a bound.

The following figures show the shape of the predicted interface for pH and temperature in response to decreasing water activity (Fig 3.1a) and increasing lactic acid concentration (Fig 3.1b) given by Eqn 3.6. The response is limited to suboptimal conditions of temperature and pH. The range of conditions modelled is pH < 7 and temperature < 37°C.

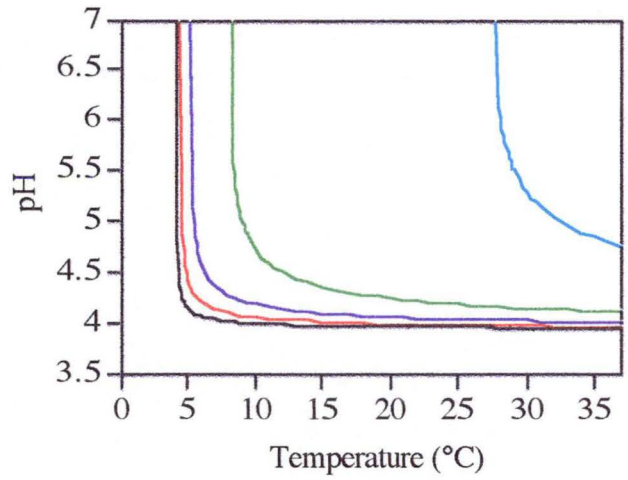


Figure 3.1a - Eqn 3.6 predictions of the growth/no growth boundary for *E. coli* M23 at *P*=0.5 for water activities of 1.000 (black), 0.985 (red), 0.975 (blue), 0.965 (green) and 0.955 (cyan) lines.

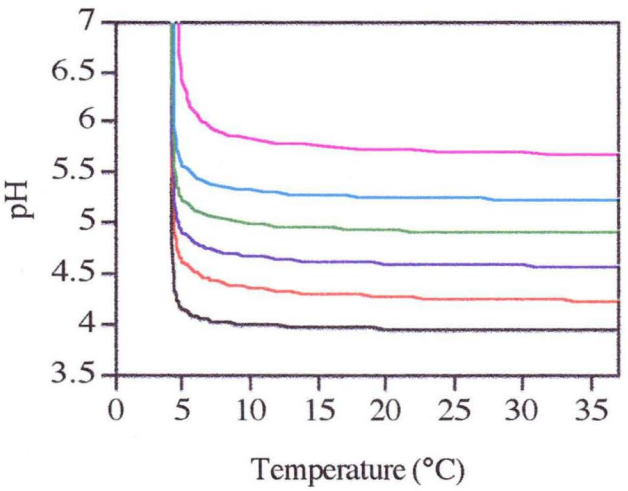


Figure 3.1b - Eqn 3.6 predictions of the growth/no growth boundary for *E. coli* M23 at *P*=0.5 for lactic acid concentrations of 0 (black), 25 (red), 50 (blue), 100 (green), 200 (cyan) and 500mM (magenta) lines.

3.4.1.2 Models with Superoptimal pH and Temperature Terms

Several growth limits models with superoptimal T_{max} and pH_{max} terms were created based on Eqn 3.2. The T_{max} term coefficient "d" (0.317) and T_{max} (49.13°C) were fixed values from a growth rate temperature model for this strain (Salter, 1998). The "cardinal" theoretical limits T_{min}, $a_{W_{min}}$, pH_{min}, pH_{max}, U_{min} and D_{min} estimated by the growth/no growth modelling process, the b coefficients as well as the standard errors for each parameter estimate are given in Table 3.4 for the dataset of 413 points. The ROC (c) was 0.979, the maximum rescaled R^2 was 0.8251 and the Hosmer Lemeshow goodness of fit characteristic was 1.3505 with 6 degrees of freedom which corresponds to an acceptable probability of 0.9688. The fitted model is given below:

$$\begin{aligned} Logit(P) = {} & 29.6 + 2.24\ln(T - 4.27) + 43.1\ln(1 - \exp(0.317(T - 49.1))) \\ & +9.36\ln(a_w - 0.935) \\ & +5.19\ln(1 - 10^{3.89-pH}) - 150\ln(1 - 10^{pH-9.05}) \\ & +18.4\ln\left[1 - \frac{LAC}{19.5(1+10^{pH-3.86})}\right] + 1.03\ln\left[1 - \frac{LAC}{539(1+10^{3.86-pH})}\right] \end{aligned} \qquad (3.7)$$

The shape of the predicted interface for pH and temperature given by Eqn 3.7 in response to decreasing water activity and increasing lactic acid concentration is shown in Figure 3.2a and 3.2b respectively. The effect of the high pH term's negative coefficient in Eqn 3.7 can be seen in the expansion in the temperature dimension of the interface conditions at high pHs compared to the restriction of the interface at low pHs (Figure 3.2).

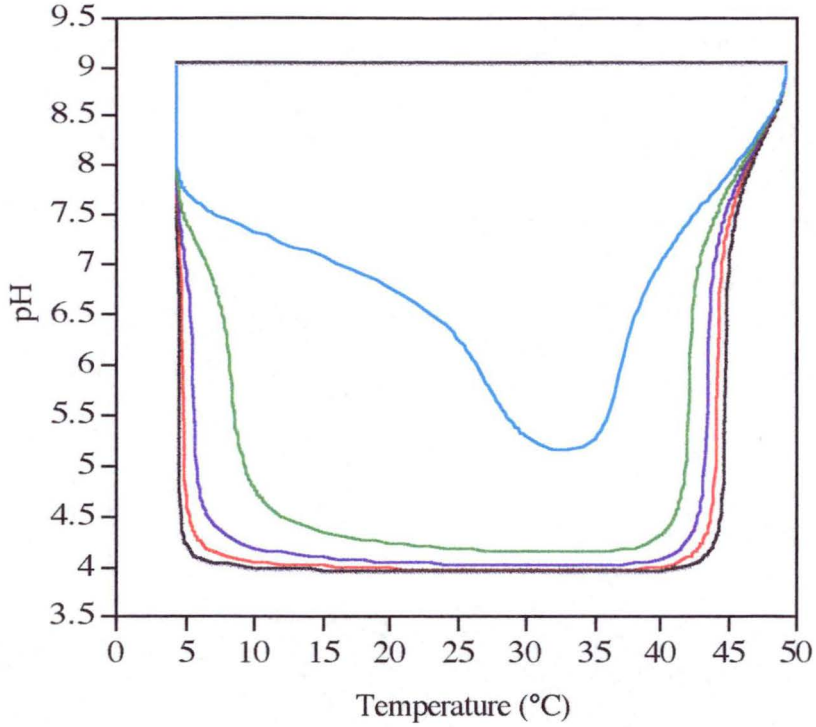


Figure 3.2a - Eqn 3.7 predictions of the growth/no growth boundary for *E. coli* M23 at *P*=0.5 for water activities of 1.000 (black), 0.985 (red), 0.975 (blue), 0.965 (green) and 0.955 (cyan) lines.

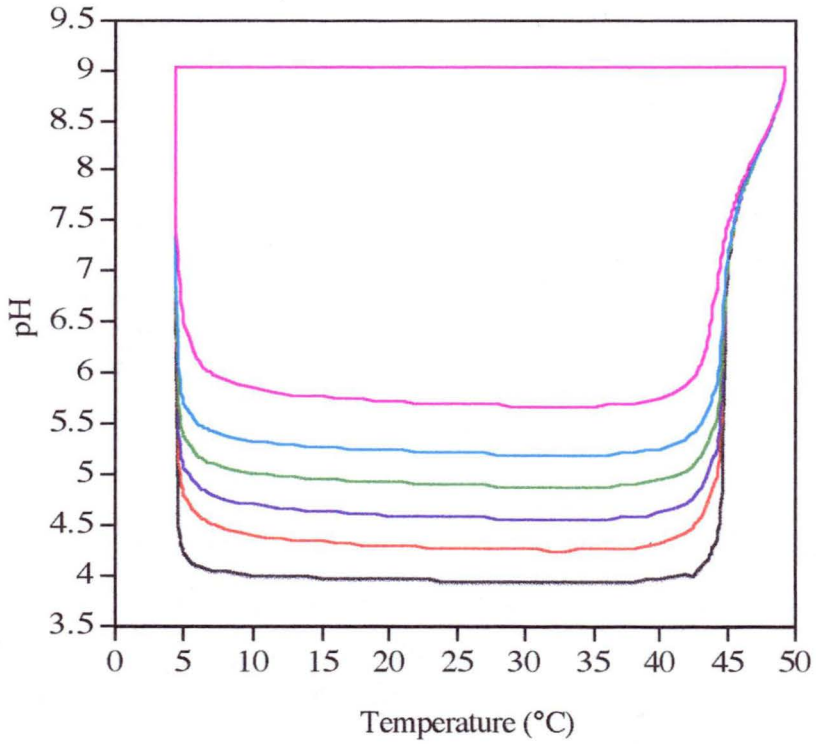


Figure 3.2b - Eqn 3.7 predictions of the growth/no growth boundary for *E. coli* M23 at *P*=0.5 for lactic acid concentrations of 0 (black), 25 (red), 50 (blue), 100 (green), 200 (cyan) and 500mM (magenta) lines.

This same type of model with the same fixed T_{max} values was fitted using the augmented dataset of 871 points, including 458 extra datapoints to yield Eqn 3.8. This dataset included the data from Experiment 2 and novel growth rate high pH data. The collection of novel high pH growth rate data allowed more realistic values for the pH_{max} estimate bounds to be used in Eqn 3.8. Also, for the fitting of Eqn 3.8 the coefficients were constrained to be greater than zero to prevent a negative coefficient for the pH_{max} term from occurring as in Eqn 3.7. The "cardinal" theoretical limits T_{min}, $a_{W_{min}}$, pH_{min}, pH_{max}, U_{min} and D_{min} estimated by the growth/no growth modelling process, the b coefficients as well as the standard errors for each parameter estimate are given in Table 3.4. The ROC (c) was 0.975, the maximum rescaled R^2 was 0.8217 and the Hosmer Lemeshow goodness of fit characteristic was 128.04 with 8 degrees of freedom which corresponds to an unacceptably low probability of 0.0001. While the ROC (c) and the maximum rescaled R^2 are similar to those obtained for Eqn 3.7, the Hosmer Lemeshow goodness of fit criterion indicates a worse fit due to the presence of significant outliers and disagreements in the expanded dataset. The fitted model is given below:

$$\begin{aligned} Logit(P) = {} & 17.0 + 1.71\ln(T - 6.00) + 53.6\ln(1 - \exp(0.317(T - 49.1))) \\ & + 5.54\ln(a_w - 0.934) \\ & + 15.2\ln(1 - 10^{3.27 - pH}) + 3.01\ln(1 - 10^{pH - 9.47}) \\ & + 8.66\ln\left[1 - \frac{LAC}{17.6(1 + 10^{pH - 3.86})}\right] + 0.493\ln\left[1 - \frac{LAC}{510(1 + 10^{3.86 - pH})}\right] \end{aligned} \qquad (3.8)$$

The shape of the predicted interface for pH and temperature given by Eqn 3.8 in response to decreasing water activity and increasing lactic acid concentration is shown in Figure 3.3a and 3.3b respectively.

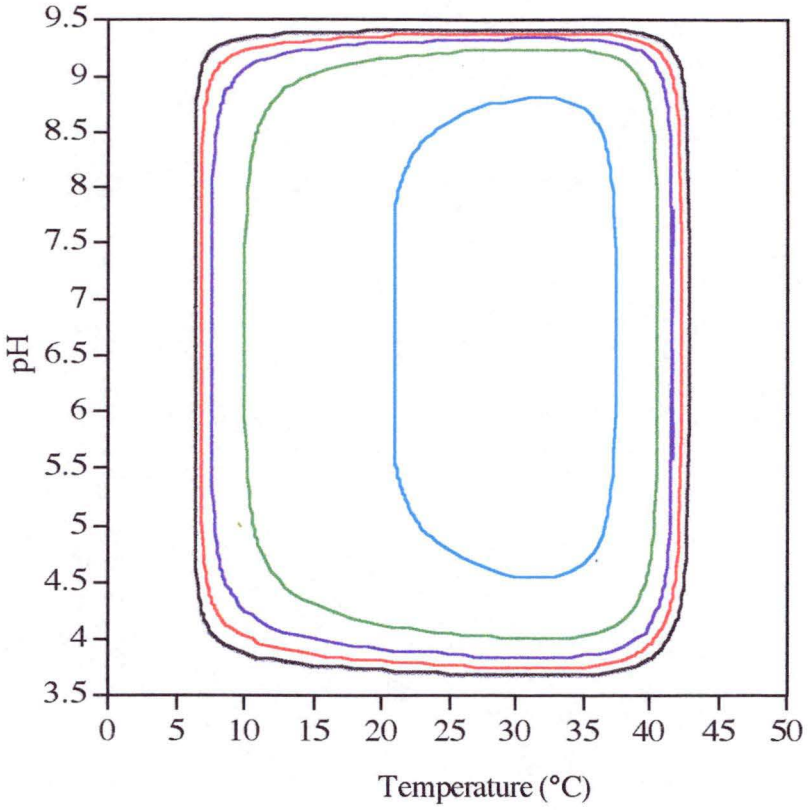


Figure 3.3a - Eqn 3.8 predictions at *P*=0.5 for water activities of 1.000 (black), 0.985 (red), 0.975 (blue), 0.965 (green) and 0.955 (cyan) lines.

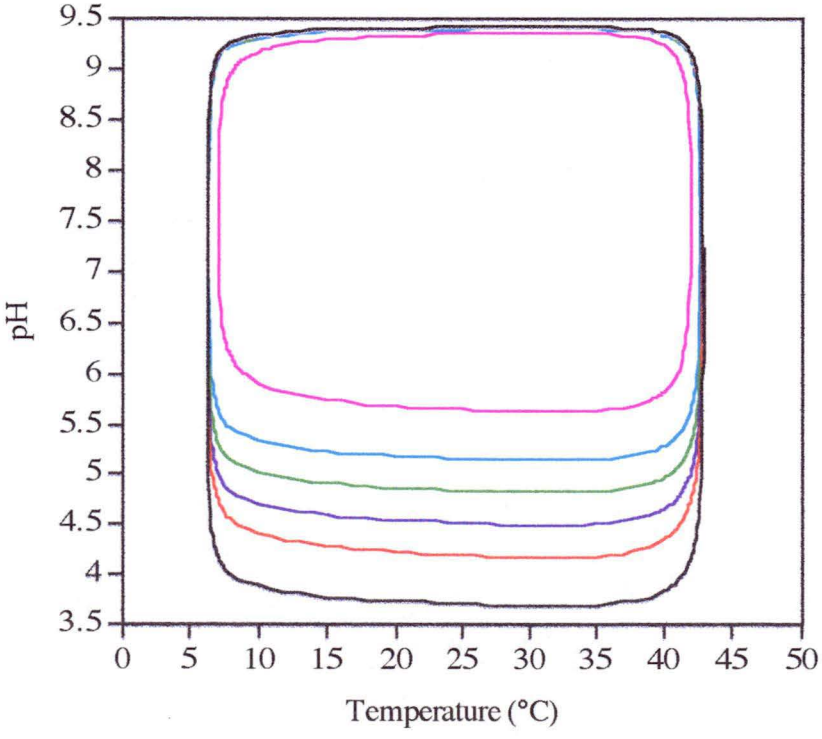


Figure 3.3b - Eqn 3.8 predictions at *P*=0.5 for lactic acid concentrations of 0 (black), 25 (red), 50 (blue), 100 (green), 200 (cyan) and 500mM (magenta) lines.

3.4.1.3 Model with Q factor

In the third type of model (Eqn 3.4) the superoptimal terms were removed and the suboptimal pH term was modified to include an extra scaling coefficient, equivalent to the Q factor in kinetic modelling (Eqn 2.10). The growth limits model shown below was created using most of the data from Experiments 1 and 2. However, significant data were removed from the dataset in order to help the model fit correctly, including the high pH data which were no longer necessary as no high pH term was included. The "cardinal" theoretical limits T_{min}, $a_{w_{min}}$, pH_{min}, pH_{max}, U_{min} and D_{min} estimated by the growth/no growth modelling process on the reduced dataset of 641 points, the b coefficients as well as the standard errors for each parameter estimate are given in Table 3.4. The ROC (c) was 0.970, the maximum rescaled R^2 was 0.8043 and the Hosmer Lemeshow goodness of fit characteristic was 5.1511 with 8 degrees of freedom which corresponds to an acceptable probability of 0.7413. The fitted model is given below:

$$\begin{aligned} Logit(P) = {} & 36.6 + 1.41\ln(T - 7.89) + 9.76\ln(a_w - 0.933) \\ & + 23.7\ln(1 - 10^{0.273(2.40 - pH)}) \\ & + 29.5\ln\left[1 - \frac{LAC}{36.4(1 + 10^{pH-3.86})}\right] \\ & + 1.20\ln\left[1 - \frac{LAC}{507(1 + 10^{3.86-pH})}\right] \end{aligned} \qquad (3.9)$$

The shape of the predicted interface for pH and temperature given by Eqn 3.9 in response to decreasing water activity and increasing lactic acid concentration is shown in Figure 3.4a and 3.4b respectively.

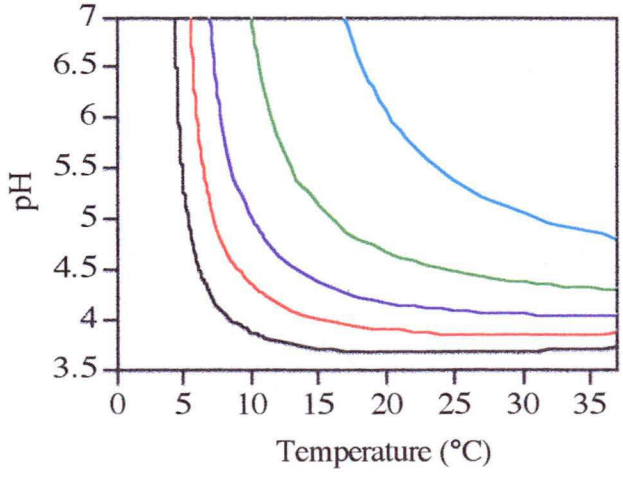


Figure 3.4a - Eqn 3.9 predictions at P=0.5 for water activities of 1.000 (black), 0.985 (red), 0.975 (blue), 0.965 (green) and 0.955 (cyan) lines.

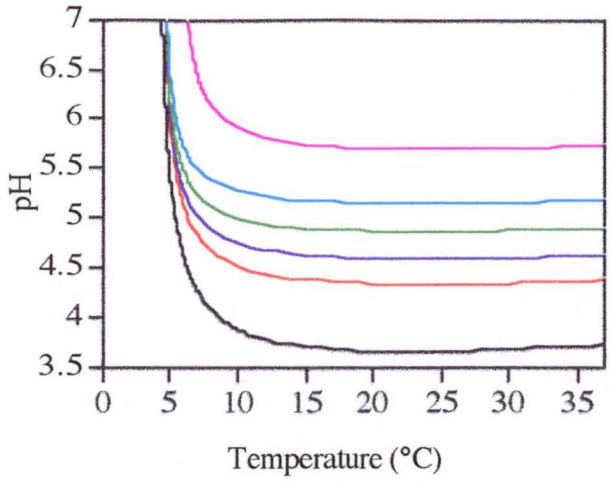


Figure 3.4b - Eqn 3.9 predictions at *P*=0.5 for lactic acid concentrations of 0 (black), 25 (red), 50 (blue), 100 (green), 200 (cyan) and 500mM (magenta) lines.

3.4.1.4 Suboptimal Temperature and pH Model with Squared and Cross Product Terms

In the final type of model described here (Eqn 3.5) the dataset of 641 points from Eqn 3.9 was used to fit a model with a cross product and squared term. The "cardinal" theoretical limits T_{min}, $a_{W_{min}}$, pH_{min}, pH_{max}, U_{min} and D_{min} estimated by the growth/no growth modelling process, the *b* coefficients as well as the standard errors for each parameter estimate are given in Table 3.4. The ROC (*c*) was 0.977, the maximum rescaled R^2 was 0.8284 and the Hosmer Lemeshow goodness of fit characteristic was 8.598 with 8 degrees of freedom which corresponds to an acceptable probability of 0.3773. The fitted model is given below:

$$\begin{aligned} Logit(P) = {} & 67.6 + 0.376\ln(T - 2.58) + 26.4\ln(a_w - 0.938) \\ & +23.7\ln(1 - 10^{0.219(2.40-pH)}) \\ & +66.0\ln\left[1 - \frac{LAC}{69.4(1+10^{pH-3.86})}\right] + 1.47\ln\left[1 - \frac{LAC}{507(1+10^{3.86-pH})}\right] \\ & -2.65\ln(T - 2.58)^2 - 5.57\ln(T - 2.58)\ln(a_w - 0.938) \end{aligned} \quad (3.10)$$

The shape of the predicted interface for pH and temperature given by Eqn 3.10 in response to decreasing water activity and increasing lactic acid concentration is shown in Figure 3.5a and 3.5b respectively.

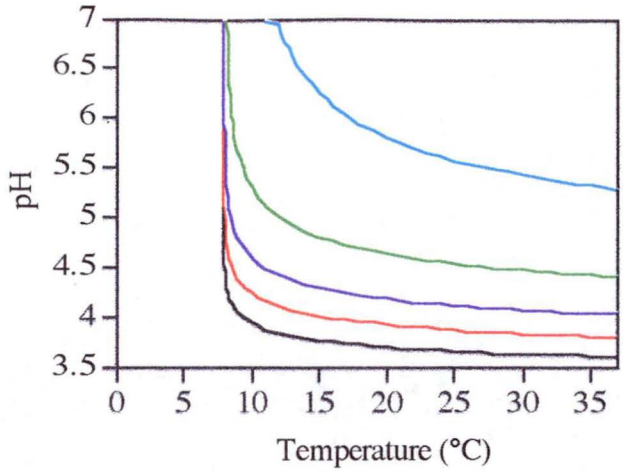


Figure 3.5a - Eqn 3.10 predictions at *P*=0.5 for water activities of 1.000 (black), 0.985 (red), 0.975 (blue), 0.965 (green) and 0.955 (cyan) lines.

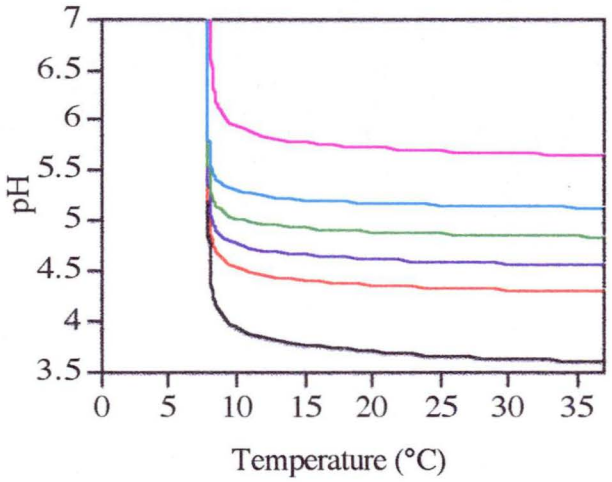


Figure 3.5b - Eqn 3.10 predictions at *P*=0.5 for lactic acid concentrations of 0 (black), 25 (red), 50 (blue), 100 (green), 200 (cyan) and 500mM (magenta) lines.

The change in probability of growth over a range of pH and temperature conditions is shown in Figure 3.6 for model Eqn 3.8 as an example. This model was used because it contained both superoptimal terms (T_{max} and pH_{max}) and so showed a complete three dimensional shape as well as being based on the full dataset of 871 points. Similar responses were noted for the other models. As the inhibition by water activity increased from none to the level of inhibition at water activity 0.965 and 0.955, the change in probability became less abrupt with the maximum probability of growth only reaching 70% at water activity 0.955 (Fig 3.6c). However, as lactic acid concentration increased the boundary did not change "shape" but shifted towards higher pH (Fig 3.6d). For Eqn 3.6 and 3.7 a very similar shape was observed. However, for Eqns 3.9 and 3.10 there was a different shape of the response at severely limiting water activity of 0.955 as shown by Fig

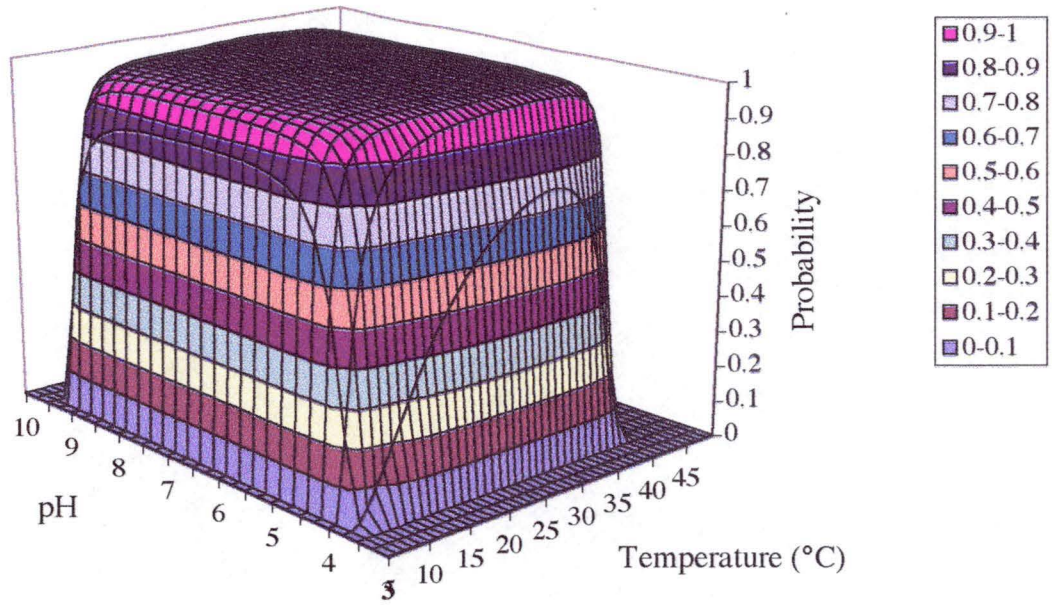


Figure 3.6a - Probabillity of growth given by Eqn 3.8 at a water activity of 1.000 with no lactic acid present

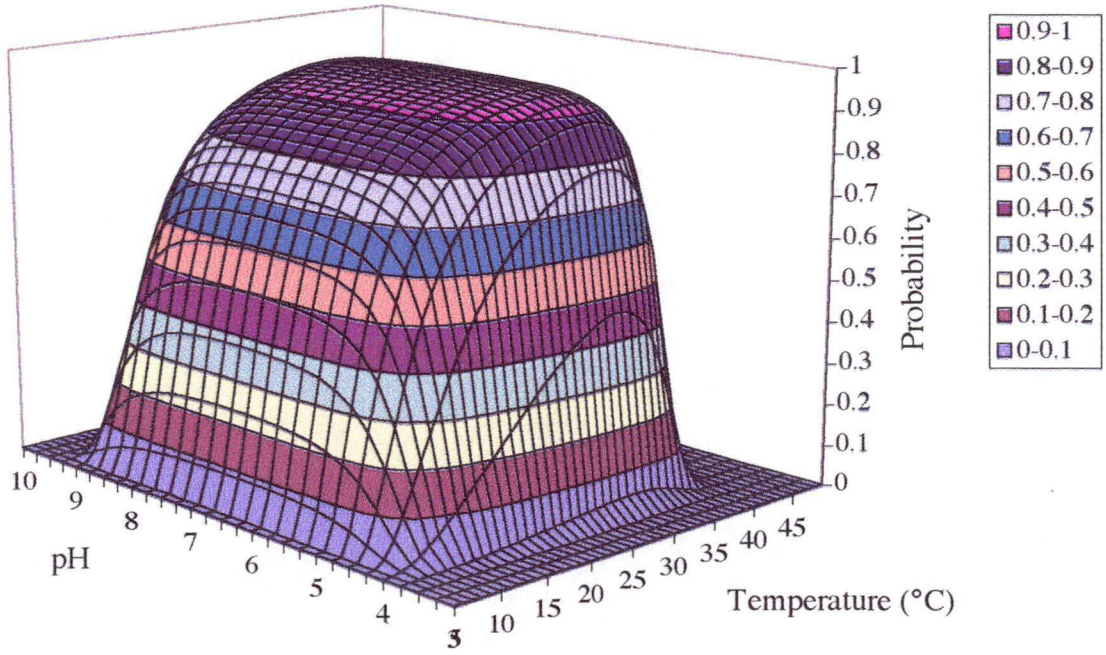


Figure 3.6b - Probability of growth given by Eqn 3.8 at a water activity of 0.965 with no lactic acid present

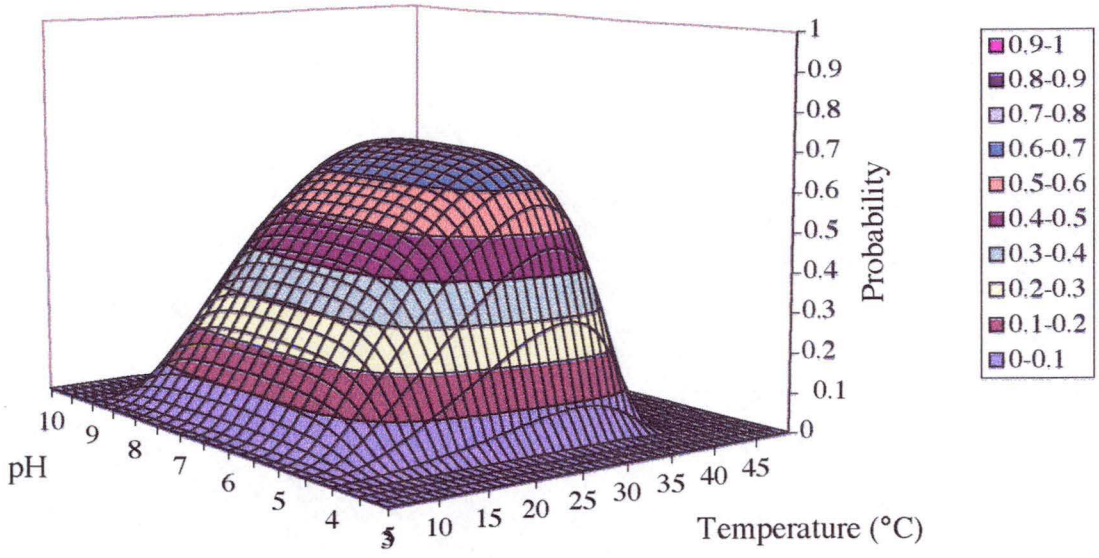


Figure 3.6c - Probability of growth given by Eqn 3.8 at a water activity of 0.955 with no lactic acid present

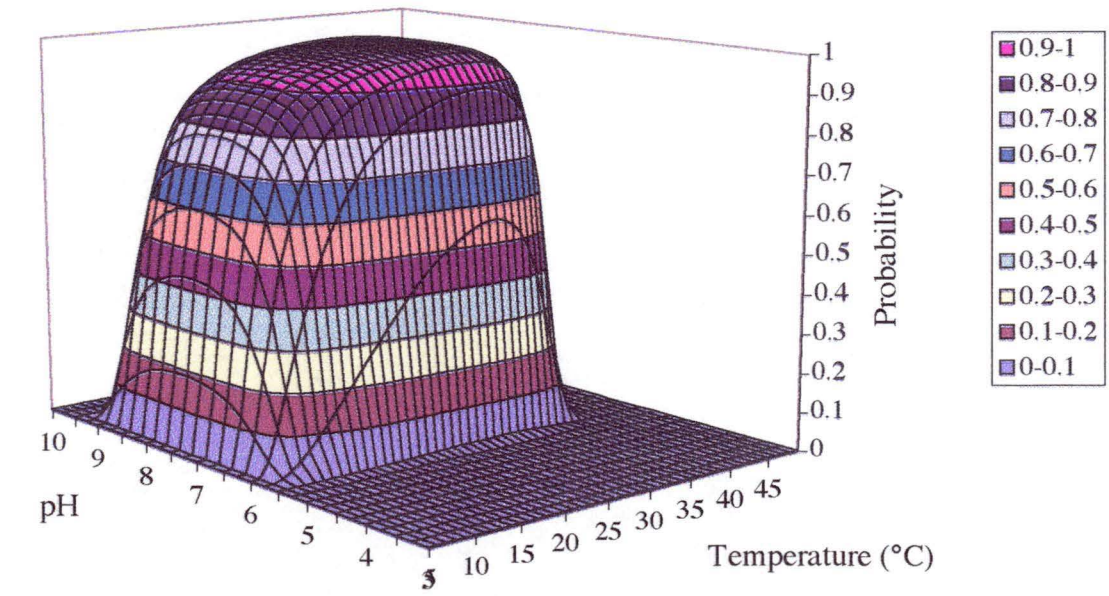


Figure 3.6d - Probability of growth given by Eqn 3.8 at 500mM lactic acid and a water activity of 0.986

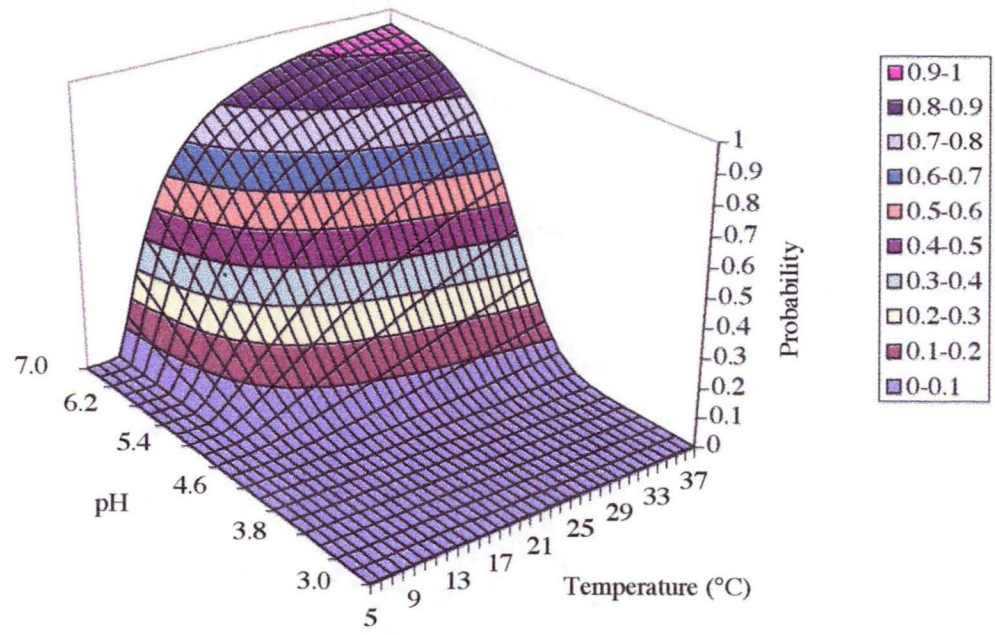


Figure 3.7 - Probability of growth given by Eqn 3.9 at a water activity of 0.955 with no lactic acid present

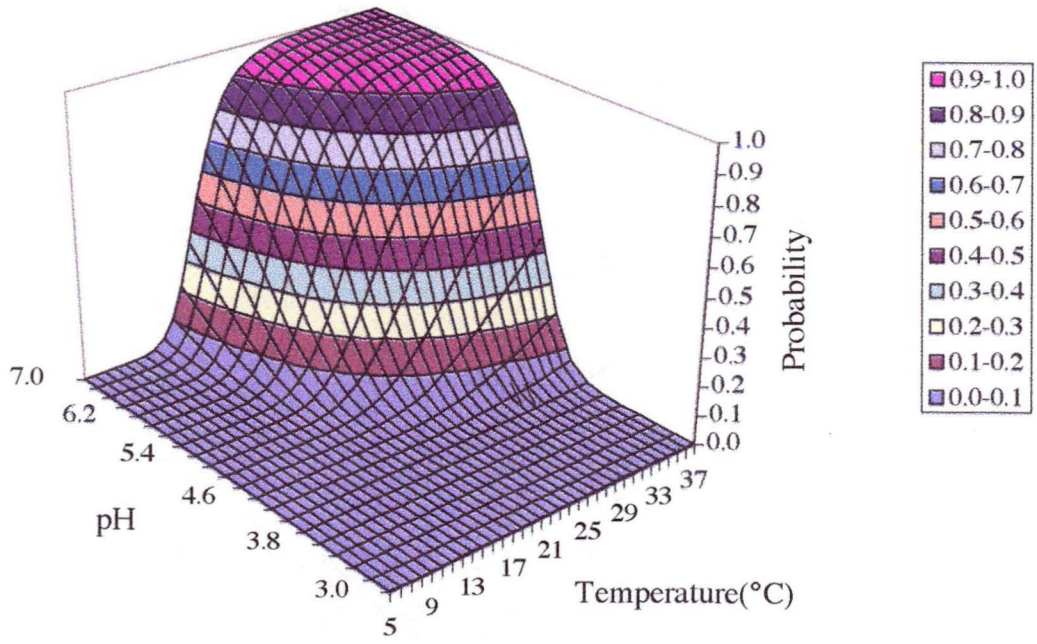


Figure 3.8 - Probability of growth given by Eqn 3.10 at a water activity of 0.955 with no lactic acid present

3.4.2 Comparisons of Models to Data: Water Activity and pH

In the following section subsets of the dataset at each water activity (~0.996, 0.985, 0.975, 0.965 and 0.955) are shown for the whole range of temperature and pH (Figures 3.6 - 3.10). Data for 10, 20, 25, 30 and 37°C are from Presser (1995) and Experiment 1 and data for 8, 12, 17, 22, 24, 27, 32 and 36°C are from Experiment 2. No growth was observed at any pH or temperature combination for a water activity of 0.945 in Experiment 2. All data that were incorrectly predicted by any model are shown in Appendix 3.2 which shows all the different models predictions for that datapoint. Also the number and percentage of under or overpredictions for two levels of stringency are given in Table 3.5 for the complete set of 871 data or for the specific subset of data used to create the individual models (Table 3.6).

Table 3.5 - Number and percentage of underpredictions (growth observed but model predicted no growth) and overpredictions (no growth observed but model predicted growth) for each model for the total dataset of 871 observations.

	Underpredictions				Overpredictions			
	$P < 0.50$		$P < 0.25$		$P > 0.50$	%	$P > 0.75$	%
Eqn 3.6	39	4.4%	13	1.5%	52	6.0%	42	4.8%
Eqn 3.7	34	3.9%	13	1.5%	42	4.8%	29	3.3%
Eqn 3.8	15	1.7%	6	0.7%	52	6.0%	17	2.0%
Eqn 3.9	25	2.9%	9	1.0%	48	5.5%	28	3.2%
Eqn 3.10	21	2.4%	11	1.3%	44	5.1%	26	3.0%

Table 3.6 - Number and percentage of underpredictions (growth observed but model predicted no growth) and overpredictions (no growth observed but model predicted growth) for each model for only the data used in fitting the models. For Eqns 3.6 and 3.7 there were 413 observations and for Eqns 3.9 and 3.10 there were 641 observations.

	Underpredictions				Overpredictions			
	$P < 0.50$		$P < 0.25$		$P > 0.50$		$P > 0.75$	
Eqn 3.6	18	4.4%	7	1.7%	18	4.4%	10	2.4%
Eqn 3.7	18	4.4%	7	1.7%	15	3.6%	8	1.9%
Eqn 3.9	25	3.9%	9	1.4%	34	5.3%	14	2.2%
Eqn 3.10	21	3.3%	11	1.7%	30	4.7%	12	1.9%

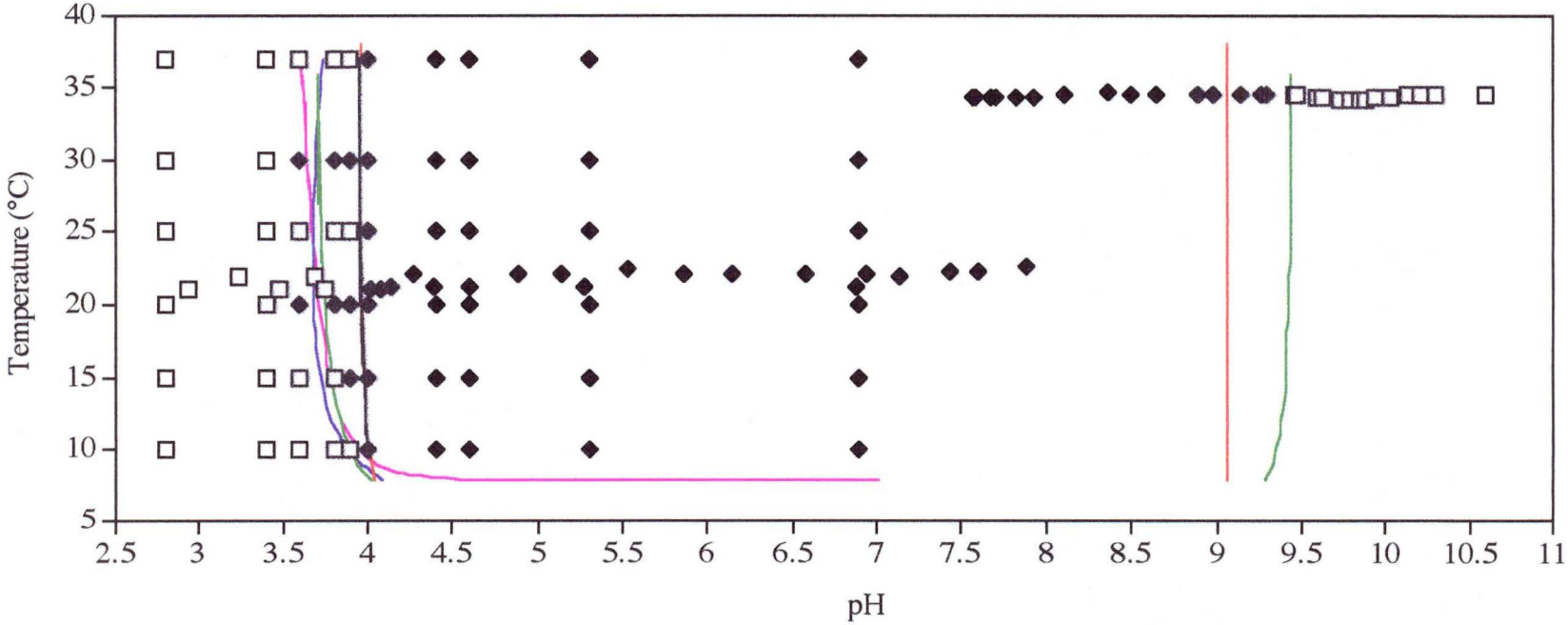


Figure 3.9a) - Data from Experiments 1 & 2 as well as additional data collected at high pH at a water activity of 0.996 with no lactic acid present. Open squares indicate no growth was observed and closed diamonds indicate growth was observed. Model predictions at $P=0.5$ are indicated by the solid lines: Eqn 3.6 (black), Eqn 3.7 (red), Eqn 3.8 (green), Eqn 3.9 (blue) and Eqn 3.10 (magenta). Eqn 3.7 (red) is not visible where it has the same prediction as Eqn 3.6 (black).

There were a number of inconsistencies in the minimum pH for growth at different temperatures for water activity of 0.996. Growth was observed at pH 3.6 at 20 and 30°C but no growth was observed at 3.9 at 25°C. This resulted in underpredictions of P <0.25 for all models for growth rate observations at pH 3.6 (20 and 30°C), underpredictions of P < 0.25 for Eqn 3.6, 3.7, and 3.8 for growth rate observations at pH 3.8 (20 and 30°C) and underpredictions of P < 0.25 for Eqn 3.6 and 3.7 for growth rate observations at pH 3.9 (15, 20 and 30°C). However, while Eqns 3.8, 3.9 and 3.10 had less underpredictions, these models overpredicted by P > 0.75 the no growth observations at pH 3.9 (25 and 37°C). Eqns 3.9 and 3.10 also overpredicted the no growth observations at pH 3.8 (25 and 37°C) and Eqn 3.10 overpredicted at pH 3.8 (15°C), but of these only for Eqn 3.9 at pH 3.8 (37°C) had P > 0.75.

As high pH data were not included in fitting Eqns 3.6, 3.9 or 3.10 these models did not correctly predict the no growth observations at high pH (pH > 9.3). Eqn 3.7 and Eqn 3.8 did include a high pH term. However, Eqn 3.7 did not model some of the high pH data correctly (9.13 <pH < 9.30) due to pH_{max} being bounded at a lower value (pH 9.045) Subsequent data collection observed in the growth/no growth boundary at between pH 9.29-9.45.

Another observation that was underpredicted P < 0.25 by Eqns 3.6, 3.7 and 3.9 was that observation of growth at pH 4 at 10°C. For these models the predictions at this "corner" point, where pH and temperature both reach limiting conditions, were less than the observed growth.

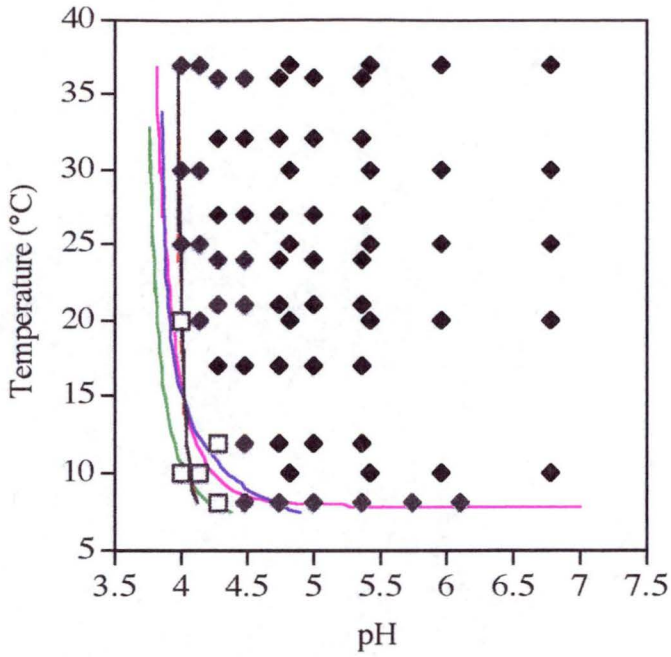


Figure 3.9b) - Data from Experiments 1 & 2 at a water activity of 0.985 with no lactic acid present. Open squares indicate no growth was observed and closed diamonds indicate growth was observed. Model predictions at p=0.5 are indicated by the solid lines: Eqn 3.6 (black), Eqn 3.7 (red), Eqn 3.8 (green), Eqn 3.9 (blue) and Eqn 3.10 (magenta). Eqn 3.7 (red) is not visible where it has the same prediction as Eqn 3.6 (black).

At water activity 0.985 growth is possible over almost the whole range of the data (Figure 3.9b). Growth was observed at all conditions except at temperatures < 21°C and pH < 4.5. Eqns 3.9 and 3.10 underpredicted the growth observations at 8°C and pH 4.48 and 4.74, with only Eqn 3.10 at pH 4.74 predicting $P > 0.25$. Eqns 3.9 also underpredicted the growth observation at 8°C and pH 5.01 at $P > 0.25$.

All models overpredicted growth at pH 4.28 and 12°C with only Eqn 3.10 predicting $P < 0.75$ probability of growth. Overpredictions occurred at pH 4.15 and 10°C for Eqns 3.6, 3.7 and 3.8 and for Eqn 3.8 alone the overprediction was $P < 0.75$. Eqns 3.8, 3.9 and 3.10 overpredicted growth at pH 4 and 20°C and in this case only Eqn 3.8 gave $P > 0.75$. Eqns 3.6 and 3.7 overpredicted the growth at 4.28 and 8°C with $P > 0.75$ but Eqn 3.8 predicted a growth probability of only 0.51.

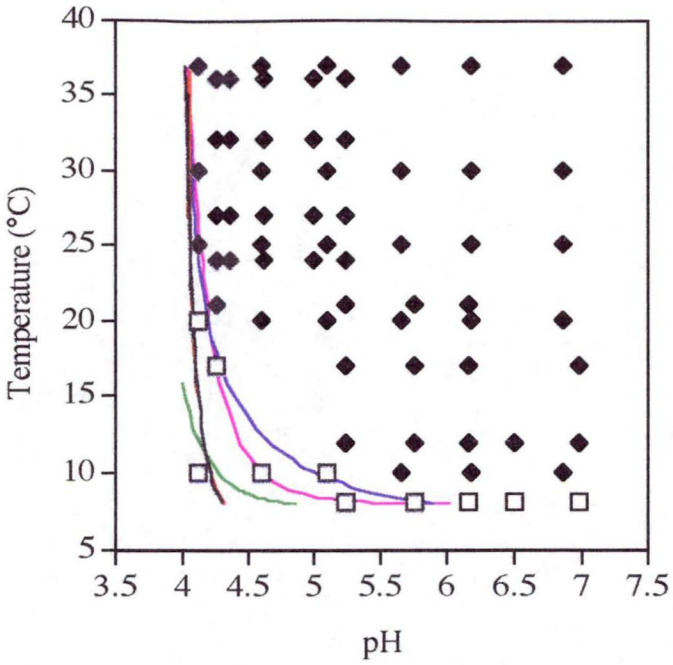


Figure 3.9c) - Data from Experiments 1 & 2 at a water activity of 0.975 with no lactic acid present. Open squares indicate no growth was observed and closed diamonds indicate growth was observed. Model predictions at p=0.5 are indicated by the solid lines: Eqn 3.6 (black), Eqn 3.7 (red), Eqn 3.8 (green), Eqn 3.9 (blue) and Eqn 3.10 (magenta). Eqn 3.7 (red) is not visible where it has the same prediction as Eqn 3.6 (black).

At water activity 0.975 the temperature and pH ranges over which growth is possible is similar to 0.985 except that for some conditions growth is prevented near the lower temperature limit (Figure 3.9c). Growth was observed even at the lowest pH except at temperatures < 21°C. Similarly growth was observed at 10°C for pH > 5.5. There was only one underprediction observed for Eqn 3.9 at pH 4.11 at 25°C but this prediction was only slightly < 0.5 ($P = 0.45$). Overpredictions occurred at 8°C at pHs 5.23, 5.75, 6.15, 6.5 and 6.98 for Eqns 3.6, 3.7, 3.8 but only at the highest three pHs for Eqns 3.9 and 3.10. Of these overpredictions those for Eqn 3.8 all had $P < 0.75$ whereas those for Eqn 3.6 and 3.7 were all greater. Eqn 3.9 overpredicted ($P > 0.75$) for the two highest pHs and Eqn 3.10 for only the highest pH. At 10°C all models overpredicted the no growth observation at pH 5.09 with $P > 0.75$, except Eqn 3.10 which predicted $P = 0.55$. Eqns 3.6, 3.7 and 3.8 also overpredicted the next lowest pH 4.6 but with Eqn 3.8 having $P <$ 0.75. In contrast this was the only model of these three that overpredicted with $P > 0.75$ for pH 4.11 at 20°C. These three models also overpredicted ($P > 0.75$) for pH 4.26 at 17°C where Eqn 3.9 slightly overpredicted ($P = 0.50$).

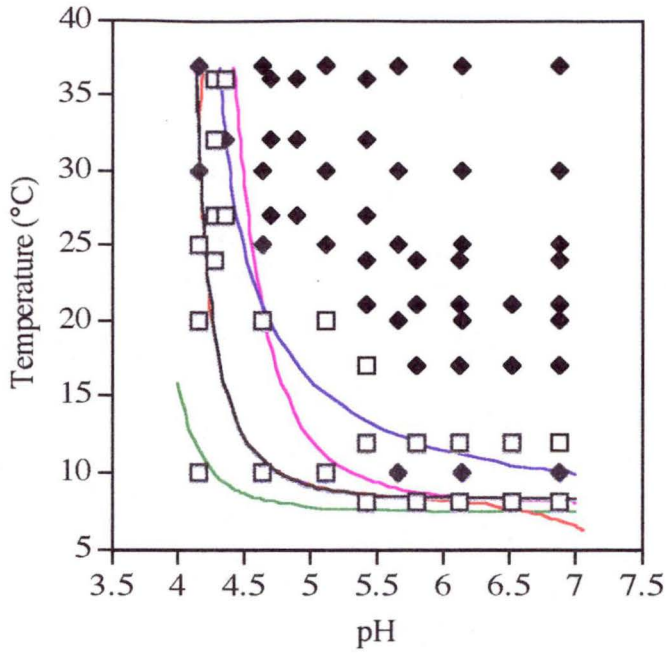


Figure 3.9d) - Data from Experiments 1 & 2 at a water activity of 0.965 with no lactic acid present. Open squares indicate no growth was observed and closed diamonds indicate growth was observed. Model predictions at p=0.5 are indicated by the solid lines: Eqn 3.6 (black), Eqn 3.7 (red), Eqn 3.8 (green), Eqn 3.9 (blue) and Eqn 3.10 (magenta). Eqn 3.7 (red) is not visible where it has the same prediction as Eqn 3.6 (black).

At water activity 0.965 there is a reduction in the growth permissive range compared to water activity 0.975. There were two regions in which it was difficult to predict the response using these models (Figure 3.9d). Firstly there was disagreement between the results of the two experiments in the area of low temperature (<15°C) and moderate pH (>5.5). The data from experiment 1 at T = 10°C and pH > 5.5 yielded growth but no growth was observed in experiment 2 at T = 12°C and pH > 5. Secondly there was disagreement between the results of the two experiments in the area of high temperature and low pH. The data from experiment 2 at a temperature of between 30 and 37°C and pH < 4.5 yielded growth but there were only observations of no growth from experiment 1 at $30 < T < 37$°C and pH < 4.5.

The inconsistency in the data caused the models to either over or underpredict the data in these regions. Eqn 3.10 underpredicted the growth rate observations at 10°C and pH 5.66, 6.14 and 6.87 with only pH 6.87 being predicted with $P > 0.25$. In the other area of inconsistency, Eqns 3.9 and 3.10 underpredicted the growth rate observations at pH 4.15 for 30°C and 37°C at $P < 0.25$. Eqn 3.9 also underpredicted the growth observation at 32°C at pH 4.36 but at $P > 0.25$. For Eqns 3.6, 3.7 and 3.8 there were underpredictions for a few of these same points but all had $P > 0.25$.

Overpredictions were common for water activity 0.965 data due to the inconsistencies in the data. All models overpredicted observations of no growth at 12°C for pH 6.12 -6.87, 17°C at pH 5.42 and 20°C at pH 5.12. For Eqns 3.6, 3.7 and 3.9 all these overpredictions had $P > 0.75$ and these models also overpredicted observations of no growth at 12°C for pH 5.42 and 5.8. Eqn 3.8 overpredicted these same points but with $P < 0.75$ with the exception of 20°C at pH 5.12. Eqns 3.6, 3.7 and 3.8 also overpredicted no growth observations at pH < 4.5 and $T > 20$°C.

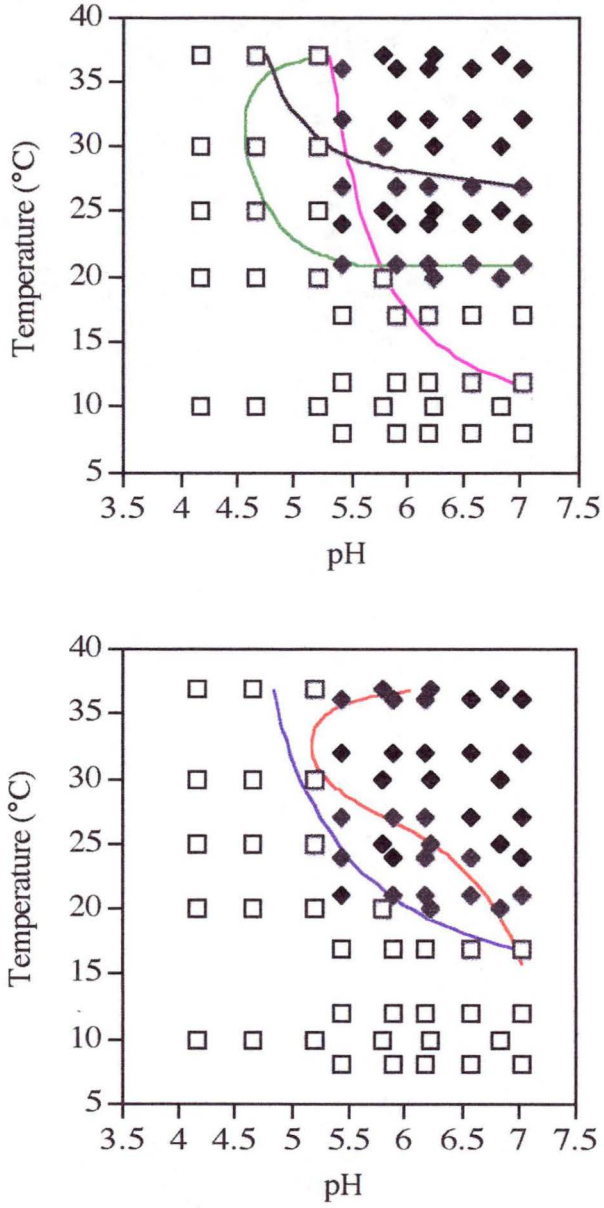


Figure 3.9e) - Data from Experiments 1 & 2 at a water activity of 0.955 with no lactic acid present. Open squares indicate no growth was observed and closed diamonds indicate growth was observed. Model predictions at p=0.5 are indicated by the solid lines: Eqn 3.6 (black), Eqn 3.7 (red), Eqn 3.8 (green), Eqn 3.9 (blue) and Eqn 3.10 (magenta).

At water activity 0.955, the stress imposed by the reduced water activity severely restricted the range of growth (Figure 3.9e). There is a distinct boundary between growth and no growth that occurs at a temperature of approximately 20°C and a pH of approximately 5.45. The square boundary shape implies that there is little interaction of pH and temperature inhibition at low water activity. An interaction should result in a curved boundary for growth, that is, cutting off the square corner with a boundary that would be at higher pH at the lowest temperature and at higher temperatures at the lowest pH.

There were a number of underpredictions of growth at temperatures 20, 21, 24, 25 and 27 for a range of pH from 5.42 - 7.02. However, only one prediction had $P < 0.25$ which was at pH 5.42 and 21°C for Eqn 3.10. Most underpredictions were made by Eqns 3.6 and 3.7. There were also overpredictions at temperatures 17, 25, 30 and 37 for a range of pH from 5.20 - 7.02. However, only two predictions had $P > 0.75$ which was at pH 5.20 and 37°C for Eqn 3.10 and at pH 7.02 and 17°C for Eqn 3.9.

3.4.3 Comparisons of Models to Data: Lactic Acid and Undissociated Acid Concentrations

In the following section the subsets of the dataset at each lactic acid concentration (25, 50, 100, 200 and 500) are shown for their whole range of temperature and undissociated acid concentration (Figures 3.10a - 3.10e). Undissociated acid is the proportion of the total lactic acid with the hydrogen ion still attached which varies according to the pH of the solution. Undissociated acid was chosen to represent the inhibition by lactic acid graphically because it allows easier comparison between different total lactic acid concentrations. It is this form of organic acid that causes the main inhibitory effects (Chapter 1).

The scale of most figures has been truncated to highlight the growth/no growth boundary in each case. For 25mM the whole range of undissociated acid is shown. For 50, 100, 200 and 500mM undissociated lactic acid concentrations, only concentrations of undissociated acid up to 25mM are shown. In all cases only observations of no growth at high undissociated acid concentrations are excluded by this truncation. All data are at temperatures of 10, 20, 21-22, 25, 30 and 37°C from dataset 1. A similar response to undissociated acid is shown at all total lactic acid concentrations. The growth/no growth boundary occurs at between 5 and 10 mM undissociated lactic acid with decreases in the boundary at temperatures < 15°C for all total concentrations for all models.

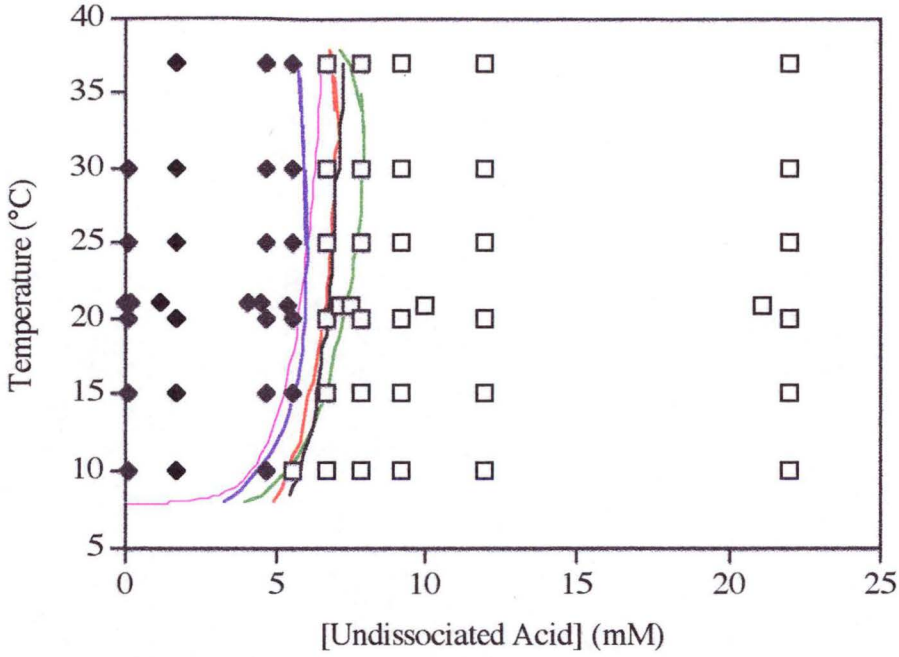


Figure 3.9a) - Data from Experiment 1 at a water activity of 0.996 with 25mM total lactic acid present. Open squares indicate no growth was observed and closed diamonds indicate growth was observed. Model predictions at p=0.5 are indicated by the solid lines: Eqn 3.6 (black), Eqn 3.7 (red), Eqn 3.8 (green), Eqn 3.9 (blue) and Eqn 3.10 (magenta).

For 25mM, Eqn 3.9 undepredicted the observations of growth at [U] of 4.5mM and 10°C and [U] of 4.4mM and 15°C. Also Eqn 3.10 underpredicted the observation of growth at pH 4.5 and 10°C but all of these underpredictions had $P > 0.25$. Overpredictions of no growth observations at [U] of 6.66mM occurred at 30 and 37°C for Eqns 3.6, 3.7 and 3.8. Eqns 3.6 and 3.8 overpredicted at 25°C and Eqn 3.8 alone overpredicted at 20°C. Of these overpredictions only Eqn 3.6 at 37°C and Eqn 3.6 at 30°C had $P > 0.75$.

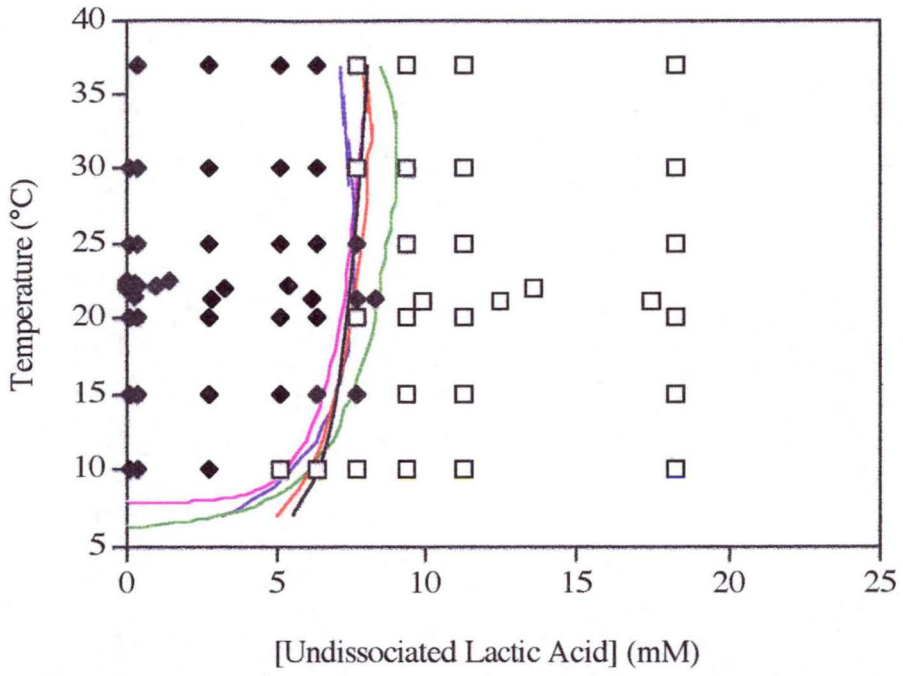


Figure 3.10b) - Data from Experiment 1 at a water activity of 0.996 with 50mM total lactic acid present. Open squares indicate no growth was observed and closed diamonds indicate growth was observed. Model predictions at p=0.5 are indicated by the solid lines: Eqn 3.6 (black), Eqn 3.7 (red), Eqn 3.8 (green), Eqn 3.9 (blue) and Eqn 3.10 (magenta).

For 50mM, all models underpredicted the observations of growth at [U] of 7.7mM at 15°C with only Eqn 3.10 underpredicting with $P > 0.25$. At this same concentration of undissociated acid, Eqns 3.6, 3.7, 3.9 and 3.10 underpredicted the observations of growth at 21.22 and 25°C but all these had $P > 0.25$. All models also underpredicted the observations of growth at [U] of 8.32mM at 22.10°C with only Eqn 3.8 underpredicting with $P > 0.25$.

Overpredictions of no growth observations at [U] of 5.15mM occurred at 10°C for all models but of these only Eqn 3.6 had $P > 0.75$. Some no growth observations at [U] of 7.7mM, at temperatures 20, 30 and 37°C were overpredicted by Eqns 3.6, 3.7, 3.8 and 3.9. However, none had $P > 0.75$.

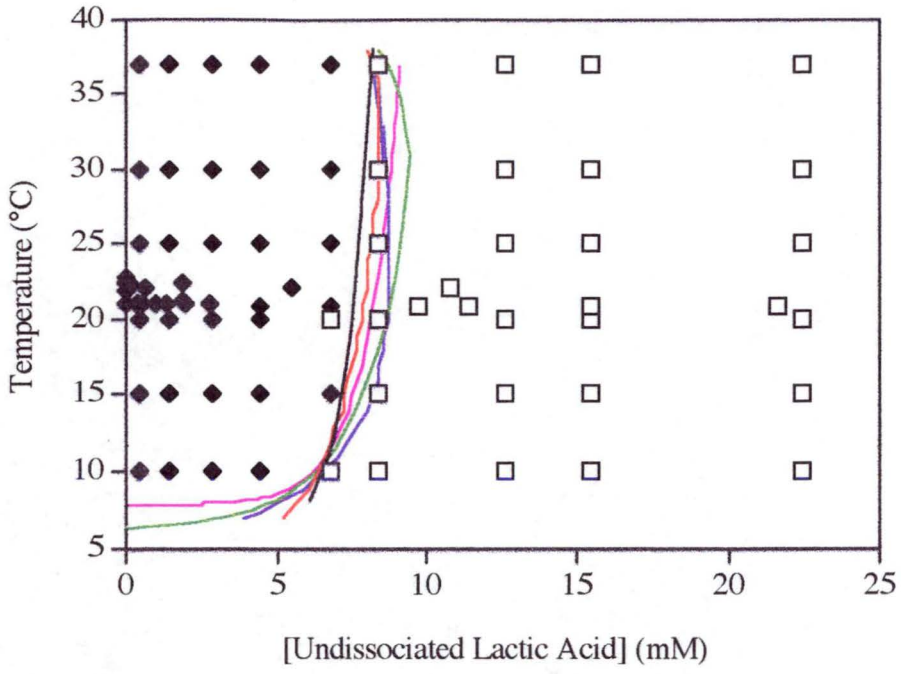


Figure 3.10c) - Data from Experiment 1 at a water activity of 0.996 with 100mM total lactic acid present. Open squares indicate no growth was observed and closed diamonds indicate growth was observed. Model predictions at p=0.5 are indicated by the solid lines: Eqn 3.6 (black), Eqn 3.7 (red), Eqn 3.8 (green), Eqn 3.9 (blue) and Eqn 3.10 (magenta).

For 100mM, there were no underpredictions by any models. Overpredictions of no growth observations at [U] of 6.76mM occurred at 20°C for all models and all had $P >$ 0.75. Some no growth observations at [U] of 8.36mM, at temperatures 20, 25, 30 and 37°C were overpredicted by Eqns 3.8, 3.9 and 3.10. However, none had $P > 0.75$.

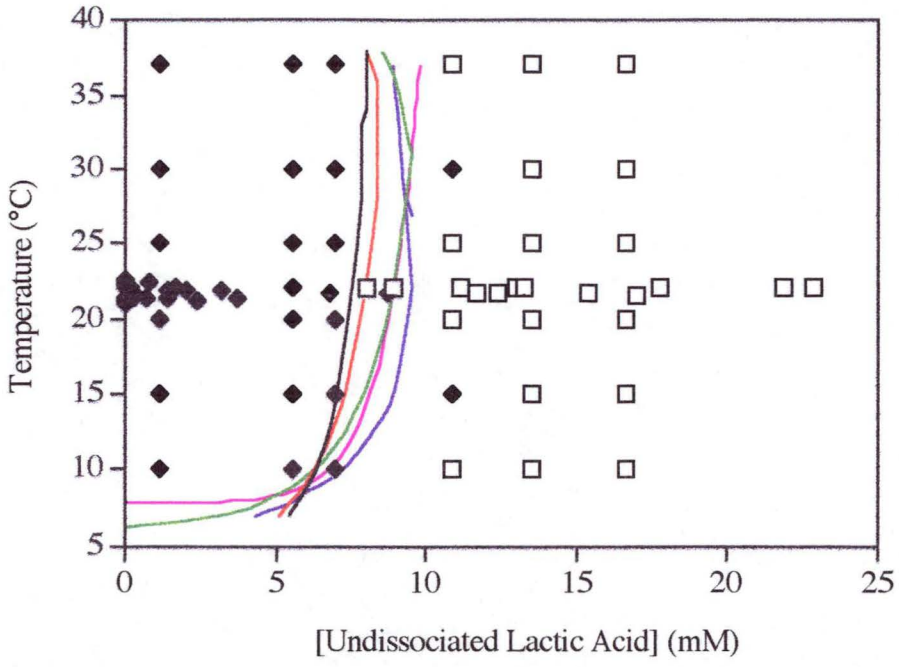


Figure 3.10d) - Data from Experiment 1 at a water activity of 0.994 with 200mM total lactic acid present. Open squares indicate no growth was observed and closed diamonds indicate growth was observed. Model predictions at p=0.5 are indicated by the solid lines: Eqn 3.6 (black), Eqn 3.7 (red), Eqn 3.8 (green), Eqn 3.9 (blue) and Eqn 3.10 (magenta).

For 200mM, all models underpredicted the observations of growth at [U] of 7.01mM at 10°C with Eqn 3.8, 3.9 and 3.10 underpredicting with $P > 0.25$. At this same concentration of undissociated acid, Eqns 3.6 and 3.7 underpredicted the observations of growth at 15°C but all these had $P > 0.25$. Also all models underpredicted the observations of growth at [U] of 10.88mM at 15 and 30°C with all models underpredicting with $P < 0.25$. Eqns 3.6, 3.7, 3.8 and 3.9 underpredicted at [U] of 8.74 at 21.7°C, with Eqns 3.8 and 3.9 predicting $P > 0.25$.

Overpredictions of no growth observations at [U] of 8.00mM occurred at 22°C for Eqns 3.8, 3.9 and 3.10 and of these only the predictions of Eqn 3.10 had $P > 0.75$. Eqn 3.10 also overpredicted growth where no growth was observed at 22°C and [U] of 8.94mM but this prediction had $P < 0.75$.

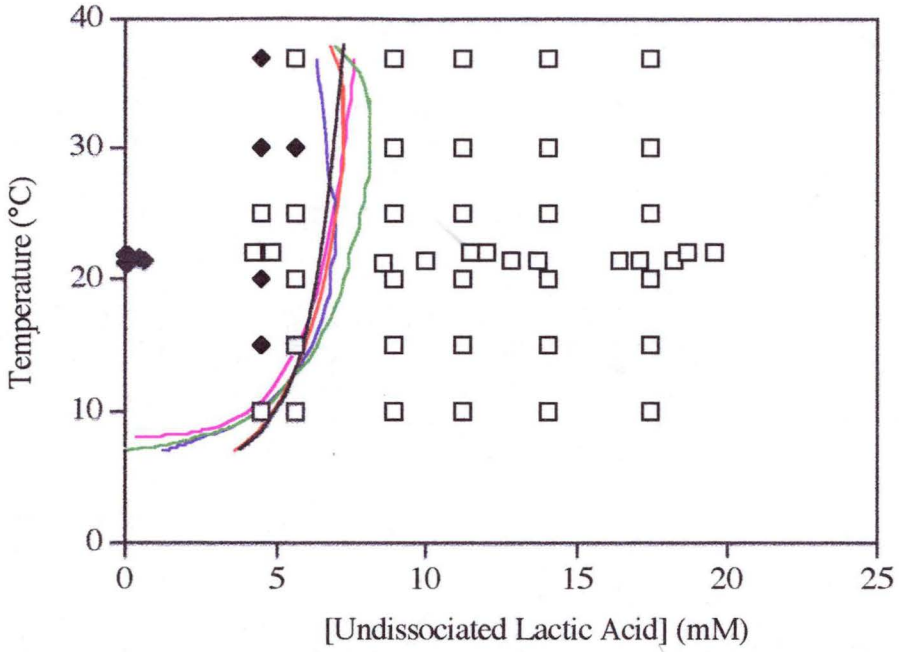


Figure 3.10e) - Data from Experiment 1 at a water activity of 0.986 with 500mM total lactic acid present. Open squares indicate no growth was observed and closed diamonds indicate growth was observed. Model predictions at p=0.5 are indicated by the solid lines: Eqn 3.6 (black), Eqn 3.7 (red), Eqn 3.8 (green), Eqn 3.9 (blue) and Eqn 3.10 (magenta).

For 500mM, all models underpredicted the observations of growth at [U] of 4.52mM at 15°C with all models underpredicting with $P > 0.25$. At [U] of 5.68mM at 30°C, Eqns 3.9 and 3.10 underpredicted the observations of growth but these also had $P > 0.25$.

Overpredictions of no growth observations occurred at combinations of [U] of 4.84mM and 22°C, [U] of 4.52mM and 25°C and [U] of 4.22mM and 22°C for all models. Eqns 3.6, 3.8 and 3.9 also overpredicted growth where no growth was observed at 37°C and [U] of 5.68mM but all these predictions had $P < 0.75$.

The variation in the observed response seems to increase with increasing lactic acid concentration. For 200mM growth was observed at undissociated acid concentrations greater than 10mM for 15 and 30°C. In contrast, for 500mM the observations of growth were restricted to lower undissociated acid concentrations.

3.4.4 Comparison of rate of change of predicted probability

The following figures show the differences between the predictions of the five models at two example pH levels with no lactic acid present as the level of the predicted probability changes from 0.25 to 0.75. Unlike the previous figures which showed only the position of the interface at $P = 0.5$, these figures show the rate of change predicted of boundary conditions of temperature and water activity as these factors become increasingly stringent. For all models there is an increase in the spread of the lines of probability at pH 4 in comparison to pH 7 (Figure 3.11-3.15). However, this effect is most extreme in Eqn 3.10 and is least seen in Eqn 3.8. For Eqn 3.10 at pH 4 there is an upwards curve at the highest temperatures showing that the addition of the cross product and squared terms for temperature give an equivalent shape to those models with a T_{max} term (Eqn 3.7 and Eqn 3.8).

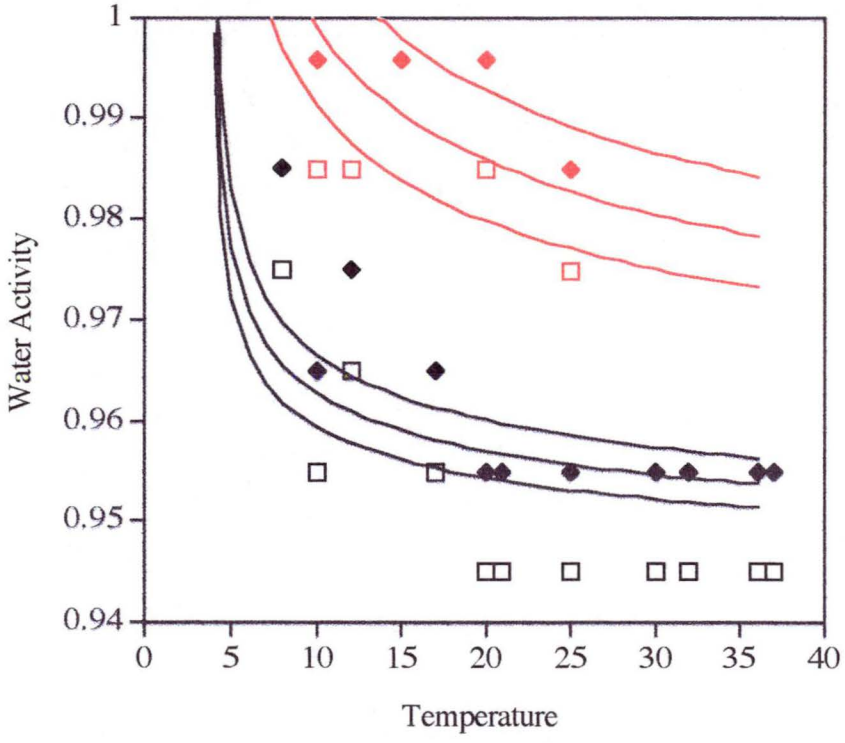


Figure 3.11 - Predictions for Eqn 3.6 for water activity and temperature at pH 7 (black) and pH 4 (red) at P = 0.25, 0.5 and 0.75 (lowest to highest line) in comparison with growth (solid diamonds) and no growth observations (open squares).

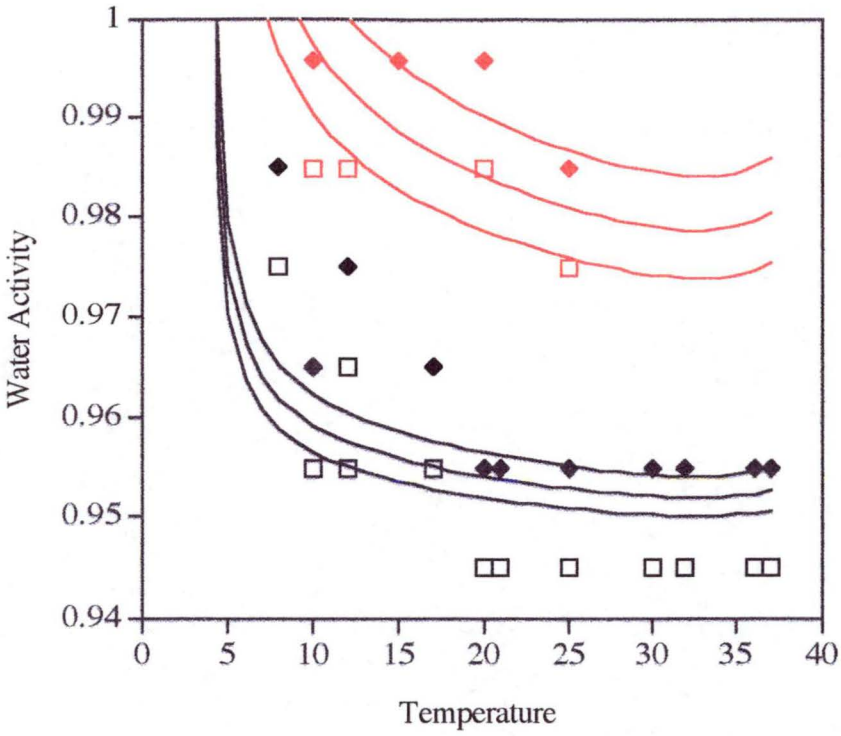


Figure 3.12 - Predictions for Eqn 3.7 for water activity and temperature at pH 7 (black) and pH 4 (red) at P = 0.25, 0.5 and 0.75 (lowest to highest line) in comparison with growth (solid diamonds) and no growth observations (open squares).

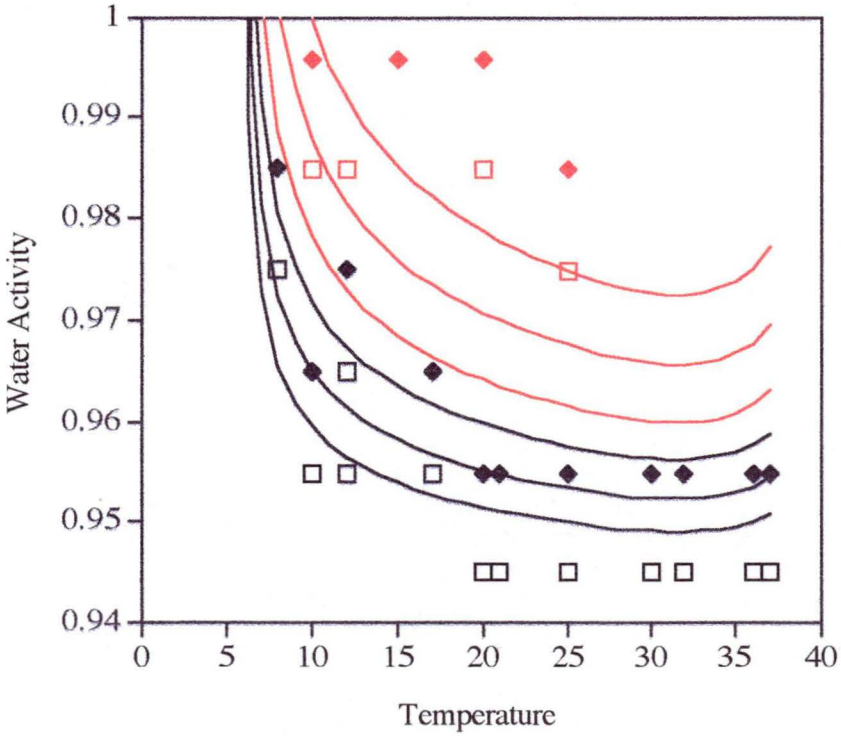


Figure 3.13 - Predictions for Eqn 3.8 for water activity and temperature at pH 7 (black) and pH 4 (red) at P = 0.25, 0.5 and 0.75 (lowest to highest line) in comparison with growth (solid diamonds) and no growth

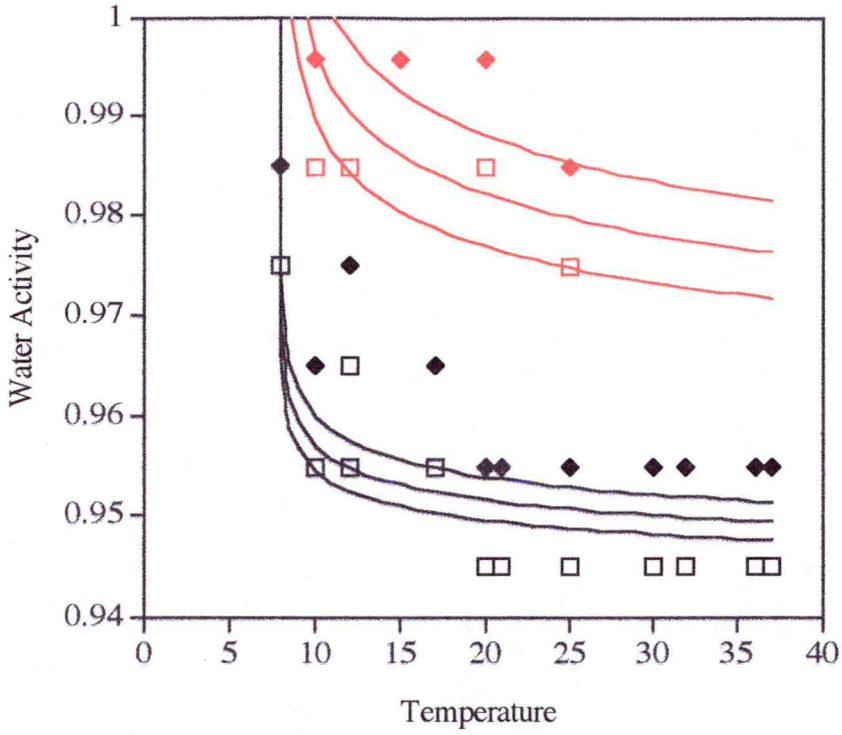


Figure 3.14 - Predictions for Eqn 3.9 for water activity and temperature at pH 7 (black) and pH 4 (red) at P = 0.25, 0.5 and 0.75 (lowest to highest line) in comparison with growth (solid diamonds) and no growth observations (open squares).

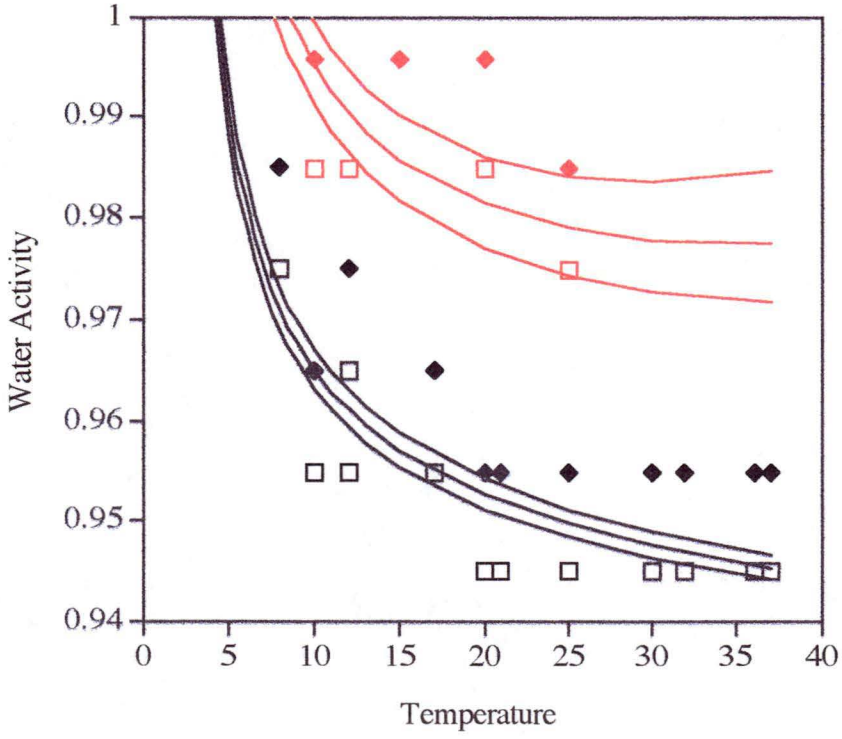


Figure 3.15 - Predictions for Eqn 3.10 for water activity and temperature at pH 7 (black) and pH 4 (red) at P = 0.25, 0.5 and 0.75 (lowest to highest line) in comparison with growth (solid diamonds) and no growth

3.5 Growth/No Growth Discussion

The fitting of the growth rate model using growth rate data and the reasons for the development of novel terms for pH and lactic acid inhibition are described in Chapter Two. Growth/no growth models (Eqn 3.6 - 3.10) were created using the growth rate models such as (Eqn 2.4 and 2.6) and they successfully described the inhibition of *E. coli* due to pH, organic acid and water activity over a range of suboptimal temperatures. This type of microbial growth limits modelling began with the work of Ratkowsky and Ross (1995). They used published growth rate data to demonstrate the use of a logistic regression model for defining the growth/no growth interface at specified probabilities. Subsequently, experiments were designed specifically to determine the growth/no growth response and fitting models to these data confirmed the method also worked for data not limited by time. These data were at narrow intervals of the independent variables, e.g. pH or temperature, especially near the predicted growth/no growth interface. This type of modelling can make further use of existing data such as observations of no growth which otherwise are not used in growth rate modelling. The mathematical expression used to model the growth/no growth interface has undergone significant development as described in this chapter. However, further developments are necessary to optimise the effectiveness of this type of modelling. Nevertheless, growth limits modelling is an promising new way of tackling the problems of food safety and spoilage (Presser, 1999; McMeekin *et al.*, 2000).

A growth/no growth model can be used in two ways. In the first and simplest case the model can be used to predict a probability of growth at a specific set of environmental conditions. For example, pH = 5, a_W = 0.975 and T = 20°C are entered into the fitted equation and logit (*P*) calculated (see Appendices 3.1 and 3.2 for tables of calculated predictions). Secondly, the model equation can be solved for a particular probability of growth. For *E. coli* models which described only the effect of temperature and water activity, the value of water activity that gave a particular probability at a given temperature was easily calculated. For the more complex *E. coli* models described in this study, in order to solve the equation for a specified probability, some parameters must be held constant. By solving the equation for values over a range of one parameter such as temperature, the values of another parameter, for example pH, that give a probability of growth of 0.5 can be determined at a constant water activity and concentration of lactic acid. All the combinations of variables that give this probability, over the range modelled for each parameter, can be calculated. However, each additional parameter multiplies the number of combinations. In order to plot the growth/no growth interface at a particular probability the number of parameters varying must be restricted to a maximum of 3 for a 3 dimensional plot or 2 for a normal 2 dimensional plot. When $P = 0.5$ is used logit (*P*) equals 0 however the model equations can be solved for any probability between 0 and 1.

3.5.1 Overall Results

The predicted interfaces between conditions allowing growth and conditions where growth did not occur was abrupt where growth was limited by pH and organic acid inhibition. The predicted inhibitory effect of combinations of low water activity and pH was more variable with temperature than the response to pH and organic acid inhibition. That is, the inhibition of increasing concentrations of organic acid changed only the position of the pH of the predicted boundary to higher values and the position of the boundary was not affected by the temperature range of the data (10-37°C). Only at temperatures close to the minimum does the pH of the interface increase steeply (Figures 3.1b- 3.5b). For those models with a T_{max} term (Eqn 3.7 and 3.8) a similar effect was predicted at high temperatures (Figures 3.2b and 3.3b).

In contrast to lactic acid inhibition, lowering water activity changed the predicted interfaces to a higher pH at optimal temperatures and also the predicted interfaces were at higher temperatures over the optimal pH range to different degrees depending on the model (Figures 3.1a- 3.5a). The predicted high temperature effect was similar to low temperature inhibition although the response was not symmetrical. There was less change in the interface at high temperature, for example in Fig 3.3a, at the lowest water activity the highest temperature decreases from 39 to 37°C but the lowest temperature increases from 11 to 21°C. The growth/no growth interface given by water activity inhibition showed a wider range of pH for the transition from conditions where growth was observed to those where growth was not observed. The total dataset of 871 points did not contain any data where the growth was limited only by either suboptimal or superoptimal temperature. However, the water activity data showed a clear restriction of the growth region to higher temperatures at the lowest water activities in comparison with the highest lactic acid data which showed no restriction.

The response to increasing stringency of conditions, lower water activity or higher organic acid concentration, was similar for all models. There were only small changes in the growth/no growth interface boundary at slightly inhibitory conditions and the degree of change of the boundary conditions increased as the inhibition increased to near limiting conditions. This can be seen in Figures 3.1b - 3.5b. In the presence of lactic acid, as the lactic acid concentration doubles from 25 to 50 to 100 then 200mM, the distance between the pH increases of the interfaces remains similar especially for Eqns 3.6, 3.7 and 3.8. The position of the pH interface is also very similar for all models for all total lactic acid concentrations. This case is more complex because the pH scale is logarithmic. However, the data show (Figure 3.10) that for all total concentrations of lactic acid tested in these experiments, the undissociated concentration at which growth ceases is similar, having values between 5 and 12mM.

Apart from these similarities there were many differences in the predicted shape and position of the interface by the different models especially at low water activity. The main difference in the response predicted by the models for increasing water activity inhibition, compared to lactic acid, is an increase in the predicted minimum temperature of the growth/no growth interface at the most inhibitory condition of water activity 0.955. For all models the temperature of the growth/no growth interface at 0.955 increased as pH decreases but each to a different degree and each interface has a different shape (see Figure 3.9e). The two types of shape for the water activity interface are a constant minimum temperature at a range of optimal pH (Eqns 3.6 & 3.8) and a continuous curve to lower temperatures as pHs lowers (Eqns 3.7, 3.9 and 3.10). The unusual shape given by Eqn 3.7 (Figure 3.2a) is due to the presence of the "stimulatory" high pH term, i.e. the large negative coefficient for this term. That is, the growth region expands as pH gets higher. There is a correlation between the modelled a_{Wmin} and the restriction of the boundary at 0.955. Eqn 3.6 with the lower a_{Wmin} has a minimum temperature at 27°C compared to 21°C for Eqn 3.8. Similarly comparing the temperature of the boundary at pH 7, Eqn 3.9 has a temperature of 17°C compared with 12°C for Eqn 3.10.

The difficulty in modelling the shape of the observed interface at water activity 0.955 can be seen especially for Eqn 3.3 by the large number of underpredictions in the boundary region. However, most of the underpredictions were for data that were not included in the creation of Eqns 3.3 or 3.4. Even for Eqn 3.6 there are overpredictions in the boundary region at 17°C and the highest pH values as well as 30°C at the lowest pH.

The trends in the change in the predicted probability of growth at any fixed value of water activity or lactic acid, for the range of temperature and pH, were similar for all models except for Eqn 3.9 and 3.10, extending to P=1.0 even at a water activity of 0.955 (Figures 3.6 - 3.8). As the stringency of the fixed condition increased, the steepness of the change in probability decreased from the very steep "loaf" shape of optimal conditions to a gradual slope under limiting conditions. The lack of data with intermediate proportions of growth not exactly equal to one or zero does not allow assessment of whether these predictions for the changing probabilities is valid. More data with observed proportions of growth intermediate between all growth or all no growth are needed to test this prediction.

The data in Experiment 2 did have the confirmation tests for *E. coli* growth that were performed in Experiment 1. It was found that *E. coli* growth range was more limited in Experiment 2. For example (Figure 3.9d) where growth was not observed at $T = 12$°C $5.4 < \text{pH} < 7$ in comparison to Experiment 1 were growth was observed at $T = 10$°C. This makes it unlikely that there was any undetected contamination that caused false observations of *E. coli* growth.

There was an improvement in the goodness of fit as the modelling evolved at each step of the process. However, as different datasets were used, some types of comparisons are not possible. While Eqn 3.6 showed a good fit to the data each of the other models resulted in an improvement in at least one of the goodness of fit criteria compared to Eqn 3.6. For the two pairs of models created with the same datasets the ROC (c) characteristic improved from 0.973 (Eqn 3.6) to 0.979 (Eqn 3.7) and from 0.970 (Eqn 3.9) to 0.977 (Eqn 3.10). Similarly the maximum rescaled R^2 improved from 0.798 (Eqn 3.6) to 0.825 (Eqn 3.7) and from 0.804 (Eqn 3.9) to 0.828 (Eqn 3.10).

Predictions of Eqn 3.6 for the growth/no growth interface agreed with 90% of the total 871 experimental dataset where growth was observed if ≥ 50% of replicates recorded growth and where a model prediction of $P \geq 0.5$ was defined as growth. This increased to 94% where a prediction of $P < 0.25$ or $P > 0.75$ was used to define a poor prediction. Eqn 3.7 showed an improvement in agreement for the whole 871 dataset with 91% using 0.5 criterion and 95% if the $P < 0.25$ or $P > 0.75$ criterion was used. Eqn 3.8 showed a higher number of overpredictions (6%) than underpredictions (2%) using the 0.5 criterion. However, it still showed an improvement on the predictions of Eqn 3.6 and 3.7 with agreement with 92% (0.5 criterion) and 97% ($P < 0.25$ or $P > 0.75$ criterion).

Using the entire 871 datapoints, Eqns 3.6, 3.9 and 3.10 did not predict as well as Eqn 3.8. However, if the 14 no growth observations at high pH which Eqns 3.6, 3.9 and 3.10 all overpredicted at $P = 1$ are removed, Eqn 3.9 showed an improved agreement compared to Eqn 3.8 with 93% ($P = 0.5$ criterion) and 97% ($P < 0.25$ or $P > 0.75$ criterion). Eqn 3.10 showed a further improvement with 94% (0.5 criterion) but a similar agreement at the other level 97% ($P < 0.25$ or $P > 0.75$ criterion). However, Eqn 3.6 still has a worse agreement than Eqn 3.8 for the 857 remaining datapoints with 91% ($P = 0.5$ criterion) and 95% ($P < 0.25$ or $P > 0.75$ criterion).

When only the data used to create the model was used to evaluate the performances of the models (Table 3.6), similar rates of agreements were observed. This suggests that the total dataset was not significantly different to the dataset used to create the model and there were no dataset specific differences between the models.

3.5.2 Model Evolution

The development of growth/no growth modelling has been a cumulative process of ideas and hypothesis testing leading to new guidelines for the methods to model these data correctly . One important discovery in the development of the models was that the coefficient preceding the single model term should not be allowed to be negative. A negative coefficient for any single model term predicts a stimulatory effect for that parameter instead of an inhibitory effect. For quadratic and cross product terms the coefficient can be negative or positive. In all the models described here the terms model the relative reduction of a maximum rate due to the inhibition of that independent variable. Insufficient data have led to the fitting of negative coefficients for model terms in a few cases. In two cases in the work on *E. coli*, this occurred firstly for water activity (Presser, 1995) and secondly for high pH (Eqn 3.7). The first case was solved by the generation of new data over a range of low pH and water activity to create a model better able to describe water activity inhibition (Eqn 3.6). A good fit of the model for the high pH term was also achieved when high pH data was added and the model constrained to fit a positive coefficient for this term (Eqn 3.8). However, there was an insufficient range of data available to warrant the inclusion of the high pH term in any later models (Eqn 3.9 - 3.10).

A study on the inhibition of *Listeria monocytogenes* has also given rise to a model with negative coefficients for high pH (Tienungoon, 1998). Similarly there was either very little or no data at inhibitory high pH and no combinations of high pH with other suboptimal conditions. The fitting of a large negative coefficient for these high pH terms could mean that the high pH term was being used in the modelling process to fit inhibition by low pH better. That is, modelling a "stimulatory" effect of high pH will change the inhibitory effect modelled at low pH. In order to achieve a better fit for low pH inhibition a slope coefficient for the pH term, Q, that was developed in Chapter 2, was added to the growth/no growth interface model (Eqn 3.9). The addition of the Q factor was found to improve the model performance significantly (data not shown) and was included in subsequent models (Eqns 3.10).

Advances in logistic modelling techniques and software have allowed estimation of all coefficients and parameters in the modelling process (Presser *et al.*, 1999). This specialised nonlinear logistic regression modelling is not generally used in statistics or widely reported in the literature. However, use of this method of modelling allows nonlinear parameters such as T_{min} and $a_{W_{min}}$ to be estimated from the growth/no growth data along with the linear b coefficients in the same fitting process. For a term to be fitted correctly for any model, data must be collected over the whole range for that term with as many combinations of other conditions as possible. Only when sufficient data are collected and included in the modelling process will models be able to predict microbial responses correctly.

Some parameters could not be estimated by the model because of a lack of data, for example, T_{max} could not be estimated due to the absence of high temperature data. The data were collected for a range of temperatures nearly up to the optimum growth rate temperature for *E. coli* of 40°C. However, even at 37°C there were indications of high temperature inhibition occurring in the water activity and temperature data (Salter *et al.*, 2000). This led to the necessity to add a high temperature term, without the data available to reliably estimate T_{max}. Therefore the T_{max} term used in this case and other growth/no growth models is based on the estimates of T_{max} and the slope coefficient, d, from growth rate modelling.

Growth rate increases as temperature increases up to a point (the optimal temperature for growth rate) beyond which it decreases very rapidly (Figure 3.16). Thus the temperature at which the fastest growth rate occurs is near the upper end of the temperature range. For growth rate models the T_{max} term and its slope coefficient describes the rapid growth rate decline at temperatures greater than optimum. However, there is no reason to expect that the factors that allow the very fast growth of an organism also give the same benefit to the organism to grow under stress. It is possible that a plot of the growth/no growth interface may be more symmetrical for temperature, so that the optimal survival temperature is closer to the midrange. The effect of changing an environmental condition on survival of bacteria will not necessarily be the same as the effect of the same change on growth rate. Currently the growth/no growth interface (all models) is predicted to have a very sharp increase in the limiting water activity as temperature increases above 37°C. This is contrary to experience which reported that *E. coli*, strain R31, is able to grow at temperatures up to 45°C (Salter, 1998). This anomaly could be the result of having to fix the constant within the T_{max} term that gives the slope of the response to high temperatures at the value given by the growth rate modelling. More data are needed to resolve which type of T_{max} term or which value of the slope constant best describes the growth/no growth response at higher temperatures. Similar considerations apply to the high pH region where there is also a lack of experimental data.

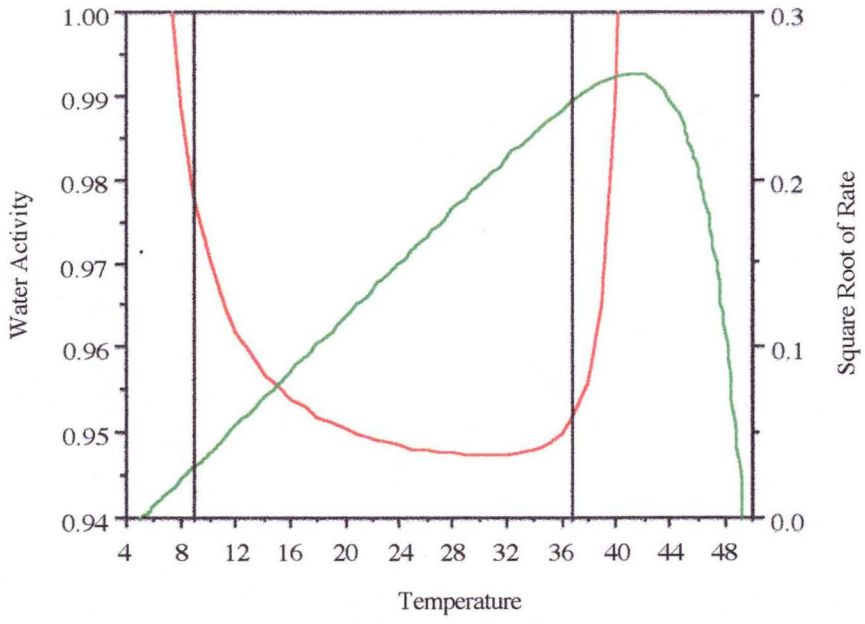


Figure 3.16 - Predicted responses to temperature for *E. coli* strain R31 (Salter *et al.*, 1998) of a growth rate model (green line - right hand Y axis) and a growth/no growth water activity interface P=0.5 (red line - left hand Y axis). Black lines indicate the range of temperature at which growth was observed in the growth/no growth dataset.

During the model fitting process it was observed that for some growth regulatory factors, e.g. water activity and low pH, the estimates would tend towards more and more inhibitory conditions until they were restricted to a "bound" minimum value entered into the modelling program. In some cases the relaxation of the "bound" value would not resolve this problem as the model estimate would again converge to the new lowest possible "bound" value. For other factors, e.g. dissociated lactic acid concentration, the estimates tended towards less inhibitory conditions and would hit "bound" values given by the data. For example, observations of growth at 499mM dissociated lactic acid. There is a need to develop an understanding of what are realistic boundary values for growth/no growth modelling. For example, the "bound" value for T_{min} was 6°C when Eqn 3.8 was fitted and the estimate "hit" this upper bound. Subsequently the "bound" for T_{min} was increased to above 7.8°C, the minimum temperature at which *E. coli* growth was reported (Shaw *et al.*, 1971). In this study growth was not observed at 8°C although this could be in part because another condition, water activity, was not optimal. The practical limit for the observation of *E. coli* growth is given as 8°C (ICMSF, 1996). However observations of *E. coli* growth have been reported at 5 and 6°C (Kauppi *et al.*, 1997; Kauppi, 1998). The upper "bound" at 7.9°C allowed estimation of T_{min} for Eqn 3.9 above the previous bound at 6°C. By restricting the estimate to below 6°C an estimate was not able to be fitted for Eqn 3.9.

Other studies (Tienungoon *et al.*, 2000; Salter *et al.*, 2000) have also found that the parameter estimates given by nonlinear logistic regression modelling can converge to much more extreme values than those estimated for analogous growth rate models.

Such observations might lead to the reconsideration of the appropriateness of growth rate terms for growth limits modelling. If the values are not the same as for growth rate modelling, then the model parameters should not be given the same names or perhaps not even the same terms. The use of a growth rate model form for growth limits modelling implies the same mechanisms that control growth rate also define the limits to growth. This may be the case for some factors but not others. Further studies are needed to resolve this.

It is important to monitor the trends in the parameter estimates to check that there are no fundamental problems in the model fitting process. A trend in parameter estimates might also highlight an incompatibility between the shape of the modelled response and the shape implicit in the model term used to fit that response. For terms such as pH and high temperature, coefficients within the model terms, such as the Q factor, are used to give a change in slope for kinetic models. Similarly in growth/no growth models different terms with such extra coefficients may be needed so the most accurate and precise descriptions of the growth/no growth response can be obtained.

The effect of the collection of data over the narrowest measurable intervals of conditions can be seen in Figure 3.9. There is a distinct boundary between the growth data and the no growth data even though they are not separated by any large differences in the conditions at which the data were collected. However, the dataset contains some anomalous points that do not fit the general trend from growth to no growth as conditions become more stringent (Figure 3.10d). In most cases, it is an arbitrary decision as to which datum of a growth or no growth pair is anomalous. For example at water activity 0.965 at temperatures between 8 and 15°C and pH > 5.5 (Figure 3.9d) there is a pattern of no growth, growth then no growth observations again as temperature changes. However, in other cases data are more clearly inconsistent with the boundary described by all the other data, for example, the growth observed at temperatures of 20 and 30°C at pH 3.6 at water activity 0.996 with no lactic acid present (Figure 3.9a). Fitting the model to the data is more difficult when anomalous points exist. A sequence of datapoints contains a "growth" observation between two "no growth" observations as one variable is changed (e.g. Figure 3.10d at LAC 200mM at a temperature of approximately 22°C between undissociated lactic acid concentrations of 8 and 9mM) is impossible to model correctly without compromise and results in at least one of the data points being on the wrong side of the predicted growth/no growth interface. These anomalous points should not be assumed to be due to experimental error but could indicate the need to collect more replicates of the data in those regions to elucidate the real variability in growth at those conditions.

The use of few replicates increases the likelihood of obtaining anomalous data points. By analogy, by sampling only one family it is possible to observe that all 4 children are male or female, despite that the odds of any one person being born male or female within the human population are approximately 50:50. If more replicates are available (for example sampling a whole school with a hundred children) the observation is more likely to reflect the real probability of a child being born male or female. A lack of resolution leads to a relatively large measurement error. Four replicates only allows for the resolution of five 'shades of grey' or observed proportions of growth to no growth in the data, that is 0/4, 1/4, 2/4, 3/4 and 4/4. Ten replicates allows for the resolution of eleven different observed proportions of growth to no growth in the data 0/10, 1/10, 2/10, 3/10, 4/10, 5/10, 6/10, 7/10, 8/10, 9/10 and 10/10. More replicates would allow a clearer picture of the real probabilities and would decrease the relative weight of any anomalous datapoint when estimating parameters which, even if non influential, would make the model appear to predict incorrectly. Increasing the number of replicates from that used in the current growth/no growth experiments to a larger number will also to lead to a better elucidation of the real shape of the interface. This should also indicate whether the probability transitions are sharp and distinct, as appears to be the case for the growth/no growth interface where pH is the limiting constraint, or more gradual as appears to be the case of the growth/no growth interface for pH at low water activities. A greater number of replicates will increases the number of 'shades of grey' observable between the probability of growth being 1 (white) and the probability of growth being 0 (black).

For some conditions the collection of more data may be problematical. There is a practical limit to the use of greater total lactic acid concentrations in this type of experiment. After the addition of high concentrations of acid the pH of the broth is very low (e.g. 2.2 for 500mM) and must be increased by several pH units back into the growth permissive region (e.g. pH > 5.6 for 500mM). This extra addition of NaOH or other alkalising agent also further lowers the water activity of the final broth. Additionally, the collection of further data at the very narrow range of conditions near the pH interface could be limited by the ability to measure the pH accurately and reproducibly. Large numbers of replicates at the boundary may be more difficult because the interface is apparently abrupt relative to the precision with which pH conditions can be measured. However, in most cases, where the boundary is more gradual, replicates over a narrow range near the interface should be possible.

During some of the modelling, data were excluded in a systematic way from the dataset used to create the model. There were several valid reasons for this. The first is that the data far away from the boundary contributes little to defining the position of the boundary. However, it was difficult to find an arbitrary level to use when deciding which data could be removed. Another subset of data that was removed in some modelling attempts were those points outside the fitted values, that is, below the minimum pH and above the critical undissociated acid concentration. For other terms no data were available below their minima, such as T_{min} or a_{Wmin}. That is, inhibition occurred due to a combination of limiting factors of temperature, water activity and other conditions as opposed to the cases of pH and undissociated acid where they each were the sole factor limiting growth. This is due to the mathematical impossibility of calculating the logarithm of a negative number which would result when the experimental conditions are below any minimum or above any maximum value. When the minima or maxima are set so that there are experimental conditions in the dataset that are outside them, the model will still fit the data using SAS PROC NLIN (SAS Institute Inc., 1989) but errors and delays will be encountered in the fitting process. Alternatively, data can be removed. However, this gives the same problems as defining arbitrary limits on the data used, and in the worst case would result in the removal of most of the no growth data for particular combinations of conditions. Another strategy recently employed is to set the minima outside the boundaries of the data. This results in a good model fit but also has the problem that these model estimates are completely dependent on the range of the data used to create them.

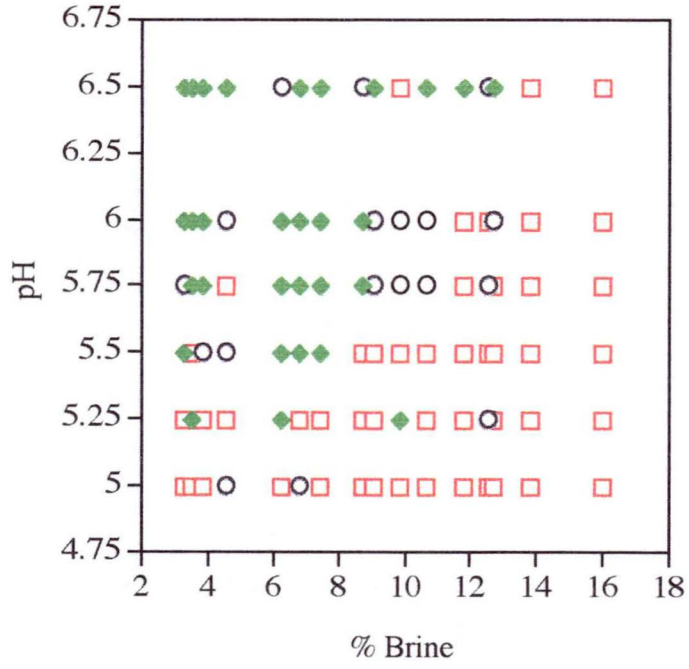
3.5.3 Comparison to other growth/no growth models

Similar growth limits models have been developed for a pathogenic strain of *Escherichia coli* (R31) inhibited by suboptimal temperature and water activity (Salter *et al.*, 2000). This was the fastest growing of a range of pathogenic strains modelled for growth rate and was selected as a representative for growth/no growth modelling (Salter, 1998). Growth/no growth models were also developed for two strains of *Listeria monocytogenes* (Scott A and L5) for the effects of suboptimal temperature, water activity, pH and lactic acid (Tienungoon, 1998). In those two studies several growth/no growth models were created following a similar evolutionary process to that undertaken for the models in this thesis. Those models show a similar response to the models described here with some notable exceptions. For example, the addition of a dissociated acid term was found not to be necessary to fit the model. Those *Listeria* models were also found to benefit from the addition of new terms. However, the new terms were squared terms for temperature and for pH (Tienungoon *et al.*, 2000). Those models had the following goodness-of-fit measures: ROC (*c*) characteristics of 0.976 (Scott A) and 0.991 (L5); maximum rescaled R^2s of 0.832 (Scott A) to 0.908 (L5) with the model for strain L5 also showing a better Hosmer Lemeshow goodness of fit measure than the model for strain Scott A.

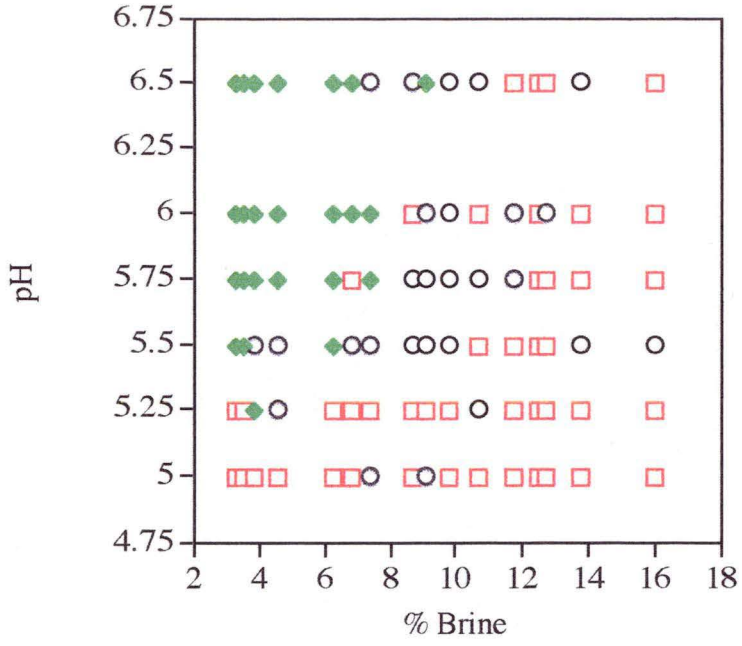
Different types of growth/no growth models for other organisms have also been published. Organisms studied include the spoilage yeast *Zygosaccharomyces bailii* (Jenkins, 2000; Lopez-Malo and Palou, 2000), *Saccharomyces cerevisae* (Lopez-Malo *et al.*, 2000) and *Listeria monocytogenes* (Bolton and Frank, 1999). These authors modelled inhibition by suboptimal water activity, pH, preservatives, organic acid constraints and temperature. Published models also differ from those described in this thesis in that polynomial models and time limited data are used, creating different models for different datasets at different incubation times (Bolton & Frank, 1999; Lopez-Malo *et al.*, 2000) or modelling time to growth instead of probability of growth (Jenkins *et al.*, 2000).

These published data are also limited by the number of replicates, for example Lopez-Malo *et al.* (2000) and Bolton and Frank (1999) have only 3 replicates. The number of replicates of data at the interface needed to fully elucidate the shape of the changing predicted probabilities across the growth/no growth interface could be as many as 100 depending on the degree of accuracy required. Model predictions of Bolton and Frank (1999) for probability of growth, survival and death are given up to three significant figures. An example of the variability shown in the three replicates at each condition is given in Figure 3.17 below where each part (a, b, c) shows a replicate for each condition.

a)



b)



c)

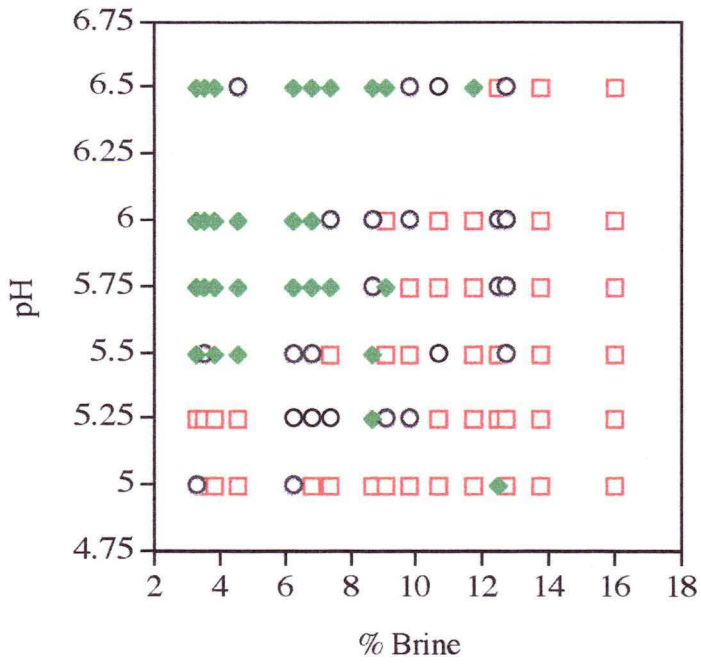


Figure 3.17 a) b) and c) are replicate observations of growth (green diamonds), survival (black circles) and death (red squares) of *Listeria monocytogenes* in Mexican style cheese after 42 days of incubation at 10°C adapted from Bolton and Frank (1999). Growth and death were defined as a greater than 0.5 log increase or decrease in bacterial numbers respectively and survival was defined as less than 0.5 log change in bacterial numbers.

While there is a consistent pattern of growth in the upper left hand corner and death in the lower right, there are many observations that are inconsistent with this general trend. For example, growth was observed at pH 5 and 12.5% Brine (Fig 3.17c) and death occurred at pH 5.75 and 7.41% Brine (Fig 3.17b). This gives an uncertain result for the growth/no growth interface even when all three replicates are put together (Figure 3.18). A much larger number of replicates would be needed to create models that could predict the observed responses to the degree of accuracy proposed by Bolton and Frank (1999). These data need to be on or close to the interface where growth occurs at $P < 100\%$, but $> 0\%$. The modelling process was developed to fit graduated data, that is observed proportions of growth at 40%, 70% etc., rather than binary all growth or all no growth data. Binary data contain less information and are less useful in creating models (Jenkins *et al.*, 2000).

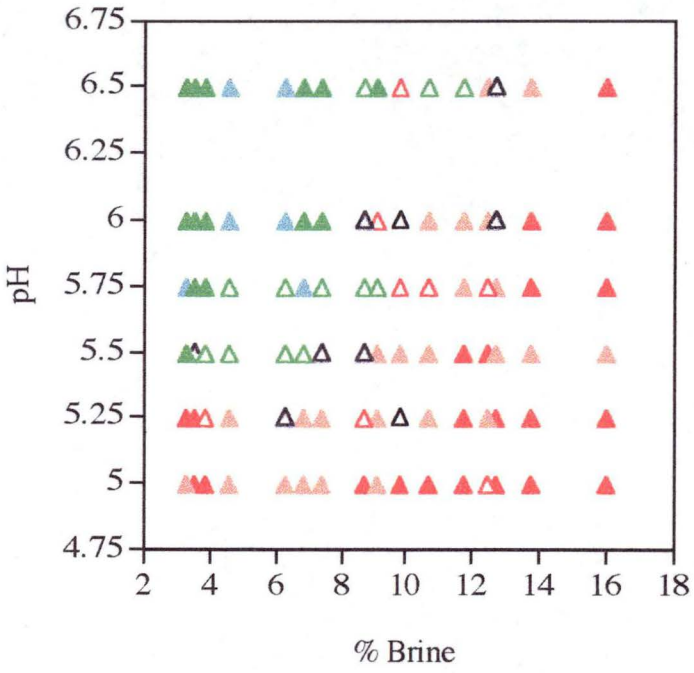


Figure 3.18 - Combined observations of three replicates of *Listeria monocytogenes* in Mexican style cheese after 42 days of incubation at 10°C adapted from Bolton and Frank (1999). All 10 possible combinations of the 3 replicates were reduced to seven categories from growth to death. These categories plotted were: 3/3 were growth (solid green); 2/3 were growth & 1/3 was survival (solid blue); 1/3 were growth & 2/3 were survival or 2/3 were growth & 1/3 was death (open green); 3/3 survival or 1/3 each of survival, growth and death (black); 1/3 was death & 2/3 were survival or 2/3 were death & 1/3 was growth (open red); 2/3 were death & 1/3 was survival (solid orange); and 3/3 were death (solid red). Growth and death were defined as a greater than 0.5 log increase or decrease in bacterial numbers respectively and survival was defined as less than 0.5 log change in bacterial numbers.

Bolton and Frank (1999) used a real food system (Mexican style cheese) rather than laboratory broth to create their growth/no growth models which they claim should produce a more realistic model, although the model produced is still "fail safe" in comparison to other literature data. Lopez-Malo and Palou (2000) also used a real food (mango puree) to create their model. This process, while needed for validation, can limit the ability to replicate the data to the required level. There could also be uncontrollable variability inherent in the food itself, for example, the ripeness or variety of the mangos obtained to make the puree (Lopez-Malo & Palou, 2000). Also at a fundamental level this requires a new rigorous study of this type to be carried out to develop a model for every different type of food. This is more time consuming and costly than creating a laboratory broth based model and then testing its range of applicability and validating the model in a variety of foods.

Extrapolation from knowledge of the effect of an environmental condition on growth rate to its effect on survival can lead to incorrect conclusions. For example, lower temperatures slow down growth rate but lead to an increase in survival times. This has implications for the use of growth/no growth data in multiple hurdle systems for stopping bacterial growth (McMeekin *et al.*, 2000). Decreasing temperature increases safety because it slows growth rate, but if the conditions are on the other side of the growth/no growth interface, decreasing temperature will slow the death rate. Microorganisms are more resistant, that is die more slowly, when exposed to a lethal stress, such as pH, when held at lower temperatures (Zhao and Doyle, 1994). However, despite this evidence, some investigators still draw unsubstantiated conclusions by extrapolating the results obtained for conditions within the growth range to conditions outside the growth range. For example Tomicka *et al.*, (1997) hypothesized that lower temperatures such as 8°C may accelerate elimination of *E. coli* O157:H7 from fermented sausage and that higher temperatures (30-42°C) promote survival. Other examples include the increase in death rate observed on the addition of compatible solutes to non-growing cultures of *E. coli*, substances which enhance the growth rate and temperature range of growing cultures (Krist, 1998). Conversely the effectiveness of the growth inhibitory substance, nisin, is not observed on the no growth side of the bacterial interface for *Listeria monocytogenes* (D. Miles, *pers. comm.*).

Descriptions of the death rate and growth rate just beyond the interface on each side should be combined with studies of the shape of the interface itself. In practical situations it is very important to determine more information about the effect of changing conditions. For example, when an environmental stress is increased, knowledge indicating whether the microorganisms start to die quickly or are able to survive for a long time without changing from their initial population level is important for controlling spoilage or ensuring the absence of microbial pathogens.

Models have been created which attempt to cross the boundary between growth and death (Peleg, 1995; Baranyi *et al.*, 1996). However, it is problematical with rate models to deal with a region of stasis, i.e. neither growth nor death, at the growth/no growth interface (Baranyi *et al.*, 1996). Stasis has been observed in growth/no growth studies over a wide range of conditions of pH and brine concentrations (Bolton & Frank, 1999). Also a region of very slow growth or death rates was observed for *Klebsiella* at the high temperature growth boundary (Niemela and Oivanen, 1992).

In the boundary between growth and death there is no single correct answer that is reproducible at any given set of conditions and this is reflected by the use of probability modelling. It is possible that the extension of varying probabilities of growth, survival or no growth events beyond the obvious growth/no growth boundary is the cause of unusual irreproducible observations of growth rates and death rates. If this is the case, it suggests the need for an even stronger link between probability and growth rate models, as well as death rate modelling. A complete description of a microbial response at limiting conditions might be given using information from both growth/no growth models and rate models. For example at a set of conditions near the growth/no growth interface the probability of growth is 70% and if growth occurs it will be at a rate of 0.002 (1/generation time(min)). There is also the possibly of modelling the presence or absence of toxin production under a similar range of conditions to growth/no growth modelling in unusual cases where knowledge of the production of toxin is more important than knowledge of the growth of the organism (Nunez, 2000).

Another strategy which will give more information about the interface is the use of smaller inoculum sizes. As well as reflecting most real food contamination, small inocula give better discrimination of the interface. As the inoculum decreases in size, assuming that only a proportion of the population is capable of growing, it is less and less likely that growth will be observed in any given trial. The extreme limit of this approach are studies that attempt to observe the response of a single cell (Stephens *et al.*, 1997; Nebe-von Caron, 1998) instead of the response of a large bacterial population. Unfortunately such studies currently have many practical and technical difficulties such as the expense, equipment and expertise needed and the limits of detection sensitivity. If these are overcome this method may become an integral part of growth limits modelling in the future.

Finally, growth limits modelling should be fundamentally linked with the physiology of the microorganism. While both are separate and important areas of study, bringing the two together results in a synergistic increase in the knowledge gained. Physiological studies are needed to determine the mechanisms of cessation of growth such as the decrease in the normal physiological range of growth (Krist, 1998). In turn, knowledge of microbial responses to environmental conditions may help physiologists identify conditions that cause changes in physiology. An example of this synergy could be in determining whether there are vital cell processes that change in relation to the proximity to the growth/no growth interface. This would be a step along the road to understanding what it is that stops the cells growing and what causes them to die.

4 PHYSIOLOGY OF *ESCHERICHIA COLI* - INTRACELLULAR PH MEASUREMENTS

4. Physiology of *Escherichia coli* - Intracellular pH Measurements

4.1 Summary

A technique of intracellular pH measurement using the fluorescent probe 5 (and 6-) -carboxyfluorescein succinimidyl ester (cFSE) was applied to the determination of the relationship of intracellular pH (pH_i) to conditions of pH and organic acid stress for *E. coli*. The technique, previously developed for Gram-positive bacteria was ineffective for labelling the Gram-negative bacterium, *Escherichia coli*. Adaptation of the technique was required when applied to Gram-negative bacteria to include a treatment to overcome the outer membrane barrier so that the bacterium took up the fluorescent probe. Compounds and techniques used to permeabilise Gram-negative bacteria were trialled. These included addition of EDTA or calcium chloride (i.e. as in preparation of competent cells to enable them to take up DNA) and electroporation of cells. Methods were developed which successfully labelled the cells but following any type of treatment no intracellular pH response was apparent. It was considered that this could be due to permeabilisation of the cells or even attachment of the probe to an exterior part of the cell. Further experimentation is required to resolve these problems. It is concluded that this technique still shows much promise as a simple and effective way of exploring the physiology of pH stress.

4.2 Introduction

It is important to be able to measure physiologically significant parameters such as the intracellular pH to determine how organic acids act to inhibit microbial growth and metabolism.

Many methods of estimating the intracellular pH of microorganisms exist (Kashket, 1985). Many rely on the partitioning of radiolabelled weak organic acids inside and outside the cells to determine the difference between the lower pH outside and higher pH inside the cell (Ita and Hutkins, 1991). Examples of acids used include acetic and acetylsalicylic (Kashket, 1985), benzoic (Kashket *et al.*, 1980), and salicylic acids (Young and Foegeding, 1993). These methods rely on the use of an acid whose pK_a is lower than the exterior pH so that the accumulation of labelled organic acid inside the cell is large enough to infer an accurate estimate of intracellular pH (Kashket, 1985). These methods also rely on the assumptions that only the lipophilic undissociated acid can pass through the membrane (Kashket, 1985) and that the acid is biochemically inert (Padan *et al.*, 1981). That is, the acid will not affect the physiological state of the cell, either by inhibition or by being metabolised by the cell. These basic assumptions have been questioned for acids such as salicylic and acetylsalicylic acid where there is evidence of active transport, for benzoic acid which is a potent inhibitor of bacterial metabolism and for acetic acid which can be used by bacteria such as *E. coli* as a sole carbon source (Booth, 1985). However, it may be that at the low concentrations used to calculate intracellular pH these effects are not significant.

Another method involves measuring the chemical shift in nuclear magnetic resonance, which is a function of pH, of ions such as the ^{31}P phosphate ion (Padan *et al.*, 1981). These studies produce values of intracellular pH which agree with other methods, such as those using weak acids. However, the equipment needed to measure nuclear magnetic resonance is not available in most standard microbiology laboratories. pH electrodes have also been used to measure changes in pH caused by metabolism (Kashket, 1985) however studies using microelectrodes have been limited by the small size of bacterial cells in comparison to plant or animal cells. Recent developments have allowed the measurement of ion flux by creation of a confluent layer of bacterial cells at a surface(Shabala, *pers. comm.*).

Finally there are many different fluorescent molecules, some of which can be used as pH probes. For example 9-aminoacridine has been used to measure pH in vesicles (Kashket, 1985) however its use is limited for intact cells. Fluorescent probes are easy to use and allow rapid, real time measurement of changes in intracellular pH (pH_i) (Breeuwer *et al.*, 1996). However many fluorescent probes which can enter cells easily also leak out of the cell during the course of experiments and create a high level of background signal making pH_i measurement difficult.

This problem can be prevented by the use of more polar fluorescent compounds, such as BCECF (biscarboxyethyl-carboxy-fluorescein) and 8-hydroxy-1,3,6-pyrene-trisulfonic acid (pyranine). However these are much more difficult for bacterial cells to take up because they are negatively charged molecules. Methods such as electroporation and acid shock have been used to aid incorporation. Another strategy is to incorporate fluorescent probes as (non-fluorescent) acetoxymethyl or diacetyl esters. These esters permeate through the membrane and once in the cytoplasm are cleaved by esterases releasing the negatively charged fluorescent form which accumulates inside the cell.

A new intracellular pH probe, 5 (& 6-) carboxyfluorescein succinimidyl ester (cFSE), which accumulates in this way has been described by Breeuwer *et al.* (1996). The probe is added to cells in the form of a diacetate ester cFDASE which is then converted by an intracellular enzyme to cFSE. Breeuwer *et al.* (1996) hypothesised that after incorporation the succinimidyl group forms conjugates with aliphatic amines which bind the probe inside the cell. This binding is an additional advantage because even less leakage occurs (Breeuwer *et al.*, 1996). Unbound probe is removed from the cell in a process that requires a short incubation of the cells in the presence of a fermentable sugar. For most bacteria glucose is used but for *Lactococcus lactis* lactose is used. It is likely that the bacteria utilises the energy from the fermentable sugar to actively transport the unbound probe outside the cell.

cFSE produces two fluorescent signals, one which is dependent on pH and used for intracellular pH measurement (490-500nm) and another whose fluorescent signal is independent of intracellular pH (440nm) (Breeuwer *et al.*, 1996). The ratio of the two signals is used as a control for the level of labelling of the cells. Comparison of results between individual experiments is allowed by standardisation of the signal ratio in order to correct for differing levels of incorporation (Breeuwer *et al.*, 1996). Breeuwer's technique was adapted to determine *Listeria monocytogenes* (LM1) viability (Simpson *et al.*, 1999).

Use of this probe and technique was more complex for Gram-negative organisms. Breeuwer *et al.*, (1996) measured the internal pH of *Lactococcus lactis*, *Listeria innocua*, *Bacillus subtilus* and *E. coli*. They employed an EDTA treatment (5mM) to facilitate incorporation of the probe through the Gram-negative outer membrane. After this treatment a small pH gradient (pH_i 7.3 at an external pH of 7.0) could be observed after the addition of glucose (10mM). However this intracellular pH value was lower than previous studies (pH_i 7.8 at an external pH of 7.0 (Booth, 1985)) and suggested that the vitality of *E. coli* was decreased by the EDTA treatment. Therefore a treatment needed to be developed
that would allow the uptake of the probe into Gram-negative bacteria without injuring the cell. Alternatively a method that allowed the bacteria to recover from any injury or stress caused by the treatment was required.

4.3 Materials and Methods

The following experiments were carried out during a three month research visit to Wye College, Kent, U.K. under the supervision of Professor Nick Russell and with the assistance of Dr. Ryan Simpson and colleagues. The spectrofluorometer (Appendix 1) needed to carry out the fluorescence assays was available for only this period of time.

4.3.1 Determination of the intracellular pH using a fluorescent probe - Protocol for *Listeria monoctyogenes*

4.3.1.1 Cell Preparation

1ml of an overnight culture (37°C) was placed in 10ml of nutrient broth (Appendix 1) and incubated at 37°C with shaking for 3 - 4 hours to obtain an exponential phase culture. 1.5ml fractions of the exponential phase culture were placed in eppendorfs. These were centrifuged in an eppendorf centrifuge at 130, 000rpm for 3 minutes, the supernatant was aspirated and the pellet resuspended in 1ml potassium phosphate buffer (pH 7) (Appendix 1) by vortexing. These steps was carried out twice to wash the cells.

4.3.1.2 Incubations with Probe and Glucose

10µl of probe solution (Appendix 1) (diluted 1 in 100 in acetone) was added to each eppendorf, vortex mixed and incubated for 5 minutes at 37°C. The cells were then washed again as above.

20µl of 1M glucose (Appendix 1) was added to each eppendorf and vortex mixed and incubated for 30 minutes at 37°C. The cells were then washed as above and incubated with glucose again as above. After a final wash the cells were placed on ice, until assayed.

4.3.1.3 Fluorescence Assay

The fluorescence assay steps are designed to determine the physiological state of the cells and determine the intracellular pH of the cells. In the first step the background fluorescence in the buffer and cuvette is measured at both wavelengths and then subtracted from all other readings. The addition of cells gives an initial intracellular pH reading. The addition of glucose is used to determine the degree of physiological activity of the cells. In the presence of such a carbon source, physiologically active cells will raise their internal pH level relative to the exterior. The addition of valinomycin permeabilises the cells to potassium ions. This causes the cell to rapidly remove both potassium and hydrogen ions which gives rise to a further increase in intracellular pH when the exterior pH is lower than the interior (pH < 8). The addition of nigericin completely permeabilises the cell and therefore the intracellular pH equals the exterior buffer pH. Finally the removal of the cells enables the determination of the level of signal attached to the cells, the level of incorporation of the probe into the cells.

3ml of buffer (50mM buffers of either citric acid for pH 4 and 5, potassium phosphate for pH 6, 6.5, 7 and 7.5 or glycine for pH 8, 9 and 10 - Appendix 1) was placed in a cuvette maintained at 37°C and fluorescence readings recorded at the emission wavelengths of 440nm and 500nm for background levels. 150µl of cell preparation was added to the cuvette, stirred, and readings recorded alternately at the emission wavelengths 440nm and 500nm. 30µl of 1M glucose was added to the cuvette, stirred and readings recorded alternately at the emission wavelengths 440nm and 500nm. 20µl of valinomycin solution (Appendix 1 - diluted 1 in 20 in absolute alcohol) was added to the cuvette, stirred, and readings recorded alternately at the emission wavelengths 440nm and 500nm. 20µl of nigericin solution (Appendix 1-diluted 1 in 20 in absolute alcohol) was added to the cuvette, stirred, and readings recorded alternately at the emission wavelengths 440nm and 500nm. For some assays extra additions and larger volumes (up to 100µl) of glucose, valinomycin or nigericin were added. The contents of the cuvette were then filtered through a 0.2µm disposable filter into a clean cuvette. Readings were taken to determine the level of fluorescent signal in the solution outside cells using the emission wavelength 440nm. A background level of less than 20% of the fluorescence independent signal was considered acceptable (Breeuwer *et al.*, 1996).

Spectrofluorometer (Appendix 1) settings were as follows: Excitation Wavelength 1 = 440nm, Emission Wavelength 1 = 530nm, Excitation Wavelength 2 = 500nm, Emission Wavelength 2 = 530nm, Slit widths - Excitation = 5nm, Emission = 10nm. The slit was closed to check that the spectrofluorometer showed zero fluorescence. Fluorescence is measured in a scale of arbitrary units. The spectrophotometer could multiply the reading by a factor of 1, 2 or 5 to fit between a scale of 0-1000. For this machine the time was also in arbitary units as the switching between wavelengths and recording of readouts occurred manually. The time for each reading could be kept relatively constant by a skilled operator and was of the order of 3-5 seconds.

4.3.2 Protocol for *Escherichia coli* - No Treatment

Initially the *Listeria* protocol was used on an overnight culture of *E. coli* with no permeability treatment to determine if a permeability treatment was necessary. Variations on the *Listeria* protocol were the extension of probe incubation times to 15, 30 and 60 minutes at 37°C. Details of conditions are given in Table 4.1 (A1-A4).

4.3.3 Protocol for *Escherichia coli* - EDTA

In this set of experiments (C - Table 4.1) EDTA was used as the agent to temporarily permeabilise the cells to allow the probe entry. Along with a control (no permeability treatment), the method, as described by Breeuwer *et al.,* (1996), was used. Variations on the *Listeria* protocol were the extension of probe incubation times to 15 or 30 minutes at 37°C in HEPES buffer + 5mM EDTA (pH 7- Appendix 1) and the use of HEPES buffer (pH 7 - Appendix 1) to resuspend cells initially and the use of potassium phosphate buffer + 10mM $MgCl_2$ (pH 7 - Appendix 1) to resuspend the cells for all steps following EDTA treatment. Details of these treatments are given in Table 4.1 (C1-C6). The treatments were plate counted (4.3.8) on nutrient agar and nutrient agar with 4% NaCl (Appendix 1) to determine the survival and injury rates of the treatments.

4.3.4 Protocol for *Escherichia coli* - Calcium Chloride

In this set of experiments calcium chloride was used as the agent to temporarily permeabilise the cells to allow the probe entry. This was the most extensively trialled permeability treatment which was undertaken in 5 experiments (B, D, E, F, G - Table 4.1). Details of these treatments are given in Table 4.1. Variations on the *Listeria* protocol included the use of overnight cultures incubated for 16 hours at 37°C. In some cases 1ml of cell suspension per eppendorf was divided into 2 eppendorfs (0.5ml) which were then treated as previously (4.3.1). In one experiment 0.1% peptone water (Appendix 1) was used as an alternative to phosphate buffer. Initially two concentrations of $CaCl_2$ 10 and 100mM were used for two incubation times of 5 and 20 minutes on ice or at 37°C (B1-B8). Two different concentrations of probe were also used, 5 and 10µl (B1-B8). The higher concentration of $CaCl_2$ was used for all further experiments (D1-D4, E1-E12, F1-F8 and G1-G4).

Variations in the time (1 or 1.5 hrs - F) and temperature (37°C or on ice - D) of the probe incubation and the time (1, 2, 3 up to 24 hrs - D, E, F) of the permeability treatment were tested to try and determine the least severe treatment that would still result in labelled cells. Different heat shock and recovery protocols used in molecular biology methods to return competent cells to normal physiologically activity were trailed in all these experiments (B, D, E, F, G). The cells were heat shocked at 42°C for 2 minutes and recovered by addition of 800µl complex nutrient broth + 10µL magnesium salts and 14µL glucose (1g in 2ml) (Appendix 1) and incubation for 45, 60 or 120 minutes at 37°C, or for 60 minutes at 5°C. Glucose incubations were performed as for the *Listeria* protocol (4.3.1) but not always included. The treatments were plate counted (4.3.8) on nutrient agar or nutrient agar and MacConkey agar (Appendix 1) to determine the survival and injury rates of these treatments.

4.3.5 Protocol for *Escherichia coli* - Electroporation

In this set of treatments (H5 and H6 - Table 4.1) electroporation was used as the agent to temporarily permeabilise the cells to allow the probe entry. Variations on the *Listeria* protocol were the concentration of the cell suspension from 1.5 to 1ml per eppendorf which was divided into 2 eppendorfs (0.5ml). The volume of probe added was either 10µl or 50µl. Cells were electroporated as described in Pena *et al.* (1995). The resistance used was 200 ohms and the capacitance 25 microfarads. The cuvette gap was 0.4cm, electroporation was performed at 2500 volts for 2.2 milliseconds. Two glucose incubations were performed as for the *Listeria* protocol (4.3.1) but with incubation times extended to 40 minutes. Details of these treatments are given in Table 4.1 (H5 and H6).

4.3.6 Protocol for *Escherichia coli* - Probe Concentrations

During acid shock experiments (described in Section 4.3.7) it was observed that higher concentrations of probe solution were found to be more effective at labelling the cells. An experiment was designed to explore this observation further using the same protocol as the acid shock experiments (4.3.7). 20, 40, 50, 60, 80 or 100µl of probe solution was added to the cell suspension. Cells were incubated for 1 hour at 37°C. The details of variations in this treatment are given in Table 4.1 (I1-I6).

4.3.7 Protocol for *Escherichia coli* - Acid Shock

In this set of treatments (H1-H4, J1-J12 - Table 4.1) acid shock was used as the agent to temporarily permeabilise the cells to allow the probe entry. Variations on the *Listeria* protocol were the concentration of the cell suspension by resuspending the pellet from 1.5ml of exponential culture in 1ml phosphate buffer during the washing and then in 100µl for the acid shock step or 200µl for the "no acid" control. For some acid shock experiments 0.25ml 10% Nutrient broth plus 0.25ml "spent" media (supernatant removed from overnight culture spun at 13,000 for 3min) was used instead of phosphate buffer. The volume of probe added was either 10µl or 50µl. Cells were acid shocked as described in Molenaar *et al.*, (1991). 10µl or 25µl of 0.1M HCl was added to cell suspensions and incubated for 5 minutes at room temperature. Two "no acid" treatments with the same two volumes of probe (10µl or 50µl) were incubated for 1 hour at 37°C. At the end of incubation 1ml of potassium phosphate buffer was added to rapidly raise the pH of the solution for the acid shocked cells. Two glucose incubations were performed as for the *Listeria* protocol (4.3.1) but with incubation times extended to 40 minutes (H1-H4) or various numbers of sets of two glucose incubations were performed (J1-J12). Details of these treatments are given in Table 4.1 (H1-H4, J1-J12).

4.3.8 Protocol for *Escherichia coli* - Viable Counts

Viable counts were performed for some treatments to compare the initial number of cells before the treatment with those remaining after each variation of treatment. In order to determine the relative severity of the treatments, plate counts were also performed on selective media, MacConkey agar (Appendix 1), as well as Nutrient Agar (Appendix 1) containing added salt (4, 5 or 6%) (Appendix 1).

Samples (1ml) were taken as soon as possible after treatment and if necessary stored at 5°C in order to minimise any further death or recovery. Samples were diluted in 0.9ml of sterile saline in eppendorfs and then 0.1ml of the, 10^{-5}, 10^{-6}, 10^{-7} serial dilutions were spread onto the agar and incubated at 37°C and counted after overnight incubation. Any plates with no visible growth were reincubated for up to 72 hours.

Table 4.1 - All Combinations of Treatments Trialled for Intracellular pH Measurements.

Treatment & Date	Cell culture	Permeation method	Probe incubation	Glucose incubations	Heat Shock and/or Recovery	pH_i	Plate Counts
A1 29/9/97	overnight	none	5 min at 37°C, 10µl probe	1	none	none	none
A2 29/9/97	overnight	none	15 min at 37°C, 10µl probe	1	none	yes	none
A3 29/9/97	overnight	none	30 min at 37°C, 10µl probe	1	none	none	none
A4 29/9/97	overnight	none	1 hr at 37°C, 10µl probe	1	none	yes	none
B1 30/9/97	overnight	$CaCl_2$ 100mM, 5 min at 37°C	30 min on ice, 10µl probe	none	1	yes	none
B2 30/9/97	overnight	$CaCl_2$ 100mM, 5 min at 37°C	30 min on ice, 5µl probe	none	1	yes	none
B3 30/9/97	overnight	$CaCl_2$ 100mM, 20 min on ice	30 min on ice, 10µl probe	none	1	yes	none
B4 30/9/97	overnight	$CaCl_2$ 100mM, 20 min on ice	30 min on ice, 5µl probe	none	1	yes	none
B5 30/9/97	overnight	$CaCl_2$ 10mM, 5 min at 37°C	30 min on ice, 10µl probe	none	1	yes	none
B6 30/9/97	overnight	$CaCl_2$ 10mM, 5 min at 37°C	30 min on ice, 5µl probe	none	1	yes	none
B7 30/9/97	overnight	$CaCl_2$ 10mM, 20 min on ice	30 min on ice, 10µl probe	none	1	yes	none
B8 30/9/97	overnight	$CaCl_2$ 10mM, 20 min on ice	30 min on ice, 5µl probe	none	1	yes	none
C1 6/10/97	exponential	5mM EDTA	15 min at 37°C, 10µl probe	1	none	yes	yes
C2 6/10/97	exponential	5mM EDTA	30 min at 37°C, 10µl probe	1	none	yes	yes
C3 6/10/97	exponential	5mM EDTA	none	1	none	none	yes
C4 6/10/97	exponential	5mM EDTA	none	1	none	none	yes
C5 6/10/97	exponential	none	30 min at 37°C, 10µl probe	1	none	yes	yes
C6 6/10/97	exponential	none	none	1	none	none	yes
D1 7/10/97	exponential	$CaCl_2$ 100mM, overnight 5°C	1 hr on ice, 10µl probe	none	60min at 37°C	yes	yes
D2 7/10/97	exponential	$CaCl_2$ 100mM, overnight 5°C	1 hr at 37°C, 10µl probe	none	60min at 37°C	yes	yes
D3 7/10/97	exponential	$CaCl_2$ 100mM, overnight 5°C	1 hr on ice, 10µl probe	1	none	yes	yes
D4 7/10/97	exponential	$CaCl_2$ 100mM, overnight 5°C	1 hr at 37°C, 10µl probe	1	none	yes	yes

Table 4.1 - All Combinations of Treatments Trialled for Intracellular pH Measurements (continued).

Treatment & Date	Cell culture	Permeation method	Probe incubation	Glucose incubations	Heat Shock and/or Recovery	pH_i	Plate Counts
E1 14/10/97	exponential	$CaCl_2$ 100mM, 1 hr at 5°C	1 hr at 37°C, 10µl probe	1	none	yes	yes
E2 14/10/97	exponential	$CaCl_2$ 100mM, 1 hr at 5°C	1 hr at 37°C, 10µl probe	1	1 hr at 37°C	yes	yes
E3 14/10/97	exponential	$CaCl_2$ 100mM, 1 hr at 5°C	1 hr at 37°C, 10µl probe	1	2 hr at 25°C	yes	yes
E4 14/10/97	exponential	$CaCl_2$ 100mM, 1 hr at 5°C	1 hr at 37°C, 10µl probe	1	4 hr at 10°C	yes	yes
E5 14/10/97	exponential	$CaCl_2$ 100mM, 3 hr at 5°C	1 hr at 37°C, 10µl probe	1	none	yes	yes
E6 14/10/97	exponential	$CaCl_2$ 100mM, 3 hr at 5°C	1 hr at 37°C, 10µl probe	1	1 hr at 37°C	yes	yes
E7 14/10/97	exponential	$CaCl_2$ 100mM, 3 hr at 5°C	1 hr at 37°C, 10µl probe	1	2 hr at 25°C	yes	yes
E8 14/10/97	exponential	$CaCl_2$ 100mM, 3 hr at 5°C	1 hr at 37°C, 10µl probe	1	4 hr at 10°C	yes	yes
E9 14/10/97	exponential	$CaCl_2$ 100mM, 1 day at 5°C	1 hr at 37°C, 10µl probe	1	none	yes	yes
E10 14/10/97	exponential	$CaCl_2$ 100mM, 1 day at 5°C	1 hr at 37°C, 10µl probe	1	1 hr at 37°C	yes	yes
E11 14/10/97	exponential	$CaCl_2$ 100mM, 1 day at 5°C	1 hr at 37°C, 10µl probe	1	2 hr at 25°C	yes	yes
E12 14/10/97	exponential	$CaCl_2$ 100mM, 1 day at 5°C	1 hr at 37°C, 10µl probe	1	4 hr at 10°C	yes	yes
F1 22/10/97	exponential	$CaCl_2$ 100mM, 2 hr at 5°C	1 hr at 37°C, 10µl probe	1	1.5 hr at 37°C	yes	yes
F2 22/10/97	exponential	$CaCl_2$ 100mM, 2 hr at 5°C	1 hr at 37°C, 10µl probe	1	2.5 hr at 37°C	yes	yes
F3 22/10/97	exponential	$CaCl_2$ 100mM, 3 hr at 5°C	1.5 hr at 37°C, 10µl probe	1	1.5 hr at 37°C	yes	yes
F4 22/10/97	exponential	$CaCl_2$ 100mM, 3 hr at 5°C	1.5 hr at 37°C, 10µl probe	1	2.5 hr at 37°C	yes	yes
F5 22/10/97	overnight	$CaCl_2$ 100mM, 3 hr at 5°C	1.5 hr at 37°C, 10µl probe	1	1.5 hr at 37°C	yes	yes
F6 23/10/97	exponential	$CaCl_2$ 100mM, 3 hr at 5°C	1.5 hr at 37°C, 10µl probe	1	1 day at 25°C	yes	yes
F7 23/10/97	exponential	$CaCl_2$ 100mM, 3 hr at 5°C	1.5 hr at 37°C, 10µl probe	1	1 day at 10°C	yes	yes
F8 23/10/97	overnight	$CaCl_2$ 100mM, 3 hr at 5°C	1.5 hr at 37°C, 10µl probe	1	1 day at 10°C	yes	yes

Table 4.1 - All Combinations of Treatments Trialled for Intracellular pH Measurements (continued).

Treatment & Date	Cell Culture	Permeation method	Probe incubation	Glucose incubations	Heat Shock /Recovery	pH_i	Plate Counts
G1 30/10/97	exponential	$CaCl_2$ 100mM, 3 hr at 5°C	1 hr at 37°C, 10µl probe	1	1 hr at 37°C	yes	yes
G2 30/10/97	exponential*	$CaCl_2$ 100mM, 3 hr at 5°C	1 hr at 37°C, 10µl probe	1	1 hr at 37°C	yes	yes
G3 30/10/97	exponential slow	$CaCl_2$ 100mM, 3 hr at 5°C	1 hr at 37°C, 10µl probe	1	1 hr at 37°C	yes	yes
G4 30/10/97	exponential slow*	$CaCl_2$ 100mM, 3 hr at 5°C	1 hr at 37°C, 10µl probe	1	1 hr at 37°C	yes	yes
H1 7/11/97	exponential	Acid Shock 10µl 0.1M HCl	10µl probe	2	none	yes	none
H2 7/11/97	exponential	Acid Shock 10µl 0.1M HCl	50µl probe	2	none	yes	none
H3 7/11/97	exponential	Acid Shock 25µl 0.1M HCl	10µl probe	2	none	yes	none
H4 7/11/97	exponential	Acid Shock 25µl 0.1M HCl	50µl probe	2	none	yes	none
H5 7/11/97	exponential	Electroporation	10µl probe	2	none	yes	none
H6 7/11/97	exponential	Electroporation	50µl probe	2	none	yes	none
H7 7/11/97	exponential	none	1 hr at 37°C, 10µl probe	2	none	yes	none
H8 7/11/97	exponential	none	1 hr at 37°C, 50µl probe	2	none	yes	none
I1 14/11/97	exponential	Acid Shock 25µl 0.1M HCl	1 hr at 37°C, 20µl probe	2	none	yes	none
I2 14/11/97	exponential	Acid Shock 25µl 0.1M HCl	1 hr at 37°C, 40µl probe	2	none	yes	none
I3 14/11/97	exponential	Acid Shock 25µl 0.1M HCl	1 hr at 37°C, 50µl probe	2	none	yes	none
I4 14/11/97	exponential	Acid Shock 25µl 0.1M HCl	1 hr at 37°C, 60µl probe	2	none	yes	none
I5 14/11/97	exponential	Acid Shock 25µl 0.1M HCl	1 hr at 37°C, 80µl probe	2	none	yes	none
I6 14/11/97	exponential	Acid Shock 25µl 0.1M HCl	1 hr at 37°C, 100µl probe	2	none	yes	none

slow = spin speed 3300rpm, * 0.1% peptone water used instead of phosphate buffer

Table 4.1 - All Combinations of Treatments Trialled for Intracellular pH Measurements (continued).

Treatment & Date	Cell Culture	Permeation method	Probe incubation	Glucose incubations	Heat Shock /Recovery	pH_i	Plate Counts
J1 17/11/97	exponential	Acid Shock 25µl 0.1M HCl, 1 min	10µl probe#	1	none	yes	none
J2 17//11/97	exponential	Acid Shock 25µl 0.1M HCl, 5 min	10µl probe#	1	none	yes	none
J3 17//11/97	exponential	Acid Shock 25µl 0.1M HCl, 5 min	10µl probe#	1	none	yes	none
J4 22//11/97	exponential	Acid Shock 25µl 0.1M HCl, 1 min	10µl probe#	2 sets	none	yes	none
J5 17//11/97	exponential	Acid Shock 25µl 0.1M HCl, 5 min	10µl probe#	2 sets	none	yes	none
J6 17//11/97	exponential	Acid Shock 25µl 0.1M HCl, 5 min	10µl probe#	2 sets	none	yes	none
J7 22//11/97	exponential	Acid Shock 25µl 0.1M HCl, 1 min	10µl probe#	3 sets	none	yes	none
J8 17//11/97	exponential	Acid Shock 25µl 0.1M HCl, 5 min	10µl probe#	3 sets	none	yes	none
J9 17/11/97	exponential	Acid Shock 25µl 0.1M HCl, 5 min	10µl probe#	3 sets	none	yes	none
J10 22/11/97	exponential	Acid Shock 25µl 0.1M HCl, 1 min	10µl probe#	4 sets	none	yes	none
J11 17/11/97	exponential	Acid Shock 25µl 0.1M HCl, 5 min	10µl probe#	4 sets	none	yes	none
J12 17/11/97	exponential	Acid Shock 25µl 0.1M HCl, 5 min	10µl probe#	4 sets	none	yes	none

More concentrated probe solution : 50µl of stock probe solution in 950µl acetone compared to previous 10µl of stock probe solution in 980µl acetone. Also 0.25ml 10% Nutrient broth plus 0.25ml "spent" media (supernatant removed from overnight culture spun at 13,000 for 3min) used instead of phosphate buffer.

4.4 Results

4.4.1 Intracellular pH of *Listeria monocytogenes*

A typical example of the changes in fluorescence signal strength at both 440 and 500nm is shown in Figure 4.1. The size of the responses in this example are small because they were performed at ambient temperatures (20-25°C) instead of 37°C due to the breakdown of the cuvette heater during these preliminary experiments on *L.monocytogenes*.

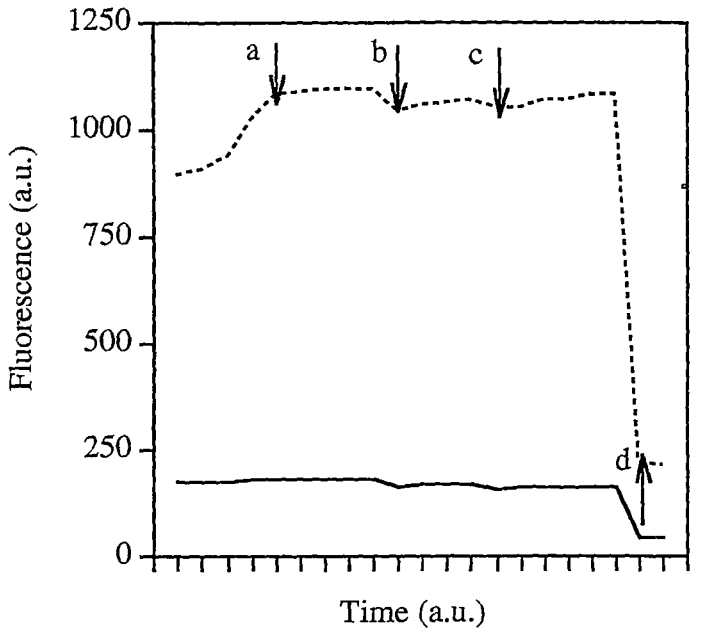


Figure 4.1 - Example of the changes in fluorescence over the course of a fluorescence assay for cFSE labelled *Listeria monocytogenes* cells in buffer of pH 9. a) addition of glucose, b) addition of valinomycin, c) addition of nigericin, d) filtering of cells (fluorescence signal of supernatant). Dotted line is the fluorescent signal at 500nm and the solid line is the fluorescent signal at 440nm.

The intracellular pH of *Listeria monocytogenes* was determined over a range of pH using different buffers at pH 4, 5, 6, 6.5, 7, 7.5, 8, 9 and 10. The value of the intracellular pH was taken as equal to the exterior buffer pH when the cells had been permeabilised with valinomycin and nigericin. The value of the ratio of the fluorescent signals at the pH dependent wavelength 500nm to the pH independent wavelength 440nm corrects for the variation in signal strength between experiments. This calibration curve (Figure 4.2) enabled the calculation of the intracellular pH of *Listeria monocytogenes* given the ratio of the fluorescent signals at the pH dependent wavelength 500nm to the pH independent wavelength 440nm. This figure is similar to other results for *Listeria* (Simpson, pers. comm.), other Gram-positive organisms (Breeuwer *et al.*, 1996) and even fungal cells (Breeuwer *et al.*, 1997). However, it is not possible to compare it to the calibration for *E. coli* as this information was not presented by Roindet (1997).

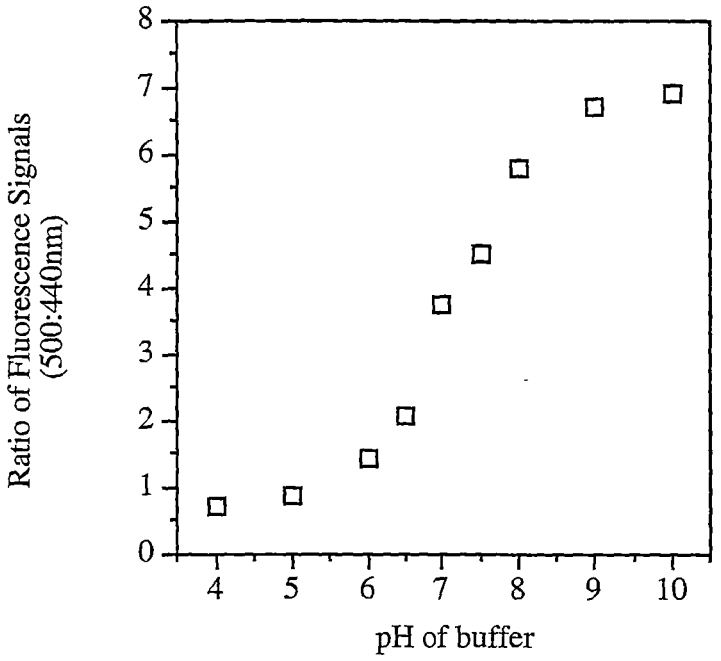


Figure 4.2 - Calibration curve of ratio of fluorescence signals at the pH independent (440nm) and pH dependent (500nm) wavelengths to pH of the external buffer for cFSE labelled *Listeria monocytogenes* cells.

4.4.2 Intracellular pH of *Escherichia coli*

As described in the Materials and Methods section, many different types and combinations of treatments were trialled to measure the intracellular pH of *E. coli*. For many attempts the most basic criterion for a successful measurement of pH_i was not met. That criterion is that the level of incorporation, i.e. the percentage of the probe associated with the cells, is greater than 80%. A certain level of background signal from probe in solution is unavoidable but if the level is too great the measurement of pH_i will not reflect the true intracellular pH of the cells (Breeuwer *et al.*, 1996). The following sections (Tables 4.2- 4.4) relate the treatments performed (Table 4.1) to the resulting levels of fluorescent probe incorporation and signal strength after glucose addition. Only 34% of all treatments achieved greater than 80% incorporation.

4.4.2.1 No Treatment and EDTA Treatments

When *E. coli* cells received no treatment (A) negligible incorporation occurred. Also none of the EDTA treatments (C) showed a successful level of incorporation of the fluorescent signal within the *E. coli* cells. That is, at the end of the fluorescence assay the level of signal remaining in the supernatant after the cells had been removed by filtration was higher than 20% of the total signal, indicating that a significant amount of the fluorescent signal was extracellular.

Table 4.2 - Variation of signal strengths (after addition of glucose) and incorporation of fluorescent probe for no treatment and EDTA treatments.

Treatment & Date (Table 4.1)	Signal Strength at 440nm min - max (av.)	Signal Strength at 500nm min - max (av.)	Incorporation %
A2 29/9/97	7-8 (8)	19-20 (20)	0
A4 29/9/97	37-40 (39)	34-37 (37)	10
C1 6/10/97	14-15 (15)	36-37 (36)	37
C2 6/10/97	26-32 (29)	67-72 (70)	43
C5 6/10/97	15-17 (17)	34-36 (34)	24

4.4.2.2 Calcium Chloride Treatments

Some calcium chloride treatments showed an acceptable level of incorporation of the fluorescent signal within the *E. coli* cells. Initial experiments with 10 or 100mM calcium chloride incubated with the probe on ice (B) showed very little incorporation of the probe. Exponential cells that were incubated in 100mM calcium chloride at 5°C overnight prior to incubation with the probe for an hour at 37°C with no heat shock and recovery step (D4) showed a 91% incorporation. Subsequent treatments (E) used the same probe incubation and investigated variations on the time of incubation in calcium chloride and the time and temperature of the recovery. All treatments where the cells were incubated for 3 hours or more in calcium chloride (E5-E12) showed at least 78% incorporation, however there was no recovery protocol which consistently gave better incorporation.

Table 4.3 - Variation of signal strengths (after addition of glucose) and incorporation of fluorescent probe for calcium chloride treatments.

Treatment & Date (Table 4.1)	Signal Strength at 440nm min - max (av.)	Signal Strength at 500nm min - max (av.)	Incorporation %
B1 30/9/97	12-13 (12)	16-17 (17)	58
B2 30/9/97	13-14 (14)	11-12 (12)	38
B3 30/9/97	7	11-12 (11)	50
B4 30/9/97	7-8 (8)	9-10 (9)	38
B5 30/9/97	18-20 (19)	27-28 (28)	15
B6 30/9/97	10	10	55
B7 30/9/97	18-20 (19)	28-30 (29)	47
B8 30/9/97	17-19 (18)	19-20 (19)	26
D1 7/10/97	132-138 (135)	851-877(868)	49
D2 7/10/97	168-171 (170)	744-773 (758)	65
D3 7/10/97	28-30 (29)	138-145 (143)	66
D4 7/10/97	101-104 (103)	444-456 (450)	**91**
E1 14/10/97	119-132 (125)	494-543 (520)	76
E2 14/10/97	178-192 (183)	770-883 (744)	71
E3 14/10/97	213-243 (231)	818-941 (865)	69
E4 14/10/97	126-133 (130)	504-539 (532)	77
E5 14/10/97	126-140 (131)	501-577 (542)	**85**
E6 14/10/97	211-224 (217)	811-901 (852)	**84**
E7 14/10/97	151-157 (154)	558-589 (575)	78
E8 14/10/97	272-304 (270)	1049-1179 (1103)	**86**
E9 14/10/97	78-93 (82)	310-330 (322)	80
E10 14/10/97	98-150 (118)	370-545 (444)	**94**
E11 14/10/97	71-98 (84)	275-378 (317)	**95**
E12 14/10/97	94-160 (126)	379-596 (464)	**97**

The levels of incorporation marked in **bold** are those >80%.
Those signal strengths underlined were measured at multiplication factors of x1 or x2 not x5.

Further variations on the time of incubation in calcium chloride (2 or 3 hours), the time of probe incubation (1 or 1.5 hours) and the time of recovery at 37°C (1.5, 2.5 or 24 hours) showed some treatments (F) achieved incorporation. All treatments where the cells recovered for 1.5 or 2.5 hours showed at least 78% incorporation (F1-F5) in comparison to poor incorporation for the 24 hour recovery period. The best treatment combination was 3 hour incubation in calcium chloride, 1.5 hour probe incubation and 1.5 hour recovery (F3 and F5) which showed better results on the overnight cell culture than the exponential phase cells. The final experiment using calcium chloride as a permeability treatment tested whether the use of a slower spin speed (3300rpm) during the centrifugation steps might result in less injured cells. This speed was selected as the slowest speed which still pelleted the cells. However, for this experiment the previous type of calcium chloride treatment did not result in incorporation for either the normal or slower spin speeds (G1 and G3). There was also a much lower level of incorporation when peptone water (G2 and G4)was used instead of phosphate buffer (G1 and G3).

Table 4.3 (cont.) - Variation of signal strengths (after addition of glucose) and incorporation of fluorescent probe for calcium chloride treatments.

Treatment & Date (Table 4.1)	Signal Strength at 440nm min - max (av.)	Signal Strength at 500nm min - max (av.)	Incorporation %
F1 22/10/97	195-204 (199)	692-710 (701)	78
F2 22/10/97	184-192 (187)	655-666 (66)	78
F3 22/10/97	285-300 (295)	1128-1158 (1145)	80
F4 22/10/97	265-273 (269)	950-970 (962)	79
F5 22/10/97	188-197 (191)	701-725 (712)	**82**
F5 22/10/97 Buffer pH 5	165-175 (170)	202-225 (211)	**87**
F6 22/10/97	245-250 (248)	904-915 (907)	72
F7 22/10/97	298-315 (306)	1110-1168 (1126)	73
F8 22/10/97	191-204 (196)	656-679 (666)	64
G1 30/10/97	66-69 (68)	153-164 (157)	53
G2 30/10/97	125-131 (126)	106-121 (110)	10
G3 30/10/97	25-28 (26)	56-59 (57)	36
G4 30/10/97	150-156 (154)	134-148 (136)	15

The levels of incorporation marked in **bold** are those >80%.
Those signal strengths underlined were measured at multiplication factors of x1 or x2 not x5

4.4.2.3 Acid Shock, Electroporation and Probe Concentration Treatments

Neither of the electroporation treatments showed any significant incorporation (H5 and H6). Five minute acid shock treatments with 50µl or more of original strength probe all showed successful incorporation (H2, H4, I3, I4, I5, I6). It was suspected this could have been because the solvent for the probe was acetone which was permeabilising the cells. In the final experiment, a five times more concentrated probe solution was made so that the same amount of probe as the previous experiments could be added by adding only 10µl of solution. These also showed good incorporation when acid shocked for 5 minutes (Treatments J2, J3, J5, J6, J8, J9, J11, J12).

Table 4.4 - Variation of signal strengths (after addition of glucose) and incorporation of fluorescent probe for acid shock, electroporation and probe concentration treatments.

Treatment & Date (Table 4.1)	Signal Strength at 440nm min - max (av.)	Signal Strength at 500nm min - max (av.)	Incorporation %
H1 7/11/97	154-158 (156)	109-110 (109)	10
H2 7/11/97	106-115 (109)	442-447 (444)	**83**
H3 7/11/97	143-147 (145)	95-98 (96)	17
H4 7/11/97	176-179 (178)	828-836 (834)	**84**
H5 7/11/97	136-141 (139)	115-118 (116)	31
H6 7/11/97	127-136 (136)	152-173 (161)	22
H7 7/11/97	28-34 (29)	29-32 (31)	0
H8 7/11/97	90-94(92)	356-354 (360)	78
H8 7/11/97	103-106 (105)	357-361 (360)	67
I1 14/11/97	80-83 (81)	71	45
I2 14/11/97	61-62 (61)	131-134 (132)	48
I3 14/11/97	114-118(116)	526-547 (535)	**87**
I3 14/11/97 Buffer pH 6	107-113 (110)	254-267 (259)	**89**
I4 14/11/97	82-86 (84)	364-367 (365)	**90**
I5 14/11/97	159-164 (161)	726-741 (735)	**88**
I6 14/11/97	187-202 (196)	866-885 (878)	**87**
J1 17/11/97	100-115 (115)	245-315 (290)	31
J2 17/11/97	720-770 (745)	2855-2960 (2901)	**86**
J3 17/11/97	975-995 (990)	4190-4255 (4215)	**81**
J4 17/11/97	255-265 (260)	950-960 (955)	50
J5 17/11/97	640-650 (645)	2705-2755 (2728)	**88**
J6 17/11/97	775-790 (780)	3195-3295 (3232)	**83**
J7 17/11/97	200-205 (200)	490-515 (510)	34
J8 17/11/97	465-480 (470)	1825-1900 (1856)	**92**
J9 17/11/97	835-850 (840)	3195-3305 (3227)	**90**
J10 17/11/97	290-310 (295)	1265-1365 (1320)	67
J11 17/11/97	385-395 (385)	1475-1550 (1507)	**93**
J12 17/11/97	725-735 (730)	2640-2675 (2655)	**91**

The levels of incorporation marked in **bold** are those >80%.

Those signal strengths underlined were measured at multiplication factors of x1 or x2 not x5.

4.4.2.4 Variation of Signal Strength during Fluorescence Assay.

The second criterion to successfully determine the pH_i was that the labelled cells were physiologically active. To test this in the assay protocol a fermentable carbohydrate carbon source is added, in this case glucose. A rise in pH_i would be observed in buffers of pH < 8, if the cell is active because bacteria can use the energy provided to raise their pH_i towards its optimal level (for *E. coli* approximately 7.8 (Booth, 1985)). For all treatments only small changes in pH were measured on the addition of glucose. Also only small changes were noted when valinomycin or nigericin were added (Figure 4.3 - 4.11). It was hypothesised that the lack of response was because the cells were killed or injured by the labelling treatment and therefore the cells were unable to respond to the assay conditions.

Table 4.5 - Fluorescence ratios for treatments with incorporation >80%.

Treatment & Date (Table 4.1)	pH of Buffer	I/I*	Δ with Val#	Δ with Nig#	Figure No.
D4 7/10/97	7	4.37	+2.5%	+2.2%	4.3
E5 14/10/97	7	4.14	+6.8%	+10.7%	4.4
E6 14/10/97	7	3.93	-3.9%	-6.9%	4.5
E8 14/10/97	7	4.09	+0.5%	+2.5%	4.6
E10 14/10/97	7	3.76	-	-	-
E11 14/10/97	7	3.77	-	-	-
E12 14/10/97	7	3.68	-	-	-
F5 22/10/97	7	3.73	+1.4%	+6.6%	4.7
F5 22/10/97	5	1.24	-2.4%	-8.5%	4.8
H4 7/11/97	7	4.69	0%	-0.1%	4.9
I3 14/11/97	7	4.61	-0.2%	-0.6%	4.10
I3 14/11/97	6	2.35	-1.5%	-4.6%	4.11
I4 14/11/97	7	4.35	+0.5%	+0.5%	-
I5 14/11/97	7	4.57	+0.7%	+1.1%	-
I6 14/11/97	7	4.48	+1.5%	+1.8%	-
J2 17/11/97	7	3.89	+2.4%	+3.3%	-
J3 17/11/97	7	4.26	+1.2%	+1.8%	-
J5 17/11/97	7	4.23	-0.9%	-0.7%	-
J6 17/11/97	7	4.14	+0.2%	+0.6%	-
J8 17/11/97	7	3.95	+1.0%	+2.6%	-
J9 17/11/97	7	3.84	+0.6%	+1.9%	-
J11 17/11/97	7	3.91	+1.0%	+3.7%	-
J12 17/11/97	7	3.64	+2.4%	+5.3%	-

* Ratio of average fluorescence signal strengths at pH dependent (500nm) and pH independent (440nm) wavelengths after addition of glucose (see Tables 4.2, 4.3 and 4.4)

Variation in average fluorescent signal strength at 500nm: a comparison between the levels after the addition of glucose and the levels after the addition of valinomycin (Val) and nigericin (Nig).

The ratio of the fluorescent pH dependent to the fluorescent pH independent signals is given in Table 4.5 (above). For all buffers trialled (pH 5, 6 and 7) the ratios were equivalent to those obtained in the calibration for *Listeria monocytogenes* (Figure 4.2). However the ratios for these calibration graphs are obtained after the permeabilisation of cells with the ionophores and is measured when pH_i equals the pH of the exterior buffer.

The lack of significant pH changes measured in all the assays implies that the fluorescent signal was only measuring the pH outside the cell. A further assumption is that the pH outside the cell was equal to the pH inside the cell and that the cells were permeabilised and were not physiologically active. Recovery protocols adapted from the competent cell techniques used in molecular biology were trialled to aid the resumption of physiological activity in labelled cells. Despite trialling many variations in the length of recovery time, the temperature and composition of the media used for recovery none of the recovery treatments trialled showed differences in the physiological activity of the cells (Figure 4.3-4.11). Most of the fluorescence traces showed a flat line for the whole course of the fluorescence assay (Figures 4.3, 4.7 and 4.10). Other traces showed variation in the fluorescence signal but this variation did not correlate to the addition of the glucose or ionophores (Figures 4.4, 4.5 and 4.6). For some traces there did seem to be a change in the fluorescence on these additions (Figure 4.5c, Figure 4.8c), however the changes were very small and not significant indications of physiological activity. Fluorescence traces at lower pH tended to show a decrease in fluorescence signal over the course of an assay (Figure 4.9 and Figure 4.11).

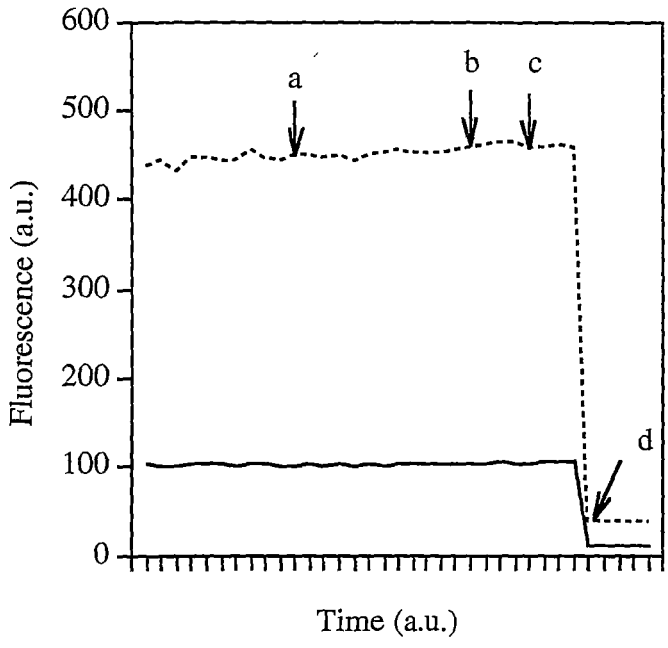


Figure 4.3 - Fluorescence signal trace from the assay of cells from treatment D4 7/10/97 in buffer pH 7. a) addition of glucose, b) addition of valinomycin, c) addition of nigericin, d) filtering of cells (fluorescence signal of supernatant). Dotted line is the fluorescent signal at 500nm and the solid line is the fluorescent signal at 440nm.

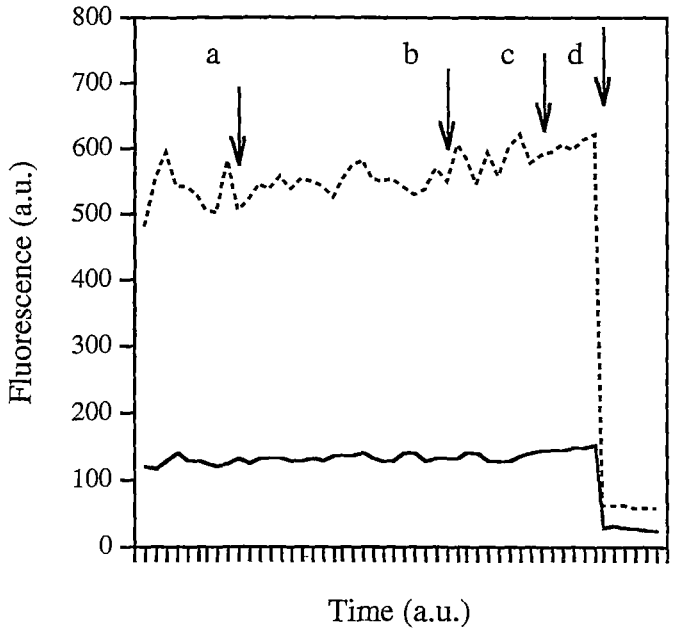


Figure 4.4 - Fluorescence signal trace from the assay of cells from treatment E5 14/10/97 in buffer pH 7. a) addition of glucose, b) addition of valinomycin, c) addition of nigericin, d) filtering of cells (fluorescence signal of supernatant). Dotted line is the fluorescent signal at 500nm and the solid line is the fluorescent signal at 440nm.

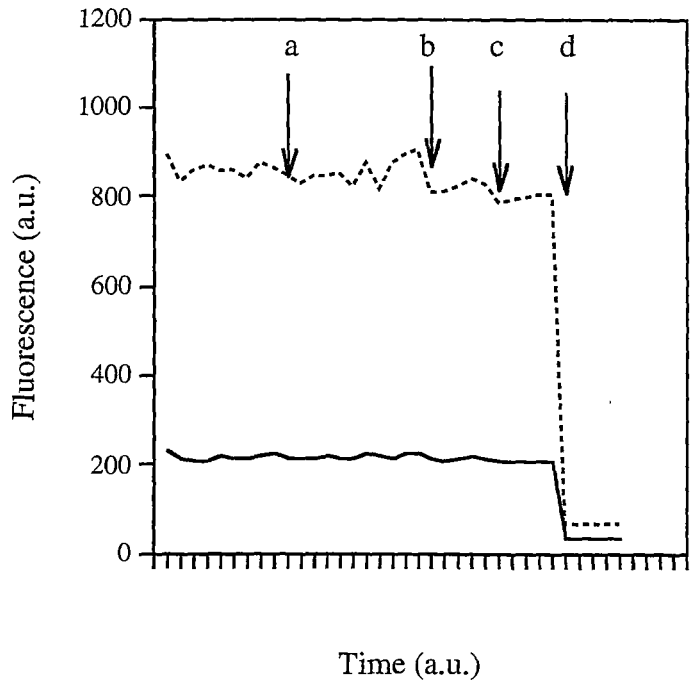


Figure 4.5 - Fluorescence signal trace from the assay of cells from treatment E6 14/10/97 in buffer pH 7. a) addition of glucose, b) addition of valinomycin, c) addition of nigericin, d) filtering of cells (fluorescence signal of supernatant). Dotted line is the fluorescent signal at 500nm and the solid line is the fluorescent signal at 440nm.

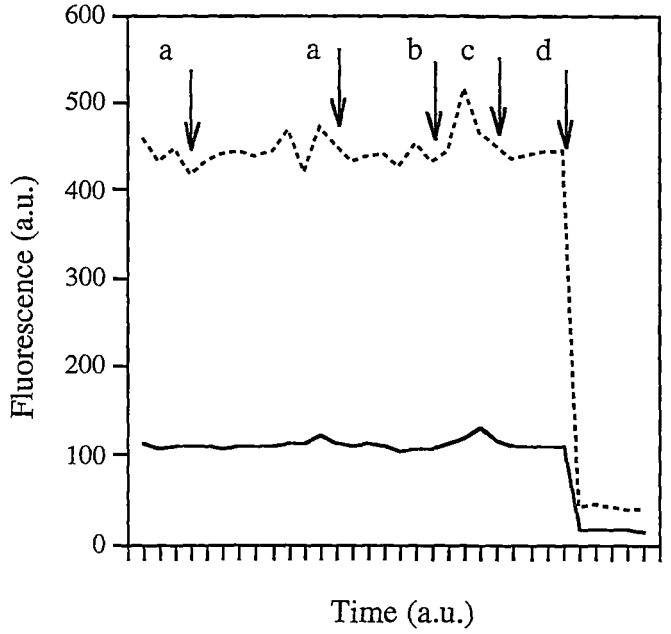


Figure 4.6 - Fluorescence signal trace from the assay of cells from treatment E8 14/10/97 in buffer pH 7. a) addition of glucose, b) addition of valinomycin, c) addition of nigericin, d) filtering of cells (fluorescence signal of supernatant). Dotted line is the fluorescent signal at 500nm and the solid line is the fluorescent signal at 440nm.

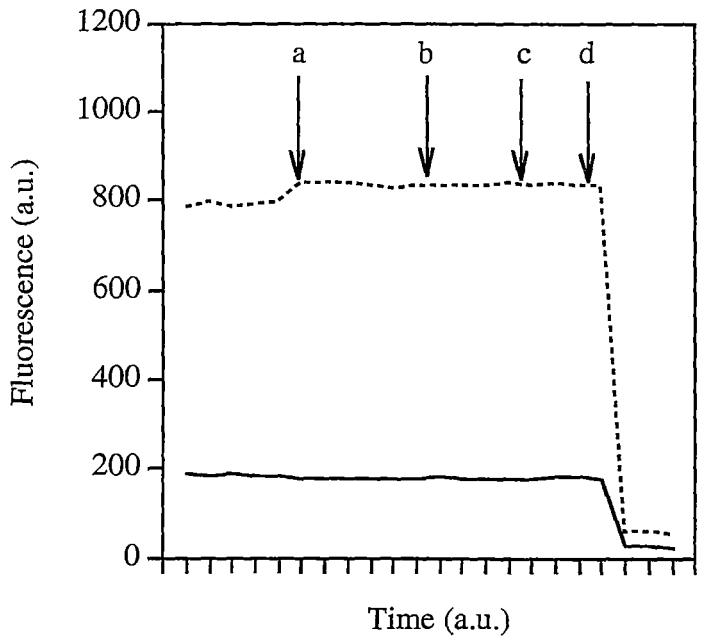


Figure 4.7 - Fluorescence signal trace from the assay of cells from treatment H4 7/11/97 in buffer pH 7. a) addition of glucose, b) addition of valinomycin, c) addition of nigericin, d) filtering of cells (fluorescence signal of supernatant). Dotted line is the fluorescent signal at 500nm and the solid line is the fluorescent signal at 440nm.

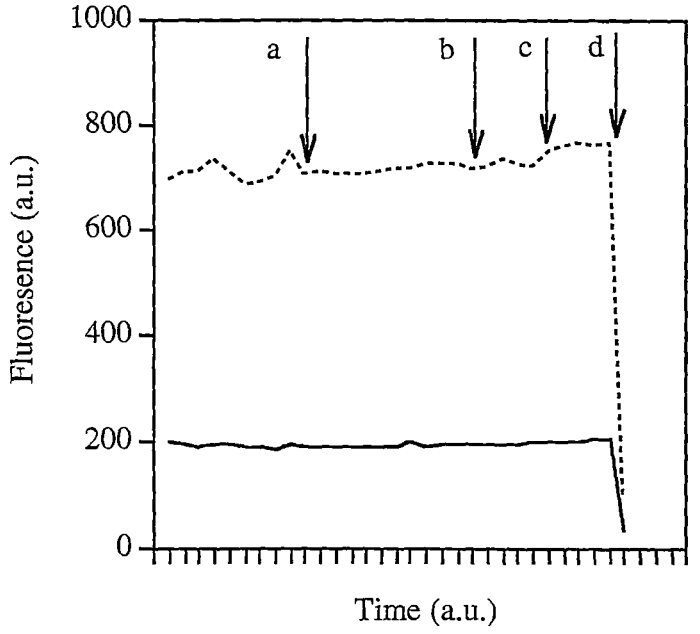


Figure 4.8 - Fluorescence signal trace from the assay of cells from treatment F5 22/10/97 in buffer pH 7. a) addition of glucose, b) addition of valinomycin, c) addition of nigericin, d) filtering of cells (fluorescence signal of supernatant). Dotted line is the fluorescent signal at 500nm and the solid line is the fluorescent signal at 440nm.

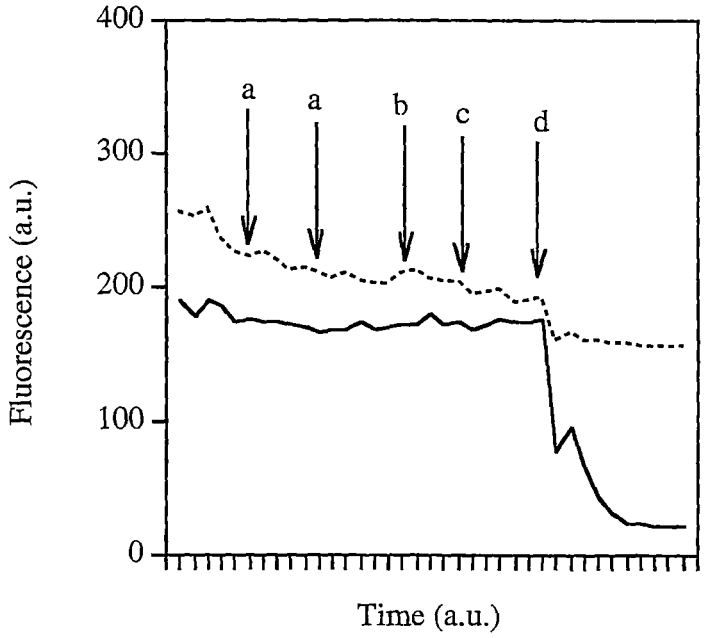


Figure 4.9 - Fluorescence signal trace from the assay of cells from treatment F5 22/10/97 in buffer pH 5. a) addition of glucose, b) addition of valinomycin, c) addition of nigericin, d) filtering of cells (fluorescence signal of supernatant). Dotted line is the fluorescent signal at 500nm and the solid line is the fluorescent signal at 440nm.

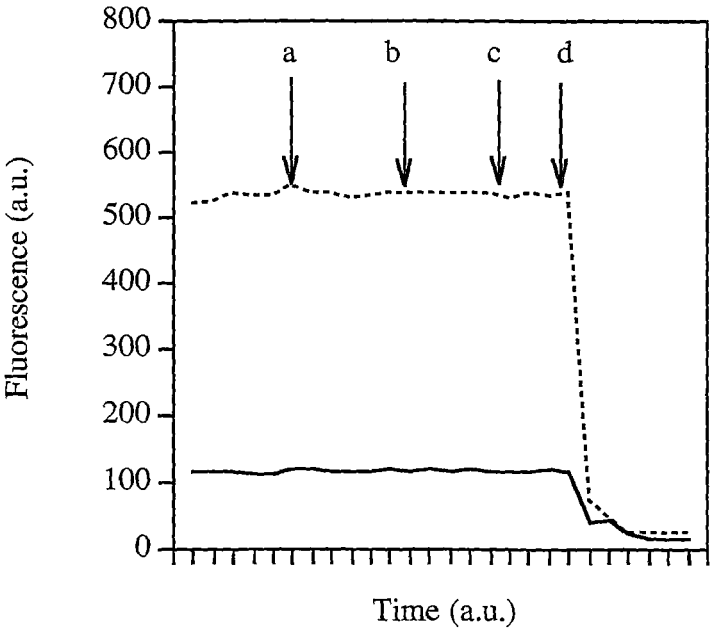


Figure 4.10 - Fluorescence signal trace from the assay of cells from treatment I3 14/11/97 in buffer pH 7. a) addition of glucose, b) addition of valinomycin, c) addition of nigericin, d) filtering of cells (fluorescence signal of supernatant). Dotted line is the fluorescent signal at 500nm and the solid line is the fluorescent signal at 440nm.

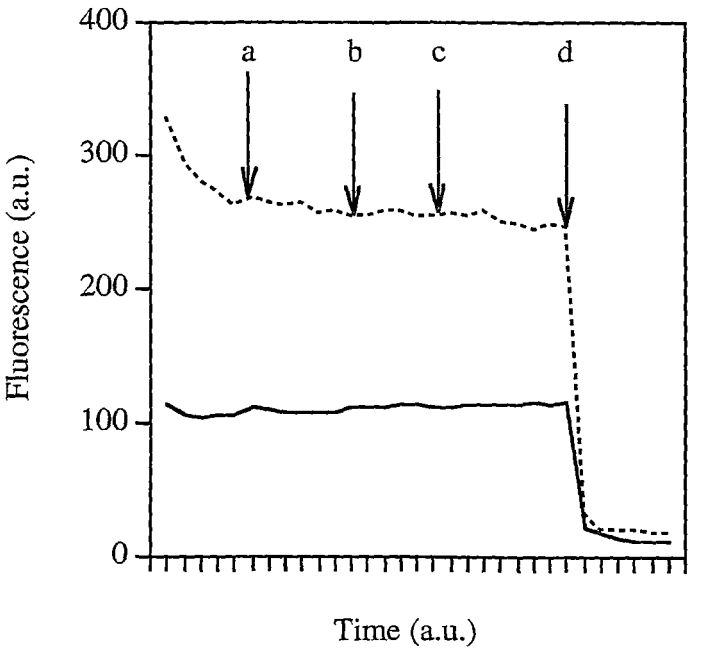


Figure 4.11 - Fluorescence signal trace from the assay of cells from treatment I3 14/11/97 in buffer pH 6. a) addition of glucose, b) addition of valinomycin, c) addition of nigericin, d) filtering of cells (fluorescence signal of supernatant). Dotted line is the fluorescent signal at 500nm and the solid line is the fluorescent signal at 440nm.

4.4.2.5 Viable Count Results for Survival of Calcium Chloride Treatment

The lack of responsive *E. coli* after calcium chloride treatment led to the hypothesis that the cells were being killed by the conditions needed to introduce the probe into the cells. In order to independently test this hypothesis viable counts of cells treated with calcium chloride were compared with cells kept in phosphate buffer at pH 7 for the same time and temperature combinations (Figure 4.12-4.14). Analysis of the data showed that the only significant variance in all the counts was that between those treated with calcium chloride and those kept in phosphate buffer at pH 7. None of the different treatment times nor the different recovery methods gave a difference to the bacterial count. Also several of the treatments trialled did not give incorporation of greater than 80%. These were all the treatments (E1-E4) in Figure 4.12, treatment E7 (Figure 4.13) and treatment E9 (Figure 4.14). The initial level of bacteria in all treatments was $1.43x10^9$.

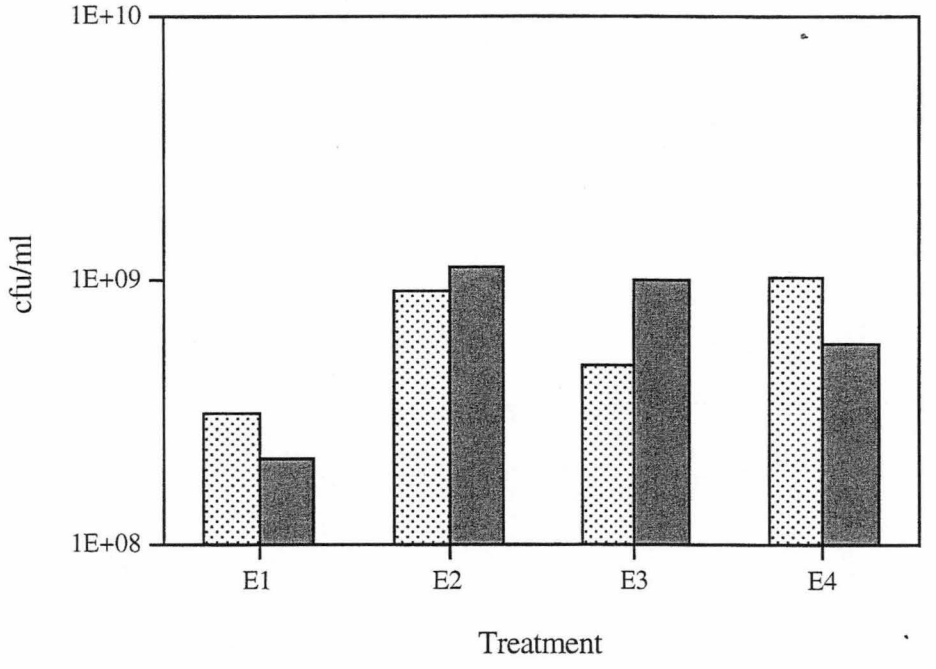


Figure 4.12 - Level of survival of *Escherichia coli* (14/10/97) between various 60min 5°C $CaCl_2$ treatments with different recovery times, E1 - none, E2 - 1 hr at 37°C, E3 - 2 hr at 25°C and E4 - 4 hr at 10°C. Dotted columns are calcium chloride treated cells, grey columns are cells suspended in phosphate buffer for the same time and temperature combinations as the calcium chloride treated cells.

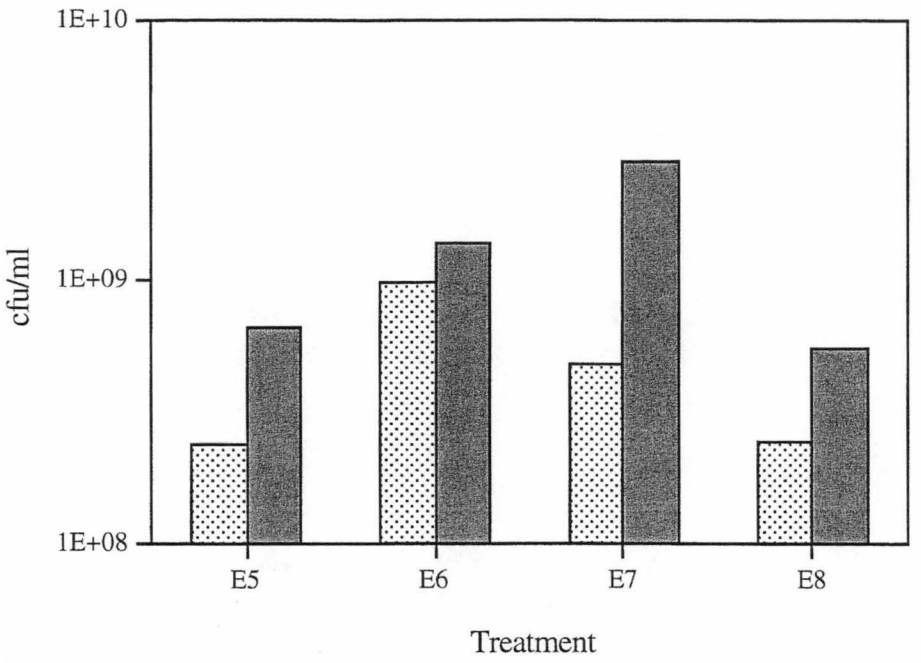


Figure 4.13 - Level of survival of *Escherichia coli* (14/10/97) between various 3 hr 5°C $CaCl_2$ treatments with different recovery times, E5 - none, E6 - 1 hr at 37°C, E7 - 2 hr at 25°C and E8 - 4 hr at 10°C. Dotted columns are calcium chloride treated cells, grey columns are cells suspended in phosphate buffer for the same time and temperature combinations as the calcium chloride treated cells.

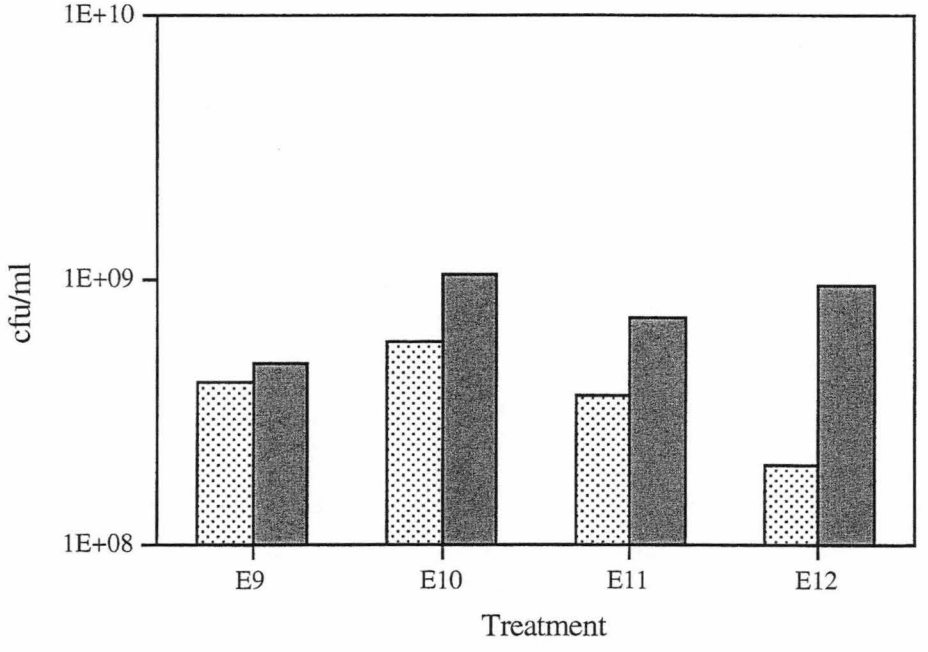


Figure 4.14 - Level of survival of *Escherichia coli* (14/10/97) between various 24 hour 5°C $CaCl_2$ treatments with different recovery times, E9 - none, E10 - 1 hr at 37°C, E11 - 2hr at 25°C and E12 - 4 hr at 10°C. Dotted columns are calcium chloride treated cells, grey columns are cells suspended in phosphate buffer for the same time and temperature combinations as the calcium chloride treated cells.

4.4.2.6 Viable Count Results for Level of Injury

Another possible explanation for the lack of physiological response was that cells were sublethally injured by the treatments and so while they still were able to grow on Nutrient Agar, they were unable to build up a pH gradient and show physiological activity during the pH_i assay. Various media were used to determine the level of sublethally injured cells. The effect of addition of NaCl to Nutrient Agar to give a water activity stress to inoculated bacteria is shown in Figure 4.15. No significant ($P > 0.05$) differences in the bacterial counts were found for any of the variables using analysis of variance.

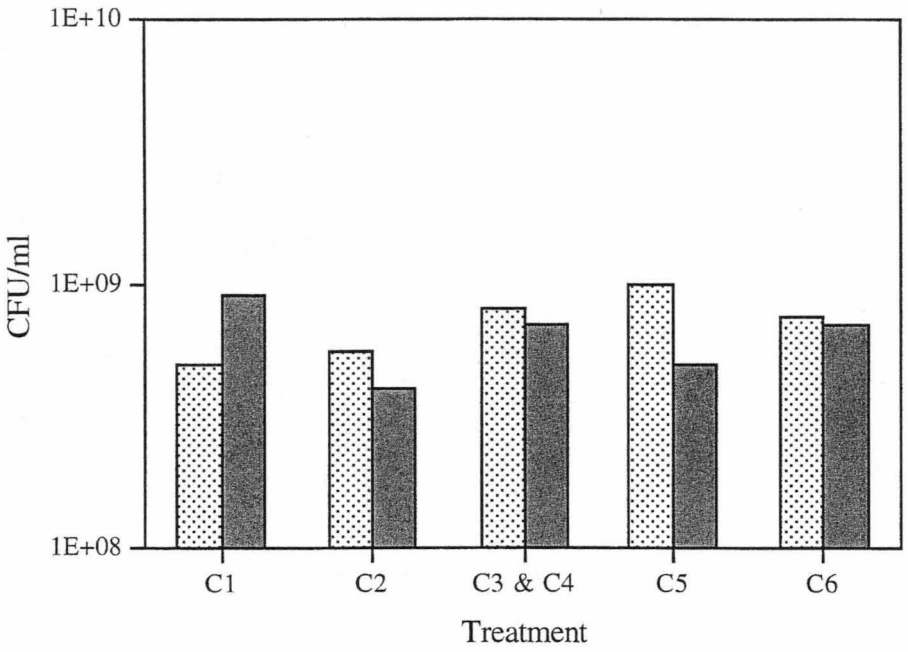


Figure 4.15 - Cell numbers (CFU/ml) of *Escherichia coli* (6/10/97) for various EDTA treatments with different probe incubation times. C1 - 15 min on ice + EDTA, C2 - 30 min on ice + EDTA, C3 and C4 - no probe + EDTA, C5 - 30 min on ice no EDTA, C6 - no probe no EDTA. Dotted columns are cells grown on Nutrient Agar and grey columns are cells grown on Nutrient Agar + 4% NaCl.

MacConkey agar was also used to show the difference in numbers between cells able to grow under optimum conditions and those able to grow under more stressful conditions. These results are shown in Figures 4.16 - 4.17. All of these treatments resulted in less than 80% incorporation except for treatment F5 (Figure 4.17). No significant ($P > 0.05$) differences in the bacterial counts were found for any of the variables using analysis of variance. The inoculum level for all treatments was 9.00×10^8.

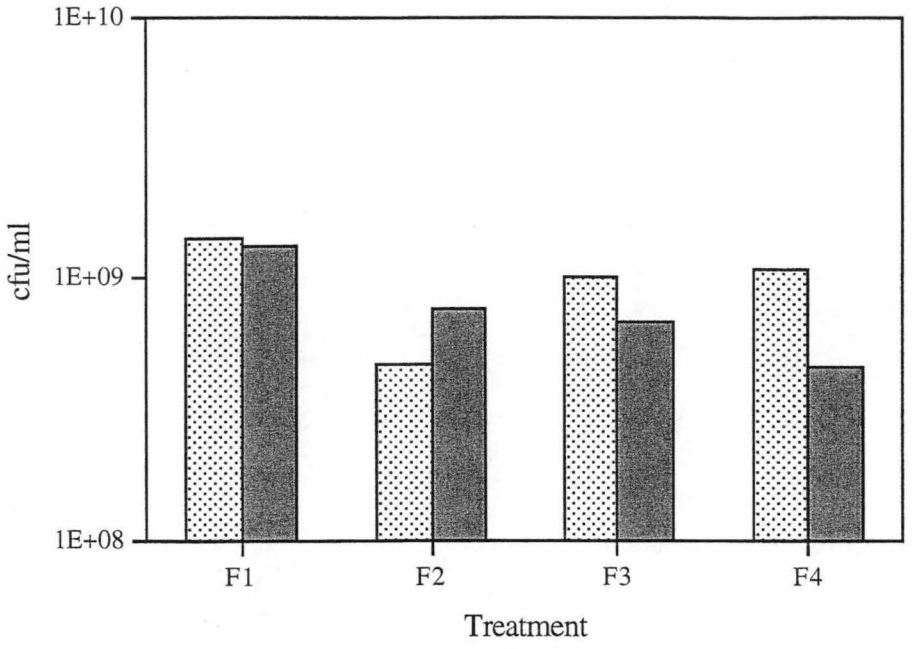


Figure 4.16 - Effect on survival of *Escherichia coli* (22/10/97) of various treatments with different recovery times. F1 - 1 hr with probe, 1.5 hr recovery at 37°C, F2 - 1 hr with probe, 2.5 hr recovery at 37°C, F3 - 1.5 hr with probe, 1.5 hr recovery at 37°C and F4 - 1.5 hr with probe, 2.5 hr recovery at 37°C. Dotted columns are cells grown on Nutrient Agar and grey columns are cells grown on MacConkey Agar.

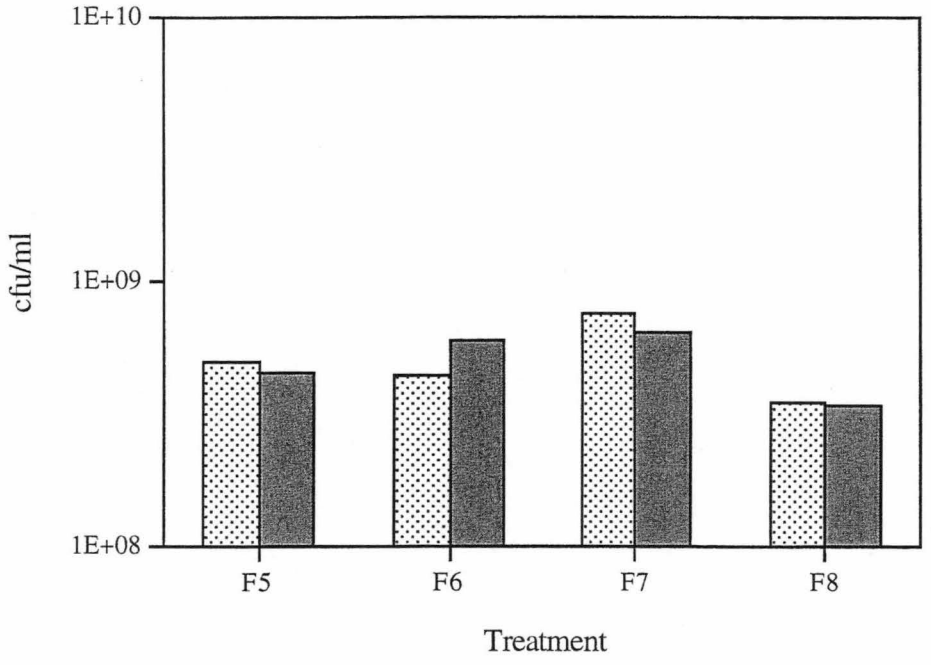


Figure 4.17 - Effect on survival of *Escherichia coli* (22/10/97) of various treatments with different recovery times. F5 - (overnight cell culture) 1.5 hr with probe, 1.5 hr recovery at 37°C; F6 - 1.5 hr with probe, 24 hours recovery at 25°C; F7 - 1.5 hr with probe, 24 hours recovery at 10°C and F8 - (overnight cell culture) 1.5 hr with probe, 24 hours recovery at 10°C. Dotted columns are cells grown on Nutrient Agar and grey columns are cells grown on MacConkey Agar.

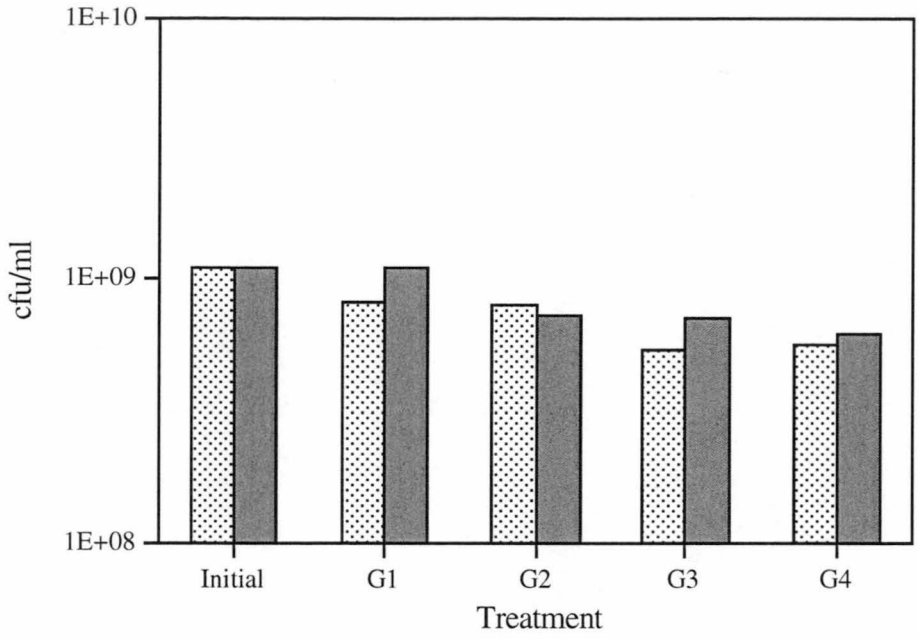


Figure 4.18 - Level of survival of *Escherichia coli* (30/10/97) between different spin speeds and diluents. G1 - phosphate buffer, normal speed; G2- peptone water, normal speed; G3 - phosphate buffer, slow speed and G4 - peptone water, slow speed. Dotted columns are cells grown on Nutrient Agar and grey columns are cells grown on MacConkey Agar.

For the final experiment the results of which are shown in Figure 4.18, MacConkey agar was used to show the difference in numbers between cells able to grow under optimum conditions and those able to grow under more stressful conditions. All of those treatments resulted in < 80% incorporation. It was suspected from the competent cell protocols that the centrifugation speeds used to pellet the cell may have caused cell death. Significant ($P < 0.05$) differences in the bacterial counts were found for the spin speed variables with the slower spin speeds leading to lower bacterial counts.

These results did not support the hypothesis as there were lower bacterial numbers with decreased spin speed which, under this hypothesis, was a more favourable condition. Also the bacterial counts themselves were very close (±0.5 log CFU), within the usual range of repeatability of plate count estimates. These results suggest that any effect of the intracellular pH treatment on the physiology of the cells did not effect their viability.

4.5 Discussion

Fluorescent techniques allow real time measurements of cytosolic conditions and their responses to the external environment (Ueckert *et al.*, 1995). Fluorescent techniques have been used successfully to measure the intracellular pH of microorganisms such as yeasts (Breeuwer *et al.*, 1994; Breeuwer *et al.*, 1995) and Gram-positive bacteria (Molenaar *et al.*, 1991; Breeuwer *et al.*, 1996; Simpson *et al.*, 1999). An example of the fluorescent signal given by a physiologically responsive *Lactococcus lactis* cells is shown in Figure 4.19. The cell is energised on the addition of a fermentable carbohydrate source, lactose. This causes the pH dependent signal to rise very quickly from 125 to 175, an increase of 40% from the original signal level (point a). The ability of the cells to control their permeability for potassium ions was damaged by the addition of the ionophore valinomycin. This leads to an efflux of hydrogen ions as the cell attempts to compensate for the lack of permeability control. Hence the pH dependent signal rises another 20% of the original signal level (point b). Then the cells were completely permeabilised by the ionophore nigericin. This leads to a drop in the signal level back to the original level before energisation (point c). The background level is determined by filtering out the cells and measuring the fluorescence of the supernatent which is less than 20% of the total signal in this example (point d).

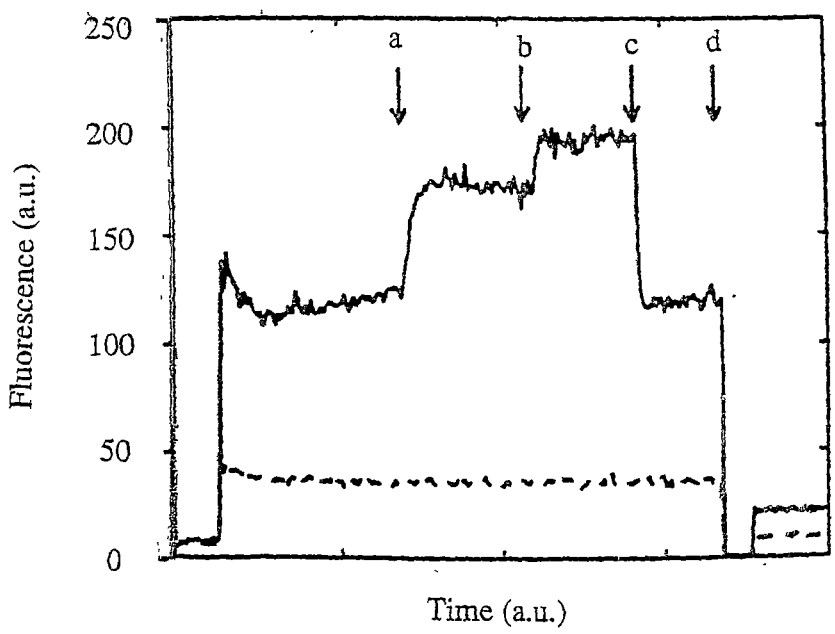


Figure 4.19 -Fluorescence at 30°C of cFSE labelled *Lactococcus lactis* cells preincubated in the presence of lactose, suspended in 50mM potassium phosphate buffer at pH 7, measured at pH dependent 490nm (solid line) and pH independent 440nm (dashed line) wavelengths with the addition of a) 10mM lactose, b) 1uM valinomycin c) 1uM nigericin and the removal d) cells filtered through a disposable disc filter (0.22um pore size). Adapted from Breeuwer *et al.*, (1996). Time for assay 20min.

Another fluorescent probe that is commonly used is BCECF. This has the advantage of being easily loaded into cells and also being highly pH sensitive. However it also has been shown to leak or be extruded from the cells, even over the short time course of an assay. This has been overcome by the use of a correction factor for the rate of leakage of the probe under specific conditions (Molenaar *et al.*, 1991) as shown in Figure 4.20. Signal A is the original trace of the fluorescence signal over time and signal B is the signal corrected for a constant rate of efflux of the probe under each set of conditions.

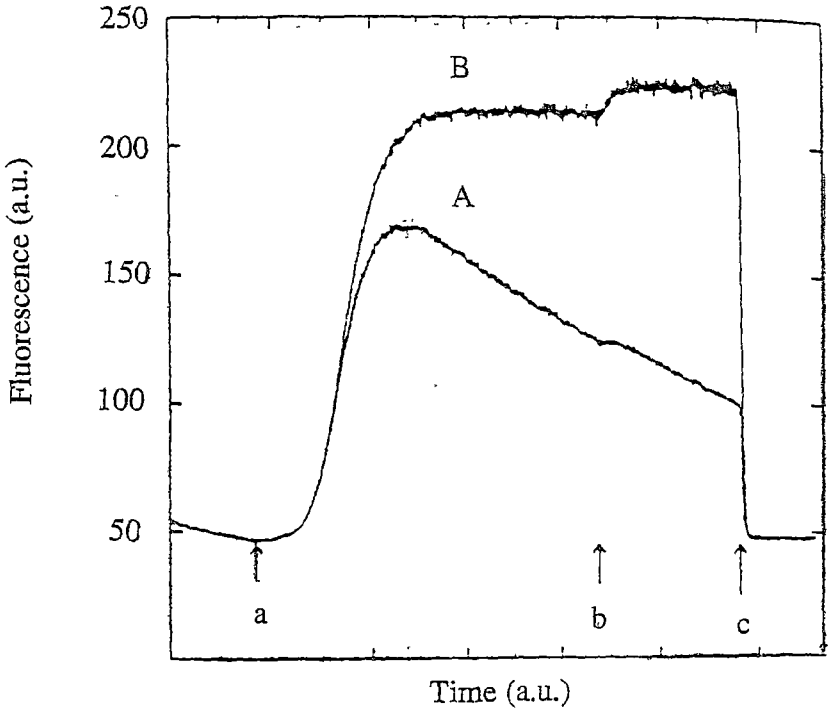


Figure 4.20 - Fluorescence at 28°C of BCECF labelled *Lactococcus lactis* cells suspended in 50mM potassium phosphate buffer at pH 6, measured at 502nm with addition of a) 2mM lactose, b) 35μM valinomycin c) 0.7μM nigericin. Adapted from Molenaar *et al.*, (1991). Time for assay 13 min.

For the fluorescent probe cFSE, the leakage problem is overcome because an enzyme reaction occurs within the cell which fixes the probe intracellularly (Breeuwer *et al.*, 1996). However, the form of the probe which is permeable for Gram-positive bacteria and yeasts is much less permeable for Gram-negatives (Breeuwer *et al.*, 1996) which have a more complex cell wall structure (Fig 4.21). Gram-negative bacteria have two membranes, the outer membrane composed of an outer lipopolysaccharide layer and an inner phospholipid layer in addition to the inner cytoplasmic membrane. Between these two structures is the periplasm, a region which contains peptidoglycan attached to lipoproteins in the outer membrane phospholipid layer but also many other proteins. These proteins are active enzymes which catalyse specific reactions which occur in the periplasm (Ferguson, 1991).

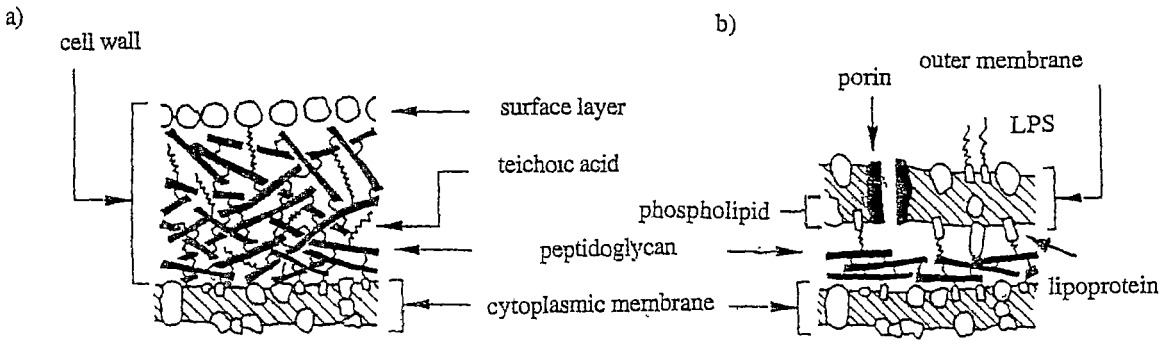


Figure 4.21 - The differences between a) Gram-positive and b) Gram-negative bacteria. The diagonal lined spaces are the membranes inside which are the white shapes of proteins embedded in the phospholipid bilayer. Adapted from Abee (1995). Not to scale.

Hence there are problems introducing the probe into the intracellular environment of Gram-negative bacteria while maintaining physiologically active cells (Breeuwer *et al.*, 1996). In the study by Breeuwer *et al.*, (1996) a treatment was used for *E. coli* to facilitate uptake of the probe. The treatment was not necessary for Gram-positive bacteria. Treatments that allow entry of the probe must by definition degrade the selectively permeable nature of the cell wall and membranes to allow the probe entry and hence cause injury to the cells. A difficult balance must be found between the permeabilisation of the cell and ensuring they retain their ability to recover and repair the damage.

Several techniques are available for the reversible permeabilisation of Gram-negative cells. Many of these are used in molecular biology to introduce DNA into bacteria, a process called the creation of 'competent' cells (Van Die *et al.*, 1983; Walker and Gingold, 1988; Castuma *et al.*, 1995). Electroporation uses an electrical pulse to disrupt the cell wall and cell membrane (Pena *et al.*, 1995). Transient incubation of these bacteria in a solution of calcium chloride ($CaCl_2$) (Walker & Gingold, 1988), concentrated acid (Molenaar *et al.*, 1991) or EDTA (Breeuwer *et al.*, 1996) causes chemical disruption of the cell wall and membranes making the cell permeable. However it is also important in these techniques that the cell is able to recover from the treatment given and subsequently return to normal physiological activity. It has been observed that strains of bacteria differ in their ability to be made permeable and then successfully return to a normal state (Murphy, *pers. comm.*).

EDTA is a chelating agent which permeabilises Gram-negative cells (Figure 4.22). At low concentrations the permeabilisation is reversible. The treatment described by Breeuwer *et al.,* (1996) involved adding 5mM EDTA to the buffer solution which contained the probe and then $MgCl_2$ to the subsequent buffer to promote recovery. The pH_i achieved by the cells using this treatment was pH 7.3 at external pH 7.0 (Breeuwer *et al.*, 1996), which was less than the normal intracellular pH of *E. coli*, pH 7.8 at external pH 7.0, when measured by other methods (Booth, 1985). While suggesting that labelling was successful, no other results for *E. coli* were given by Breeuwer *et al.,* (1996). In conclusion Breeuwer *et al.,* (1996) stated that the method required "further attention". That treatment was trialled in this study and found to be unsuccessful (Table 4.2). None of the variations of EDTA treatment resulted in the labelling of the cells. Electroporation was also unsuccessful in these studies (Table 4.4).

Calcium chloride incubations, commonly used for the acquisition of competence in molecular biology, were more successful. *E. coli* was labelled and more than 80% of fluorescence was attached to the cells, as evidenced by filtration, and no more than 20% in the buffer (Table 4.3). Acid shock treatments also resulted in the labelling of cells (Table 4.4). This success was possibly caused, not by the acid shock, but by the higher levels of acetone, as the solvent of probe solution, which were added in these experiments. Further experiments with the same amount of probe in a more concentrated solution showed a similar level of incorporation during five minute acid shock.

There were significant problems with equipment during the experiments described in this chapter. The breakdown of the cuvette heater delayed experimental work due to the much smaller response given at ambient temperatures (Figure 4.1), and the level of background fluorescence in the cuvettes occasionally interfered with measurements. Due to time and equipment constraints during a 3 month period while visiting Wye College, U.K., the opportunity to trial further treatments or different strains of *E. coli*, which may have performed better, was not available.

Under particular sets of conditions *E. coli* used in these experiments did take up label and had greater than 80% of the fluorescent signal associated with the cells. However in all cases when labelled the cells did not respond to addition of glucose, vancomycin or nigericin (Figure 4.3-4.11) even though many variations in the time and temperature of probe incubation and concentration of $CaCl_2$ were tested (Table 4.1). Experiments which reduced the severity of the treatments were not effective in bringing about normal physiological responses. Variation in time and temperature combinations of the $CaCl_2$ incubation, probe incubation and recovery were trialled. The average increase or decrease in fluorescence signals were only +6.8% and -3.9% percent of the total signal for valinomycin (Table 4.3) and +10.7% and -8.5% percent of the total signal for nigericin (Table 4.3) which is much less than the example shown in for *Lactococcus lactis* (Figure 4.19).

The lack of responses to the additions in the assay protocol implies the treatment was too harsh and had injured or permeabilised the cells. Plate counts on non-selective nutrient agar were undertaken to test this hypothesis. These showed no correlation between the small degree of injury by the $CaCl_2$ treatment (the difference in plate counts) and the level of incorporation. Some treatments which gave high levels of incorporation did show large differences in the viability of cells subjected to $CaCl_2$ and cells suspended in buffer (Figure 4.12- 4.14) however other treatments with high levels of incorporation showed little difference in the viability of cells (Figure 4.16 column A with 78% incorporation). The plate counts on selective media (MacConkey agar) were also inconclusive, and showed similar counts and even a greater count on the selective agar for one treatment (Figure 4.16 column B with 78% incorporation). This implies that the problems encountered in measuring the physiological activity of the cells might not be simply due to the lethality of the treatment or sub-lethal injuries to the labelled cells.

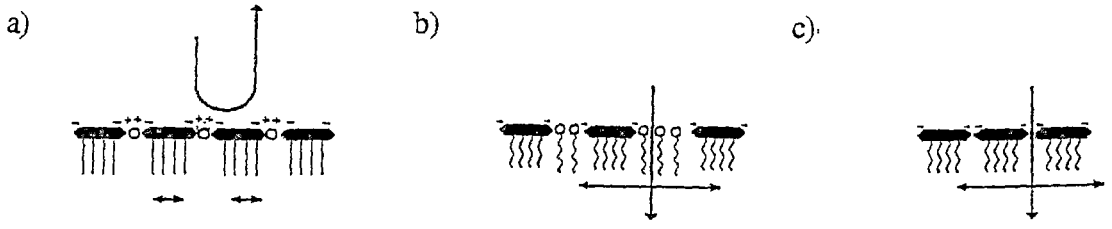


Figure 4.22 - Effect of permeabilising treatments such as EDTA on the cell outer membrane. a) a normal intact outer membrane showing lipopolysaccharide (LPS - flattened hexagons) linked by divalent cations such as Mg^{2+} or Ca^{2+} (circles) with only a small amount of lateral movement of the membrane. b) EDTA causes the removal of the divalent cations with the spaces filled by phospholipids which allow more movement of the membrane and more diffusion. c) EDTA also can cause the removal of the divalent cations and results in a disordered LPS domain which allows diffusion. Adapted from Vaara (1992).

A possible explanation for the plate count results is that the level of labelling of each cell within the population could be variable. That is, two situations may exist that were indistinguishable in the experiments undertaken. The first is that only a few cells were labelled. These may only be a small percentage of the population but are so highly and tightly labelled the level of incorporation is above the background cut off level (greater than 80%). However these highly labelled cells are injured or dead and permeabilised. Therefore the signal coming from the cells is not characteristic of the total population and does not respond to the assay conditions. This scenario is comparable to protocols for competent cells which assume that only a small proportion of the cells will successfully take up the DNA and therefore screening procedures are needed to find the transformed cells (Walker & Gingold, 1988). Alternatively all the cells in the population could be labelled at a much lower level and these labelled cells are all injured such that they do not respond to the assay conditions but they can still grow on selective media. In order to determine the subpopulation of cells which are labelled and unlabelled the use of other equipment such as a flow cytometer to count the number of cells and their degree of labelling would be required.

General conclusions from the results obtained were that a very high fluorescent signal can be caused by extracellular probe. It is possible that there was a lower signal when the probe was inside the cell because the signal was blocked or dimmed by the cell. Also the level of incorporation was very variable and could differ between essentially similar treatments which added to an inability to determine how to improve the protocol.

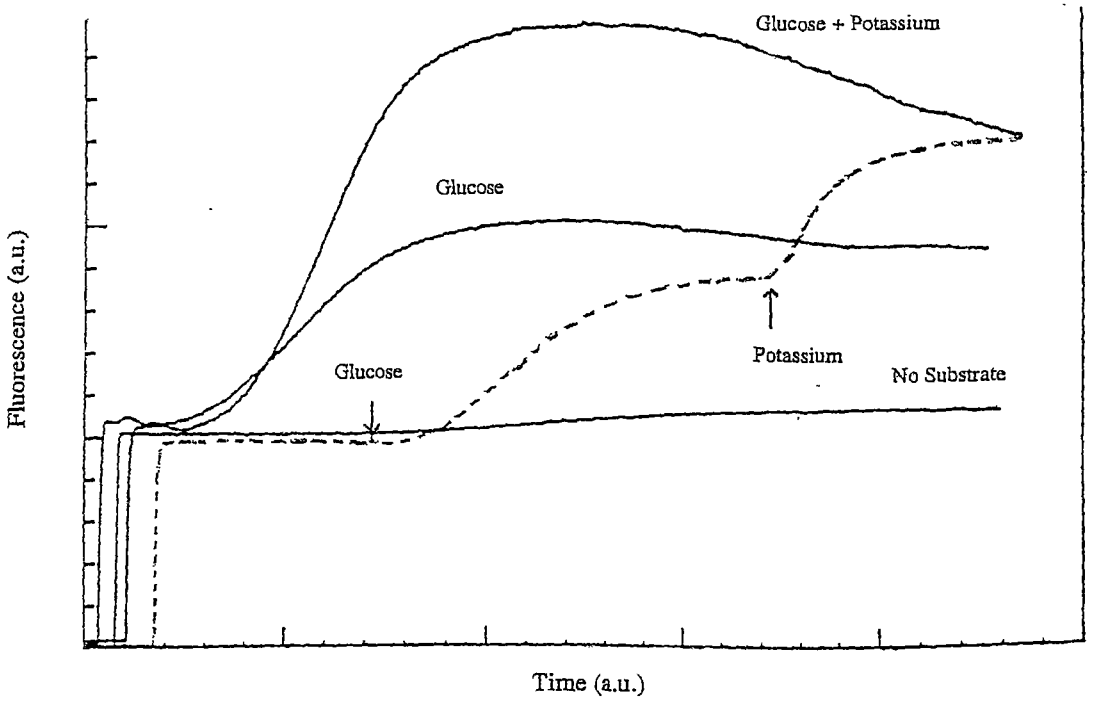


Figure 4.23 - Fluorescence at 30°C of yeast cells suspended in buffer at pH 6 measured at 460-520nm showing the addition of glucose (10mM) alone, both glucose (10mM) and potassium (10mM) and the addition of glucose (10mM) and then potassium (10mM) (dashed line). Adapted from Pena *et al.*, (1995). Time for assay 8 min.

One report exists which claims to have measured intracellular pH of Gram-negative bacteria, namely *E. coli*, using cFSE (Roindet *et al.*, 1997). Those authors altered the protocol described by Breeuwer *et al.* (1996). The pH of the probe incubation buffer was raised to 9 to help raise the intracellular pH of the cells. The reason a high pH was considered beneficial was that the probe is an amine reactive agent which reacts better at alkaline values. They also reduced the level of EDTA used as a permeability agent to 1mM, a level which they had determined by plate counts would not affect viability. During the fluorescence assay they added potassium after the addition of glucose during the assay to achieve their measurements. However other reports show that the addition of potassium alone will lead to an increase in apparent intracellular pH (Pena *et al.*, 1995). Thus the small increase (Figure 4.24) reported by Roindet *et al.*,(1997) may not really be due to the normal energised intracellular pH control by *E. coli*. Instead the increase in the pH sensitive signal may be due to the changing pH of the external environment or efflux of the probe caused by the addition of potassium. Further work is necessary to determine if the observations are reproducible and equivalent to those shown by Gram positive organisms.

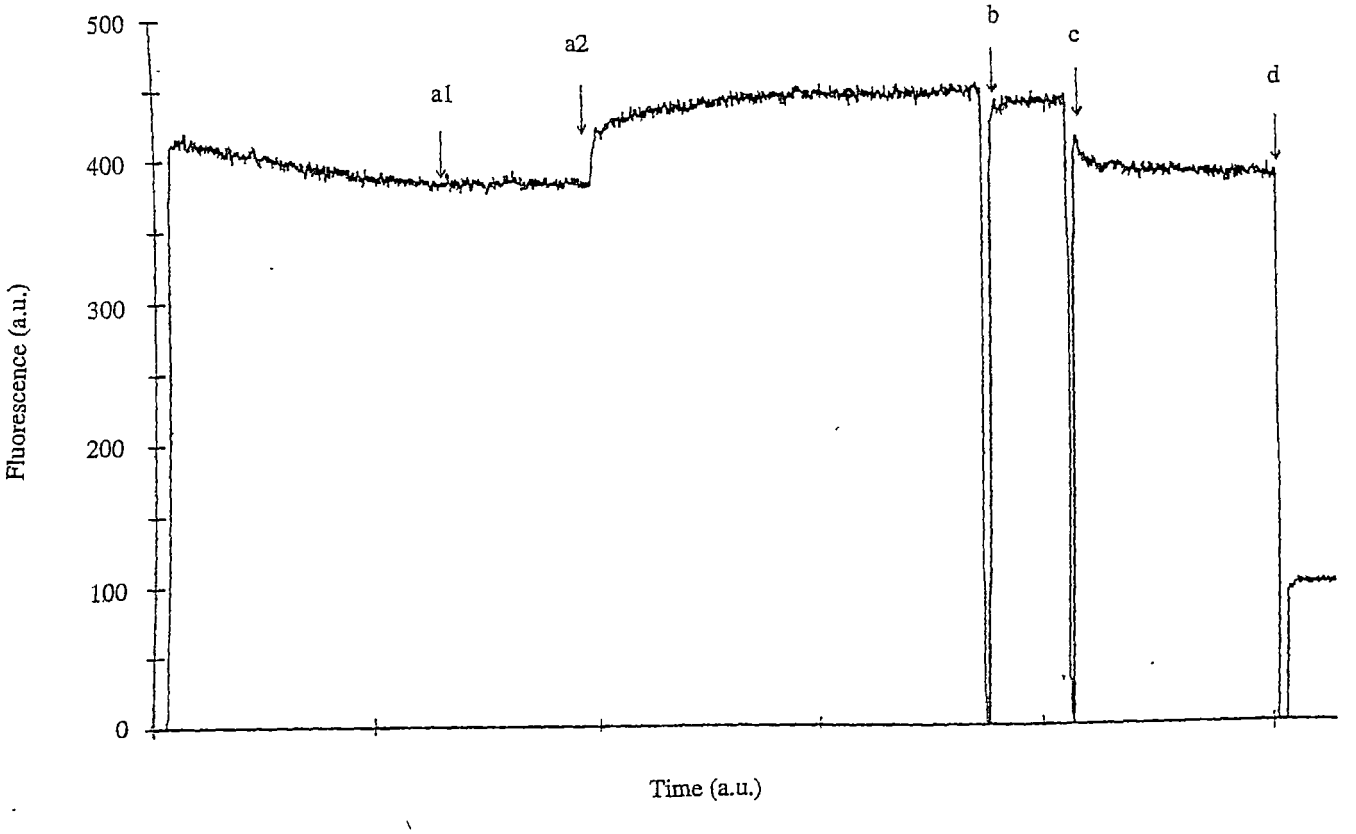


Figure 4.24 - Fluorescence at 30°C of *E. coli* suspended in buffer without potassium at pH 5 measured at 490nm with the addition of a1) 10mM glucose , a2) 50mM KCl b) valinomycin c) nigericin and the removal d) cells by filtering. Adapted from Roindet *et al.*, (1997). Time for assay 17 min.

The range of buffers used in these protocols ranged from pH 4 to 10. This is the pH growth range for *E. coli* and should not result in the leaking or death of cells when they are placed in these buffers. *L.monocytogenes* has a similar pH growth range (Tienungoon, 1998) and has been successfully labelled using this probe. Therefore, it may be assumed that the pH of buffers does not interfere with the cells ability to perform the assay. This reinforces the principle that the difference between the two species in the assay must be due to their cell wall characteristics.

A final hypothesis could explain the conflicting facts that the bacteria do not appear to be injured or dead and yet the labelled cells do not respond to the changes that could occur in the intracellular environment of a physiologically active cell. This hypothesis would also explain the difference between Gram-positive and Gram-negative cell responses other than relative permeability to the probe.

Gram-negative cell walls have a complex structure. Unlike Gram-positive bacteria there exists within the wall itself a physiologically active region called the periplasm. Also referred to as the 'periplasmic space', a name which implies an empty region outside the cell between the cell wall and the plasma membrane. This region has many constituent proteins and is the site for electron transport in denitrifying bacteria such as *Paracoccus dentrificans* (Ferguson, 1991). The composition of the periplasm is still under investigation and there is debate over the organisation and function of its constituents. It has been proposed that the periplasm exists in a gel phase that allows diffusion (Ferguson, 1991).

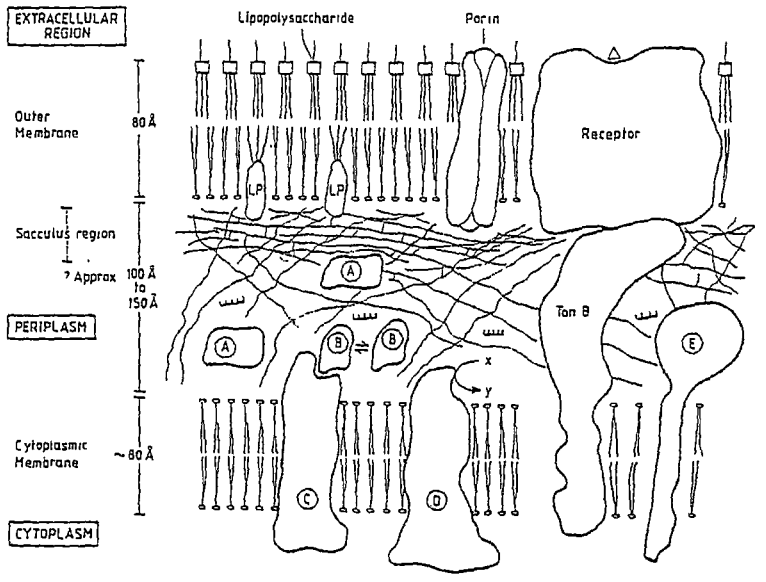


Figure 4.25 - A schematic representation of the bacterial periplasm. A-E are proteins and LP is lipopolysaccharide. Adapted from Ferguson (1991).

The existence of the complex cell wall structure in Gram-negative bacteria has been acknowledged as an important reason for the permeability problems. However, the existence of the periplasm itself could be the reason for the lack of success at intracellular pH measurements. The environment of the periplasm is not well understood but the presence of proteins in Gram-negative cells walls and not in most Gram-positive cell walls allows the possibility of the cFSE probe binding to these proteins in the periplasm instead of those inside the cells. Therefore the probe could be bound between the outer and inner membrane. The bacteria would be labelled and the probe bound and yet the probe would not be exposed to, and therefore not be able to measure, the internal pH environment of the cells. It is not known whether the pH of the periplasm is regulated and if in fact it exists as a gel phase where the pH would not be equivalent to that in an aqueous solution. This speculative hypothesis at least offers an explanation for the otherwise conflicting evidence of these experiments and further strengthens the case that further experimentation using this technique on Gram-negative bacteria is unlikely to lead to the desired outcome.

Studies on the intracellular pH response of bacteria are important to determine the mechanism of action of organic acid inhibition. Traditional descriptions of inhibition by acids have concentrated on the ability of the lipophilic undissociated acid molecule to enter the cell and, once inside, to dissociate due to the higher pH intracellularly, release protons and cause inhibition. This theory depends on the inability of the cell to counter the production of hydrogen ions or block the undissociated acid's entry to the cell. An extension of the theory is that it is the energetic drain of the removal of the excess hydrogen ions in order to maintain a high intracellular pH that restricts growth. Bracey *et al.* (1998) found that the reduction in growth rate of *Saccharomyces cerevisiae* by sorbic acid did not correlate with reduction in intracellular pH but did correlate with an increase in the intracellular ADP/ATP ratio due to an increase in ATP consumption by the cells. This implies that the organic acid induces an energetically expensive protective mechanism to maintain pH homeostasis at the cost of available energy. Krist *et al.* (1998) further support the hypothesis that energy may be important in pH inhibition as they observed that, unlike temperature or water activity stress, pH stress was energetically expensive over the range of growth and decreased final cell yield in a glucose limited batch culture.

The relationship between intracellular pH and growth has also been studied for *Listeria monocytogenes*. Both studies (Ita and Hutkins, 1991; Young and Foegeding, 1993) found a complex relationship between inhibition and intracellular pH such that the inhibition by an organic acid was not purely caused by a measured change in intracellular pH of the organism. Where the conditions were lethal, acetic acid was found to cause the greatest decrease in survival despite the fact that it did not lower the intracellular pH to the same extent as the other acids, citric and lactic (Ita and Hutkins, 1991). The effect of these organic acids on growth rate at the same intracellular pH conditions was not equal, also implying an inhibitory effect of the acids other than their ability to lower intracellular pH (Young and Foegeding, 1993) as discussed in Chapter 2. A mechanism of organic acid inhibition proposed by Russell (1993) was that maintenance of a high intracellular pH by pH homeostasis caused an accumulation of organic acid anions in the cytoplasm which was toxic to the cell's metabolism. Further studies showed a link in strains of *Escherichia coli* between resistance to acetic acid and the ability of the bacteria to survive a decreased intracellular pH which was advantageous in that it resulted in lower anion accumulation (Diez-Gonzalez and Russell, 1997).

The mechanisms by which organic acid and pH influence the metabolism and growth of bacteria and other microorganisms are not well understood. An understanding of bacterial physiological function in response to organic acid and pH could be used to better prevent growth of unwanted microorganisms in food. Studies that measure the intracellular pH of microorganisms and other indicators of their metabolic function can help to determine the mechanisms of action and suggest ways that bacterial physiology could be manipulated to prevent or inhibit growth.

5. Conclusions

The response of bacteria to environmental conditions will remain an important area of study while the problems of food spoilage and food poisoning continue. Despite modern technology the problems of food spoilage and food poisoning still occur frequently and there are new problems due to emerging pathogens and developments in the food industry such as mass production and distribution and new technologies for minimal processing. All these factors require more skills and information to provide the consumer with safe, inexpensive and palatable food.

This thesis has determined and described the response of *E.coli* to the effect of organic acids and pH. This has advanced the knowledge in this specific area and has increased the knowledge of the metabolic basis of organic acid and pH inhibition. Modelling was used to both succinctly describe the observations and to find an overall general pattern that could be used to test if the hypothesis concerning pH inhibition and the hypothesis concerning organic acid inhibition were supported by the observations.

In Chapter 2 it was found that the growth rate observations supported the hypothesis that growth rate was linearly related to hydrogen ion concentration rather than pH and that the separate additional effect of an organic acid, while complex, could be best described by the concentration of undissociated acid. The supposition that only a pH term is necessary to describe the response of bacterial growth rate to inhibition by pH and organic acid, has been made in previous attempts to model the effect of pH.This study has shown that this view is an oversimplification considering the complex interactions of pH and organic acids. By using a simple mathematical function for pH as a basis for modelling the effects of only mineral acids, previous models were limited in the conditions they could describe and were not able to describe the situation in food types where organic acids are present. Organic acids are important food preservatives and a description of their inhibitory effects is necessary to allow the development of standards for food and food handling based not on common practice or trial and error but on predictable levels of inhibition from known conditions.

In Chapter 3 the initial evolution of growth/no growth models was described. It is a process that is supported by growth rate modelling theories and practices but it is also unique with significant differences to rate modelling that need to be recognised. The current growth/no growth models are limited by their datasets. There are many examples where the collection of more data would greatly improve the knowledge of the interface. For example, for water activity 0.975 there was a gap in the data at combinations with both low temperatures (11-19°C) and low pH (4.5-5). Also in the data are anomalous observations where more replicates at these conditions should clarify the situation and give the real position of the interface.

Further the collection of many replicates of data at the narrowest range of conditions near the interface is required to allow more confident prediction that growth/no growth models really reflect the changing probability of growth and whether that change is abrupt or whether it shows a gradual change in the probability as conditions become more or less stringent.

The primary requirement to produce more and better growth rate and growth/no growth models is more data. Firstly, for the organisms for which models already exist more data needs to be collected over a larger range of conditions to fully describe all the inhibitory effects, for example those of high temperature and for high pH, for which little or no data have been collected. These conditions were a low priority because they are not common in foods but knowledge of the responses are needed for completeness and to balance the models between the inhibitory effects of suboptimal and superoptimal conditions. There are also combinations of conditions for which no data have been collected, for example for *E.coli* at low water activity in the presence of lactic acid. Combinations of interest in foods give a large scope for new experimental work that will greatly add to the usefulness and applicability of these models. Due to time and resource constraints not all combinations of factors could be determined in this study.

Finally there are many more organisms of concern to the food industry whose growth responses to temperature, water activity, pH and lactic acid could be determined using experiments such as those described here. There are also other organic acids, such as citric acid, which are used in the food industry and would be important in some foods in limiting the growth of spoilage or pathogenic organisms. Experiments similar to those described here, to collect growth rate and growth/no growth data for use in modelling studies would determine whether there was a similar response for these different organisms.

In Chapter 4 the attempt to explore the basis of pH and organic acid inhibition was unsuccessful. While the technique for measuring intracellular pH using the carboxyfluorescein probe seems robust and simple for Gram-positive organisms, the difficulties encountered for Gram-negative organisms seem to outweigh the advantages of the technique. This said, there remain many other methods available to try and determine these questions, whether the mechanism of inhibition involves the lowering of internal pH or the presence intracellularly of high concentrations of undissociated organic acids. Other probes for intracellular pH exist and new methods are under development for the measurement of ion fluxes from microbial biofilms that could help elucidate the microbial responses. The ultimate goal for predictive microbiology is to gain a through understanding of the physiology of microorganisms so that their responses can be modelled on a mechanistic rather than an empirical basis. To this end this study has added to the available knowledge and given an indication of what further needs to be learned to give a more complete understanding of microbial responses to pH and organic acids.

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Appendices

1.1 Organisms & Maintenance

E. coli BR was a commensal isolate and was obtained from Mr. Craig Shadbolt (School of Agricultural Science, GPO Box 252-54, University of Tasmania, Hobart, Tasmania, Australia).

E. coli SB 1 was a commensal isolate and was obtained from Ms. S. Bettiol (Division of Pathology, GPO Box 252-29, University of Tasmania, Hobart 7001, Tasmania, Australia).

E.coli M23 was a commensal isolate used for a long period in undergraduate teaching so therefore a laboratory strain. It was obtained from Ms. L. Hayward (School of Agricultural Science, GPO Box 252-54, University of Tasmania, Hobart 7001, Tasmania, Australia)

E.coli MJR was a recent commensal isolate from healthy infant faeces and was obtained from Dr. T. Ross (School of Agricultural Science, GPO Box 252-54, University of Tasmania, Hobart 7001, Tasmania, Australia)

E.coli FT1 and *E.coli* YY (YYC1106) were strains obtained by Ms. J. Brown (School of Agricultural Science, GPO Box 252-54, University of Tasmania, Hobart 7001, Tasmania, Australia) from Dr. Y.Y. Chang (University of Illinois, Department of Microbiology, B103 Chemical and Life Science Laboratory, MC-110, 601 South Goodwin Avenue, Urbana IL 61801). FT1 is the parental strain of YY which was a cyclopropane fatty acid (cfa) negative mutant produced by Kan insertion in the cfa gene. These strains are described in detail by Taylor, F. and Cronan, J.E., Jnr (1976) Journal of Bacteriology, **125** (2) 518-523 for FT1 and Chang, Y.Y. and Cronan, J.E., Jnr (1999) Molecular Microbiology **33** (2) 249-259.

The following strains were obtained from Mr. M. Salter (School of Agricultural Science, GPO Box 252-54, University of Tasmania, Hobart, Tasmania, Australia)

Code	Serotype	Source	Location	Toxins
R10	0126:H21	Water	Lauderdale Canal	
R31	NT*	Water	Gypsy Bay	Stxl
R91	081:H-	Pork	-	Stx2
R172	088:H-	Water	McRobies Gully	Stxl

Cultures were maintained on NA slopes and kept at 4°C, subcultured about every 2 months and plated out onto NA then EMB to check for purity.

1.2 Media

Nutrient Broth NB
OXOID CM1

Formula - "Lab-Lemco" Powder	1.0	g/L
Yeast Extract	2.0	g/L
Peptone	5.0	g/L
Sodium Chloride	5.0	g/L

pH - 7.4 ± 0.2

13g added to 1L of distilled water. Mixed well and distributed into final containers. Sterilized by autoclaving at 121°C for 15 minutes.

Nutrient Agar NA
Nutrient agar was prepared from nutrient broth (as above) with 15 gL-l Davis powdered agar added (Davis Gelatine (Australia) Co., 28 Spring St., Botany, Sydney, Australia. Mixture heated to boiling prior to autoclaving to ensure dissolution of agar.

Eosin Methylene Blue Agar (Levine) EMB
OXOID CM69

Formula - Peptone	10.0	g/L
Di-potassium hydrogen phosphate	2.0	g/L
Eosin Y	0.4	g/L
Methylene blue	0.06	g/L
Agar	15.0	g/L

pH 6.8 ± 0.2

Eosin methylene blue agar (Oxoid, CM69) was prepared and sterilised according to manufacturer' s instructions from commercially prepared dehydrated media (Oxoid,Unipath Ltd,. Basingstoke, Hampshire, England.
37.5g was suspended in 1L of distilled water and brought to boil to dissolve completely and sterilised by autoclaving at 121°C for 15 minutes. Cooled to 60°C and shaken in order to have oxidised the methylene blue (i.e. restored its blue colour) and to have resuspended the precipitate which is an essential part of this medium.

E.coli colonies - isolated colonies, 2-3mm diameter, with little tendency to confluent growth, exhibiting a green metallic sheen by reflected light and dark purple centres by transmitted light.

1.3 Software

Chemdraw™ 2.1.3: Cambridge Scientific Computing Inc, Cambridge, MA, USA
Cricket Graph 1.3.2: Cricket software, Malvern, PA, USA
Excel 5.0: Microsoft Corporation, One Microsoft Way, Redmond, WA, USA
New Cricket Graph 3: Cricket software, Malvern, PA, USA
SAS: Statistical Analysis System, SAS Institute Incorporated, SAS Circle, Cary, N.California, USA.
Ultrafit 3.0: ©Biosoft, 37 Cambridge Place, Cambridge, UK

1.4 Reagents

General reagents were obtained from a variety of chemical suppliers:

Acetic acid
Calcium chloride ($CaCl_2$)
EDTA
Magnesium chloride ($MgCl_2$)
Glucose
Hydrochloric acid (HCl)
Sodium hydroxide (NaOH)
Sodium chloride (NaCl)
Sodium lactate
HEPES buffer (50mM potassium N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)
Glycine buffer (50mM adjusted to pH with NaOH or HCl)
Potassium phosphate (50mM adjusted to pH with NaOH or HCl)
Citric Acid (50mM adjusted to pH with NaOH or HCl)

Reagents specifically obtained:

Lactic Acid (Min. 88% w/w) Univar, AR. Ajax Chemicals, Auburn, Australia.

150µM Valinomycin (Val) Sigma V0627

150µM Nigericin (Nig) Sigma V-7143

Probe Solution

100µM 5 (and 6-)-carboxyfluorescein diacetate succinimidyl ester (solution in acetone) Molecular Probes Europe, Leiden, Netherlands.

Water
All water used in the preparation of reagents and media was prepared by glass distillation of deionised water.

1.5 Consumables

Petri Dishes
Sterile plastic Petri dishes 150x860 mm (LABSERV, Australia), and 150x560 mm (Disposable Products, South Australia).

Sterile Filter Units, 25mm Activon, 0.45 µm pore size cellulose acetate membrane

Sterile Filter Units, 25mm Nalgene, 0.22 µm pore size

Sterile Well Plates
Linbro® Tissue Culture multi-well plate with cover
24 Flat bottom wells 1.7 x 1.6cm approx.
Well Capacity : 3.5ml approx., Area per well : 2.0 cm^2
Flow Laboratories, Inc. McLean, Virginia U.S.A.

1.6 Equipment

Temperature Gradient Incubator (TGI)
Two models of Advantec temperature gradient incubators were used:
Advantec TN.3, Advantec, Toyo Roshi International, California, USA. (required constant temperature room maintained at 20°C for isothermal operation)
Advantec TN-2148, Advantec MFS, Inc., 6691 Owens Drive, Pleasanton, CA 94588, USA. (capable of isothermal operation when ambient temperature is fluctuating)

L-Tubes
L- Shaped Glass Tube, 1.5cm diameter, capacity approximately 25mL.
Topped with metal cap, used to contain individual cultures grown in TGI

Thermometer
Fluke® 51K/J thermometer, John Fluke Manufacturing Co. Inc, 1150 W. Euclid Avenue, Palatine, Illinois, 60067, U.S.A. An electronic thermometer with Iron-Constantan thermocouple bead probe. Calibration was checked periodically at 0°C and 100°C.
Accuracy +/- 0.5°C
Resolution +/- 0.1°C

Spectrophotometers
Spectronic 20 (analogue display) or 20D (digital display) spectrophotometers (Spectronic Instruments Inc., 820 Linden Avenue, Rochester, NY 14625, USA) (Milton Roy Co., U.S.A.)

pH Meter
Model 250A Portable pH/Temperature/mV meter
(Orion Research Inc, Boston, MA 02129, U.S.A.)
Accuracy : +/- 0.02
Resolution : 0.01
Range : -2.00 - 19.99
with Activon AEP433 calomel sealed flat tip probe
(Activon Scientific Products Co. Pty. Ltd., 2A Pioneer Avenue, Thornleigh, PO Box 505, Pennant Hills, NSW, Australia, 2120)

Water activity meter
Aqualab Model CX2.
Decagon Devices Inc., PO Box 835, Pullman, Washington 99163, U.S.A.
Accuracy : +/- 0.003
Range : 0.03 - 1.000
Resolution : +/-0.001

Timers
Alarm clock style timers (e.g. Model 870A, Jadco, China) and were used for all growth rate experiments, timer set to zero and the real time at time zero recorded in case of timer failure.

Balances
Mettler PJ3600 DeltaRange®, Mettler Instruments AG, CH8608 Greifensee-Zurich, Switzerland

1.4 Equipment (continued)

Pipettors
A range of fixed and variable volume pipettors were used:

- Fixopet: 100 µL (fixed), 1000 µL (fixed); Pluripet: 200-1000 µL, Kartell Spa Via, Delle Industrie, 1 20082 Noviglio, Milan, Italy.
- Pipetman®: 20-200 µL, 200-1000 µL, Gilson Medical Electronics (France) S.A., B.P. 45-95400, Villiers-le-Bel, France.
- Oxford Macro-set: 5-10 mL, Oxford Labware, Division of Sherwood Medical, St Louis, MO 63103 USA.
- 'Eppendorf': 10-100 µl
- Electronic Digital Pipette 'EDP-Plus Motorized Microliter Pipette' (Rainin Instrument Co, Inc., Mack Road, Woburn, MA 0188-4026 USA). 1-10ml

Waterbaths
Ratek SWB20D shaking waterbaths, Ratek Instruments Pty Ltd, Unit 1/3 Wadhurst Drive, Boronia, VIC, Australia, 3155.

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).

Spectrofluorometer
Model 3000 fluorescence spectrometer, Perkin-Elmer, Beaconsfield, Buckinghamshire, U.K.

Centrifuge
IEC Micromax Ventilated Microcentrifuge, International Equipment Company, Needham Heights, MA 02194, U.S.A.

Spectrometer
Cecil 200 series (CE 2020)

pH meter
ELE International Limited pH meter

Appendix 2.1 - Growth rate of nonpathogenic *Escherichia coli* strains in nutrient broth at various temperatures, water activities and pHs.

Strain	pH	T (°C)	Aw	Rate (1/GT(min))
M23	4.02	21.08	0.996	0.00227
M23	4.07	21.12	0.996	0.00466
M23	4.13	21.14	0.996	0.00551
M23	4.27	22.06	0.996	0.00615
M23	4.39	21.18	0.996	0.00722
M23	4.6	21.20	0.996	0.00884
M23	4.88	22.18	0.996	0.01110
M23	5.14	22.02	0.996	0.01046
M23	5.27	21.26	0.996	0.01122
M23	5.53	22.48	0.996	0.01248
M23	5.86	22.10	0.996	0.01222
M23	6.14	22.06	0.996	0.01228
M23	6.15	22.12	0.996	0.01253
M23	6.58	22.04	0.996	0.01228
M23	6.88	21.26	0.996	0.01158
M23	6.94	22.12	0.996	0.01306
M23	7.14	22.00	0.996	0.01290
M23	7.44	22.26	0.996	0.01252
M23	7.6	22.26	0.996	0.01286
M23	7.88	22.62	0.996	0.01167
M23	4.1	21.63	0.996	0.00470
M23	4.61	21.63	0.996	0.00946
M23	4.8	21.63	0.996	0.01058
M23	5.04	21.60	0.996	0.01182
M23	5.17	21.53	0.996	0.01179
M23	5.35	21.53	0.996	0.01191
M23	5.59	21.50	0.996	0.01191
M23	5.87	21.50	0.996	0.01247
M23	6.36	21.50	0.996	0.01212
M23	6.79	21.43	0.996	0.01258
M23	7.29	21.43	0.996	0.01209
M23	7.59	21.43	0.996	0.01267
M23	8.11	21.43	0.996	0.01182
M23	4.09	21.70	0.996	0.00254

Appendix 2.1 - Growth rate of nonpathogenic *Escherichia coli* strains in nutrient broth at various temperatures, water activities and pHs.

Strain	pH	T (°C)	Aw	Rate (1/GT(min))
MJR	4.09	22.27	0.996	0.00603
MJR	4.62	22.27	0.996	0.01135
MJR	4.81	22.27	0.996	0.01179
MJR	5.04	22.17	0.996	0.01290
MJR	5.18	22.17	0.996	0.01325
MJR	5.35	22.10	0.996	0.01412
MJR	5.62	22.10	0.996	0.01455
MJR	5.85	22.07	0.996	0.01407
MJR	6.37	21.97	0.996	0.01363
MJR	6.75	21.90	0.996	0.01409
MJR	7.2	21.90	0.996	0.01415
MJR	7.59	21.80	0.996	0.01407
MJR	8.1	21.80	0.996	0.01293
MJR	4.08	21.43	0.996	0.00426
MJR	4.19	21.37	0.996	0.00614
MJR	4.3	21.33	0.996	0.00769
MJR	4.38	21.33	0.996	0.00818
MJR	4.44	21.27	0.996	0.00866
MJR	4.53	21.23	0.996	0.00830
MJR	4.74	21.17	0.996	0.01050
MJR	4.96	21.07	0.996	0.01138
BR	4.01	21.30	0.996	0.00243
BR	4.3	21.30	0.996	0.00558
BR	4.41	21.23	0.996	0.00574
BR	4.56	21.23	0.996	0.00638
BR	4.73	21.17	0.996	0.00710
BR	4.94	21.10	0.996	0.00764
BR	5.18	21.10	0.996	0.00869
BR	5.56	21.10	0.996	0.00983
BR	6.17	21.03	0.996	0.01008
BR	6.68	21.00	0.996	0.01004
BR	7.02	20.97	0.996	0.01042
BR	7.87	20.90	0.996	0.00348

Appendix 2.1 - Growth rate of nonpathogenic *Escherichia coli* strains in nutrient broth at various temperatures, water activities and pHs.

Strain	pH	T (°C)	Aw	Rate (1/GT(min))
SB	4.03	20.80	0.996	0.00366
SB	4.23	20.70	0.996	0.00673
SB	4.4	20.67	0.996	0.00805
SB	4.54	20.67	0.996	0.00868
SB	4.71	20.60	0.996	0.00994
SB	4.94	20.60	0.996	0.01078
SB	5.2	20.57	0.996	0.01219
SB	5.59	20.57	0.996	0.01262
SB	6.19	20.53	0.996	0.01286
SB	6.68	20.53	0.996	0.01314
SB	7.02	20.50	0.996	0.01279
SB	7.87	20.50	0.996	0.01279
YY - Mutant	4.6	21.63	0.996	0.00321
YY - Mutant	4.79	21.63	0.996	0.00331
YY - Mutant	4.99	21.60	0.996	0.00341
YY - Mutant	5.14	21.60	0.996	0.00380
YY - Mutant	5.29	21.60	0.996	0.00475
YY - Mutant	5.51	21.60	0.996	0.00640
YY - Mutant	5.75	21.60	0.996	0.00782
YY - Mutant	6.27	21.53	0.996	0.00813
YY - Mutant	6.69	21.53	0.996	0.00799
YY - Mutant	7.25	21.53	0.996	0.00799
YY - Mutant	7.56	21.50	0.996	0.00741
YY - Mutant	8.06	21.53	0.996	0.00727
FT1 - Parent	4.1	22.30	0.996	0.00495
FT1 - Parent	4.6	22.30	0.996	0.00739
FT1 - Parent	4.78	22.30	0.996	0.00701
FT1 - Parent	5.01	22.20	0.996	0.00742
FT1 - Parent	5.12	22.13	0.996	0.00674
FT1 - Parent	5.28	22.10	0.996	0.00673
FT1 - Parent	5.53	22.10	0.996	0.00709
FT1 - Parent	5.77	22.03	0.996	0.00757
FT1 - Parent	6.27	22.00	0.996	0.00881
FT1 - Parent	6.69	21.90	0.996	0.00841
FT1 - Parent	7.26	21.90	0.996	0.00835
FT1 - Parent	7.54	21.90	0.996	0.00847
FT1 - Parent	8.07	21.80	0.996	0.00793
FT1 - Parent	4.36	20.63	0.996	0.00348
FT1 - Parent	4.43	20.63	0.996	0.00322
FT1 - Parent	4.49	20.53	0.996	0.00402
FT1 - Parent	4.47	20.47	0.996	0.00330
FT1 - Parent	4.71	20.47	0.996	0.00405

Appendix 2.2 - Growth rate of pathogenic *Escherichia coli* strains in nutrient broth at various temperatures, water activities and pHs.

Strain	pH	T (°C)	Aw	Rate (1/GT(min))
R10	3.81	22.45	0.996	0.00267
R10	4.04	22.45	0.996	0.00428
R10	4.28	22.45	0.996	0.00668
R10	4.39	22.40	0.996	0.00864
R10	4.56	22.40	0.996	0.00860
R10	4.73	22.35	0.996	0.01063
R10	4.9	22.35	0.996	0.01210
R10	5.16	22.30	0.996	0.01299
R10	5.53	22.25	0.996	0.01370
R10	6.09	22.20	0.996	0.01473
R10	6.67	22.15	0.996	0.01407
R10	6.93	22.10	0.996	0.01473
R10	7.67	22.05	0.996	0.01357
R31	3.87	21.90	0.996	0.00213
R31	4.08	21.90	0.996	0.00451
R31	4.29	21.90	0.996	0.00739
R31	4.41	21.90	0.996	0.00865
R31	4.56	21.85	0.996	0.01009
R31	4.66	21.80	0.996	0.01166
R31	4.88	21.75	0.996	0.01224
R31	5.13	21.70	0.996	0.01345
R31	5.48	21.70	0.996	0.01347
R31	6.06	21.70	0.996	0.01468
R31	6.6	21.70	0.996	0.01457
R31	6.91	21.65	0.996	0.01485
R31	7.75	21.65	0.996	0.01430

Appendix 2.2 - Growth rate of pathogenic *Escherichia coli* strains in nutrient broth at various temperatures, water activities and pHs.

Strain	pH	T (°C)	Aw	Rate (1/GT(min))
R91	3.92	22.15	0.996	0.00369
R91	4.12	22.15	0.996	0.00537
R91	4.33	22.15	0.996	0.00822
R91	4.46	22.15	0.996	0.00984
R91	4.59	22.15	0.996	0.01157
R91	4.76	22.20	0.996	0.01208
R91	4.95	22.25	0.996	0.01323
R91	5.24	22.30	0.996	0.01378
R91	5.6	22.40	0.996	0.01476
R91	6.27	22.40	0.996	0.01592
R91	6.67	22.40	0.996	0.01574
R91	6.95	22.40	0.996	0.01612
R91	7.74	22.45	0.996	0.01521
R172	3.9	21.70	0.996	0.00273
R172	4.11	21.70	0.996	0.00538
R172	4.32	21.70	0.996	0.00716
R172	4.45	21.70	0.996	0.00756
R172	4.59	21.75	0.996	0.00981
R172	4.75	21.75	0.996	0.01041
R172	4.91	21.85	0.996	0.01179
R172	5.16	21.85	0.996	0.01226
R172	5.48	21.85	0.996	0.01337
R172	6.12	21.85	0.996	0.01427
R172	6.65	21.90	0.996	0.01436
R172	6.92	21.90	0.996	0.01399
R172	7.67	21.95	0.996	0.01307

Appendix 2.3 - Growth rate of *Escherichia coli* M23 in nutrient broth with acetic acid at various temperatures, water activities and pHs.

[AAC] mM	[U] mM	[D] mM	pH	T (°C)	Aw	Rate (1/GT(min))
0	0.00	0	4.04	20.63	0.996	0.00461
0	0.00	0	4.29	20.70	0.996	0.00641
0	0.00	0	4.43	20.67	0.996	0.00781
0	0.00	0	4.62	20.63	0.996	0.00841
0	0.00	0	4.82	20.60	0.996	0.00861
0	0.00	0	5.11	20.60	0.996	0.00991
0	0.00	0	5.49	20.57	0.996	0.01041
0	0.00	0	6.09	20.53	0.996	0.01051
0	0.00	0	6.30	20.50	0.996	0.01101
0	0.00	0	6.56	20.50	0.996	0.01101
0	0.00	0	6.70	20.47	0.996	0.01101
0	0.00	0	6.85	20.47	0.996	0.01101
0	0.00	0	6.99	20.40	0.996	0.01021
0	0.00	0	7.10	20.37	0.996	0.01081
0	0.00	0	4.09	22.43	0.985	0.00131
0	0.00	0	4.26	22.50	0.985	0.00461
0	0.00	0	4.97	22.57	0.985	0.00991
0	0.00	0	6.06	22.60	0.985	0.01311
0	0.00	0	4.21	22.13	0.975	0.00021
0	0.00	0	4.71	22.17	0.975	0.00131
0	0.00	0	5.24	22.27	0.975	0.00371
0	0.00	0	5.82	22.30	0.975	0.00391
0	0.00	0	6.28	22.40	0.975	0.00441
0	0.00	0	6.83	22.40	0.975	0.00581
0	0.00	0	5.24	21.90	0.965	0.00161
0	0.00	0	5.78	21.93	0.965	0.00171
0	0.00	0	6.78	21.93	0.965	0.00221

Appendix 2.3 - Growth rate of *Escherichia coli* M23 in nutrient broth with acetic acid at various temperatures, water activities and pHs.

[AAC] mM	[U] mM	[D] mM	pH	T (°C)	Aw	Rate (1/GT(min))
5	2.96	2.04	4.6	22.00	0.996	0.00031
5	1.20	3.80	5.26	22.00	0.996	0.00731
5	0.70	4.30	5.55	22.00	0.996	0.01011
5	0.35	4.65	5.88	22.00	0.996	0.01191
5	0.16	4.84	6.25	21.97	0.996	0.01281
5	0.07	4.93	6.63	21.93	0.996	0.01391
5	0.04	4.96	6.9	21.93	0.996	0.01381
5	2.90	2.10	4.62	20.37	0.996	0.00051
5	2.64	2.36	4.71	20.33	0.996	0.00131
5	2.36	2.64	4.81	20.30	0.996	0.00261
5	2.02	2.98	4.93	20.50	0.996	0.00381
5	1.54	3.46	5.11	20.27	0.996	0.00491
5	1.12	3.88	5.3	20.20	0.996	0.00641
5	0.71	4.29	5.54	20.20	0.996	0.00791
5	0.30	4.70	5.96	20.13	0.996	0.00921
5	0.18	4.82	6.19	20.10	0.996	0.00951
5	0.10	4.90	6.44	20.07	0.996	0.00951
5	0.09	4.91	6.51	20.07	0.996	0.00991
5	0.06	4.94	6.69	20.03	0.996	0.00891
5	0.05	4.95	6.76	20.00	0.996	0.00911
5	0.04	4.96	6.89	20.00	0.996	0.00931
5	0.03	4.97	7.03	19.97	0.996	0.00791
10	2.75	7.25	5.18	22.13	0.996	0.00131
10	1.86	8.14	5.4	22.10	0.996	0.00581
10	1.12	8.88	5.66	22.07	0.996	0.00811
10	0.19	9.81	6.48	22.07	0.996	0.01191
10	0.08	9.92	6.86	22.00	0.996	0.01341

Appendix 2.3 - Growth rate of *Escherichia coli* M23 in nutrient broth with acetic acid at various temperatures, water activities and pHs.

[AAC] mM	[U] mM	[D] mM	pH	T (°C)	Aw	Rate (1/GT(min))
15	1.89	13.11	5.6	22.30	0.996	0.00631
15	0.13	14.87	6.83	22.20	0.996	0.01351
15	2.14	12.86	5.54	20.47	0.996	0.004951
15	1.97	13.03	5.58	20.43	0.996	0.005611
15	1.36	13.64	5.76	20.40	0.996	0.006681
15	0.97	14.03	5.92	20.40	0.996	0.007611
15	0.41	14.59	6.31	20.33	0.996	0.008411
15	0.21	14.79	6.61	20.33	0.996	0.008941
15	0.13	14.87	6.81	20.30	0.996	0.009151
15	0.10	14.90	6.95	20.27	0.996	0.009221
15	0.08	14.92	7.05	20.23	0.996	0.009281
20	1.60	18.40	5.82	22.47	0.996	0.00731
20	0.54	19.46	6.32	22.43	0.996	0.01071
20	0.16	19.84	6.84	22.40	0.996	0.01331
25	2.91	22.09	5.64	20.07	0.996	0.001451
25	2.52	22.48	5.71	20.07	0.996	0.002531
25	1.96	23.04	5.83	20.03	0.996	0.003211
25	1.58	23.42	5.93	20.00	0.996	0.005421
25	1.14	23.86	6.08	20.00	0.996	0.006361
25	0.73	24.27	6.28	20.00	0.996	0.007311
25	0.49	24.51	6.46	20.00	0.996	0.007611
25	0.23	24.77	6.80	20.00	0.996	0.008711
25	0.11	24.89	7.10	20.00	0.996	0.009581
25	0.78	24.22	6.25	21.77	0.998	0.006601
25	0.30	24.70	6.67	21.73	0.998	0.009301
25	0.12	24.88	7.09	21.70	0.998	0.010001
25	0.06	24.94	7.38	21.60	0.998	0.011001
25	0.03	24.97	7.72	21.60	0.998	0.009401
25	0.02	24.98	7.90	21.57	0.998	0.008901
25	0.01	24.99	8.20	21.57	0.998	0.009501
25	0.01	24.99	8.42	21.53	0.998	0.007101
25	0.00	25.00	8.63	21.43	0.998	0.004801

Appendix 2.3 - Growth rate of *Escherichia coli* M23 in nutrient broth with acetic acid at various temperatures, water activities and pHs.

[AAC] mM	[U] mM	[D] mM	pH	T (°C)	Aw	Rate (1/GT(min))
50	1.96	48.04	6.15	21.20	0.998	0.004501
50	0.34	49.66	6.93	21.20	0.998	0.006401
50	0.18	49.82	7.21	21.17	0.998	0.007701
50	0.12	49.88	7.38	21.13	0.998	0.007301
50	0.05	49.95	7.72	21.13	0.998	0.007701
50	0.03	49.97	7.92	21.13	0.998	0.008301
50	0.01	49.99	8.33	21.13	0.998	0.006701
50	0.01	49.99	8.45	21.03	0.998	0.005801
50	0.01	49.99	8.74	21.03	0.998	0.006401
100	1.63	98.37	6.54	21.30	0.997	0.003701
100	1.16	98.84	6.69	21.27	0.997	0.003201
100	0.74	99.26	6.89	21.20	0.997	0.004501
100	0.52	99.48	7.04	21.17	0.997	0.004401
100	0.33	99.67	7.24	21.17	0.997	0.004401
100	0.17	99.83	7.52	21.17	0.997	0.005001
100	0.11	99.89	7.71	21.13	0.997	0.006101
100	0.04	99.96	8.16	21.10	0.997	0.007101
100	1.46	98.54	6.59	37.00	0.997	0.016001
100	0.56	99.44	7.01	37.00	0.997	0.020001
100	0.27	99.73	7.33	37.00	0.997	0.024001
100	0.15	99.85	7.58	37.00	0.997	0.027001
100	0.04	99.96	8.18	37.00	0.997	0.028001
100	0.01	99.99	8.64	37.00	0.997	0.020001
100	0.01	99.99	9.02	37.00	0.997	0.011001
100	0.13	99.87	7.63	20.81	0.995	0.006501
100	0.33	99.67	7.24	20.81	0.995	0.006601
100	0.48	99.52	7.08	20.93	0.995	0.005101
100	1.60	98.40	6.55	20.93	0.995	0.004201
100	0.07	99.93	7.93	20.81	0.995	0.006901

Appendix 2.4 - Growth rate of *Escherichia coli* M23 in nutrient broth with acetic acid at various temperatures, water activities and pHs not used to create acetic acid growth rate models

[AAC] mM	[U] mM	[D] mM	pH	T (°C)	Aw	Rate (1/GT(min))
15	4.13	10.87	5.18	22.30	0.996	0.00041
15	2.96	12.04	5.37	22.30	0.996	0.00561
20	4.40	15.60	5.31	22.53	0.996	0.00041
20	3.02	16.98	5.51	22.50	0.996	0.00511
25	3.09	21.91	5.61	21.80	0.998	0.00361
100	4.77	95.23	6.06	37.00	0.997	0.00711
100	3.35	96.65	6.22	20.93	0.995	0.00091
100	5.68	94.32	5.98	21.05	0.995	0.00011
15	4.93	10.07	5.07	20.53	0.996	0.00021
15	4.41	10.59	5.14	20.50	0.996	0.00031
15	3.42	11.58	5.29	20.50	0.996	0.00161
15	2.74	12.26	5.41	20.47	0.996	0.00351
25	5.11	19.89	5.35	20.13	0.996	0.00021
25	4.00	21.00	5.48	20.13	0.996	0.00081
25	3.42	21.58	5.56	20.10	0.996	0.00111
200	8.18	191.82	6.13	21.93	0.995	0.00071
200	2.12	197.88	6.73	21.87	0.995	0.00261
200	1.44	198.56	6.9	21.87	0.995	0.00371
200	1.00	199.00	7.06	21.83	0.995	0.00361
200	0.59	199.41	7.29	21.80	0.995	0.00371
200	0.40	199.60	7.46	21.77	0.995	0.00321
200	0.18	199.82	7.8	21.63	0.995	0.00431
200	0.14	199.86	7.91	21.57	0.995	0.00381
200	0.11	199.89	8.03	21.57	0.995	0.00421
200	0.05	199.95	8.33	21.47	0.995	0.00231
200	9.55	190.45	6.06	37.00	0.995	0.00161
200	1.73	198.27	6.82	37.00	0.995	0.01301
200	1.02	198.98	7.05	37.00	0.995	0.01501
200	0.52	199.48	7.34	37.00	0.995	0.02001
200	0.21	199.79	7.74	37.00	0.995	0.02201
200	0.13	199.87	7.94	37.00	0.995	0.02701
200	0.08	199.92	8.16	37.00	0.995	0.02401
200	0.06	199.94	8.28	37.00	0.995	0.02001

Appendix 2.4 - Growth rate of *Escherichia coli* M23 in nutrient broth with acetic acid at various temperatures, water activities and pHs not used to create acetic acid growth rate models

[AAC] mM	[U] mM	[D] mM	pH	T (°C)	Aw	Rate (1/GT(min))
200	0.09	199.91	8.09	20.35	0.996	0.00661
200	0.14	199.86	7.91	20.35	0.996	0.00601
200	0.32	199.68	7.56	20.47	0.996	0.00571
200	0.79	199.21	7.16	20.47	0.996	0.00481
200	3.05	196.95	6.57	20.47	0.996	0.00251
400	0.19	399.81	8.09	20.70	0.987	0.00561
400	0.69	399.31	7.52	20.70	0.987	0.00481
400	4.05	395.95	6.75	20.93	0.987	0.00051
800	3.64	796.36	7.1	20.35	0.983	0.00271
800	1.45	798.55	7.5	20.35	0.983	0.00171
0*	0.00	0.00	7.27	37	0.999	0.02941
5*	0.01	4.99	7.33	37	0.999	0.02961
10*	0.02	9.98	7.39	37	0.999	0.02841
15*	0.03	14.97	7.41	37	0.998	0.02741
20*	0.05	19.95	7.38	37	0.998	0.02741
25*	0.06	24.94	7.38	37	0.998	0.02711
30*	0.07	29.93	7.4	37	0.997	0.02561
40*	0.10	39.90	7.38	37	0.997	0.02621
50*	0.11	49.89	7.42	37	0.997	0.02731
75*	0.16	74.84	7.42	37	0.996	0.02411
100*	0.20	99.80	7.45	37	0.996	0.02361
125*	0.25	124.75	7.45	37	0.996	0.02251
150*	0.29	149.71	7.47	37	0.996	0.01951
200*	0.37	199.63	7.49	37	0.993	0.01901
250*	0.43	249.57	7.52	37	0.992	0.01781
300*	0.50	299.50	7.54	37	0.991	0.01681
400*	0.63	399.37	7.56	37	0.99	0.01521
500*	0.76	499.24	7.58	37	0.985	0.01311

* sodium acetate was added instead of acetic acid

Appendix 2.5 - Growth rate of *Escherichia coli* M23
in nutrient broth at various temperatures, water activities and pHs.

pH	T (°C)	Rate (1/GT(min))
9.29	34.45	0.02684
9.14	34.50	0.02459
8.98	34.55	0.02519
8.88	34.55	0.02556
8.64	34.60	0.02721
8.49	34.60	0.02720
8.36	34.65	0.02875
8.1	34.55	0.02790
7.92	34.35	0.02954
7.82	34.35	0.03095
7.71	34.35	0.03036
7.58	34.35	0.03129
7.51	34.45	0.02870
7.51	34.55	0.03072
7.51	34.60	0.03055
7.51	34.35	0.02967
7.51	34.35	0.02996
9.23	34.45	0.02531
9.06	34.50	0.02354
8.9	34.55	0.02531
8.77	34.55	0.02550
8.58	34.60	0.02668
8.49	34.60	0.02789
8.36	34.65	0.02721
8.1	34.55	0.02929
7.92	34.35	0.0289
7.82	34.35	0.02829
7.71	34.35	0.02803
7.61	34.35	0.02816
7.54	34.45	0.02627
7.54	34.55	0.02854
7.54	34.6	0.02865
7.54	34.35	0.02815
7.54	34.35	0.02778

Appendix 2.6 - *Vibrio parahaemolyticus* strain 38.349 from Miles (1994)
Tryptone Soy Broth with 3% salt

Data						
pH	Rate	G.T.	√Rate	Temperature	T Adjusted* G.T.	T Adjusted* Rate
4.25	0	0	0	20.48	0	0
4.37	0	0	0	20.40	0	0
4.43	0	0	0	20.52	0	0
4.57	0	0	0	20.42	0	0
4.99	0.00098	1020.41	0.03130	20.48	1090.86	0.00092
5.02	0.00100	1000.00	0.03162	20.54	1077.84	0.00093
5.45	0.00550	181.82	0.07416	20.56	196.50	0.00509
5.46	0.00570	175.44	0.07550	20.50	188.07	0.00532
5.86	0.00730	136.99	0.08544	20.56	148.05	0.00675
5.92	0.00720	138.89	0.08485	20.63	151.54	0.00660
6.20	0.00830	120.48	0.09110	20.59	130.75	0.00765
6.22	0.00740	135.14	0.08602	20.69	148.65	0.00673
6.47	0.00820	121.95	0.09055	20.73	134.87	0.00741
6.50	0.00880	113.64	0.09381	20.61	123.65	0.00809
6.77	0.00980	102.04	0.09899	20.69	112.24	0.00891
6.84	0.00910	109.89	0.09539	20.79	122.51	0.00816
7.11	0.00990	101.01	0.09950	20.71	111.41	0.00898
7.13	0.00980	102.04	0.09899	20.81	114.07	0.00877
7.45	0.01100	90.91	0.10488	20.75	100.81	0.00992
7.49	0.01000	100.00	0.10000	20.85	112.38	0.00890
7.74	0.01100	90.91	0.10488	20.79	101.35	0.00987
7.81	0.01100	90.91	0.10488	20.89	102.71	0.00974
8.08	0.01100	90.91	0.10488	20.85	102.17	0.00979
8.15	0.01100	90.91	0.10488	20.93	103.26	0.00968
8.27	0.01200	83.33	0.10954	20.87	93.90	0.01065
8.35	0.01200	83.33	0.10954	20.97	95.16	0.01051
8.55	0.01300	76.92	0.11402	20.91	87.14	0.01148
8.65	0.01200	83.33	0.10954	20.97	95.16	0.01051
8.87	0.01200	83.33	0.10954	20.97	95.16	0.01051
8.90	0.01200	83.33	0.10954	21.05	96.17	0.01040

* adjusted using the relative rate concept (McMeekin *et al.* 1993) with a T_{min} of 5.86

Source of Model	Estimates				Goodness of Fit
	c	Q	pH_{min}	pH_{max}	Chi^2
Adams '91	0.00397	-	4.55	-	0.003231
Wijtzes '95	-4.71×10^{-6}	-	4.79	11.45	0.000623
Eqn 2.10	0.00729	0.391	4.90	-	0.000133

Appendix 2.6 - *Listeria monoctyogenes Scott A* from Ross (1993)
Tryptone Soy Broth with 0.2M lactate

Data				
pHmid	Rate	G.T. (hr)	√Rate	Temperature (°C)
7.60	0.51020	1.96	0.71429	19.5
7.45	0.53476	1.87	0.73127	19.5
7.25	0.53763	1.86	0.73324	19.5
6.90	0.50000	2.00	0.70711	19.5
6.55	0.54054	1.85	0.73521	19.5
6.20	0.48309	2.07	0.69505	19.5
6.00	0.41494	2.41	0.64416	19.5
5.85	0.29326	3.41	0.54153	19.5
5.80	0.17241	5.80	0.41523	19.5
5.65	0.09434	10.60	0.30715	19.5
5.50	0	-	-	19.5

Source of	Estimates				Goodness of Fit
Model	c	Q	pH_{min}	pH_{max}	Chi^2
Wijtzes '93	0.0257	-	5.15	8.74	0.02576
Wijtzes '95	-0.000864	-	5.52	8.36	0.01564
Eqn 2.10	0.0397	1.24	5.59	-	0.00970

Listeria monoctyogenes, Scott A from Petran & Zottola (1989) Tryptic Soy Broth

Data				
pH	G.T. (min)	Rate	√Rate	Temperature (°C)
4.5	0	0	0	30
4.7	371.0	0.00270	0.05196	30
5.0	182.0	0.00550	0.07416	30
6.0	52.0	0.01920	0.13856	30
7.0	44.7	0.02240	0.14967	30
8.0	50.1	0.02000	0.14142	30
9.0	146.0	0.00680	0.08246	30
9.2	179.0	0.00560	0.07483	30
9.4	0	0	0	30

Source of	Estimates				Goodness of Fit
Model	c		pH_{min}	pH_{max}	Chi^2
Wijtzes '93	37985	b=1.66x10-8	4.16	9.97	0.00009
Wijtzes '95	1.26x10-8	b=347	4.57	9.49	0.00021
Eqn 2.14	0.0399	Q=0.00826 R=0.00835	4.54	9.40	0.00024

Appendix 2.6 - *Escherichia coli O157:H7* from Glass *et al.* (1992)
Trypticase Soy Broth

Data				
pH	G.T. (hr)	Rate	√Rate	Temperature (°C)
4.0	0	0	0	37
4.5	0.8	0.0208	0.14422	37
5.0	0.5	0.0333	0.18248	37
5.5	0.5	0.0333	0.18248	37
6.0	0.4	0.0416	0.20396	37
6.5	0.4	0.0416	0.20396	37
7.0	0.4	0.0416	0.20396	37
7.3	0.4	0.0416	0.20396	37
9.0	0.5	0.0333	0.18248	37

Source of	Estimates				Goodness of Fit
Model	c	Q	pHmin	pHmax	Chi^2
Wijtzes '95	2.71×10^{-6}	-	3.31	10.95	0.00023
Eqn 2.11	0.00626	0.57	3.98	9.66	0.00014

E.coli Nat. Collection of Type cultures No 86 (U.K.) from Gale and Epps (1942)

Data				
pH	Rate	G.T. (min)	√Rate	Temperature (°C)
4.70	0.00930	107.5	0.09645	27
4.80	0.01075	93.0	0.10370	27
5.30	0.01600	62.5	0.12649	27
6.10	0.01905	52.5	0.13801	27
6.90	0.02000	50.0	0.14142	27
7.30	0.02000	50.0	0.14142	27
7.90	0.01923	52.0	0.13868	27
8.60	0.01538	65.0	0.12403	27
9.20	0.00909	110.0	0.09535	27
4.70	0.01064	94.0	0.10314	37
5.10	0.02105	47.5	0.14510	37
5.70	0.03125	32.0	0.17678	37
6.20	0.03636	27.5	0.19069	37
7.35	0.03846	26.0	0.19612	37
8.00	0.03636	27.5	0.19069	37
8.50	0.02222	45.0	0.14907	37
8.90	0.01290	77.5	0.11359	37

Source of	Estimates				Goodness of Fit
Model	c		pHmin	pHmax	Chi^2
Wijtzes '93	34632	b=1.51	3.45	10.33	0.00222
Wijtzes '95	-4.95	-	4.16	9.63	0.00234
Eqn 2.11	0.00606	Q=0.576	4.38	9.06	0.01060

Appendix 2.6 - *Lactococcus curvatus* from Wijtzes et al. (1995)

		Data	
pH	Temperature (°C)	Rate	√Rate
4.60	29	0.425	0.65192
4.65	29	0.100	0.31623
4.75	29	0.330	0.57446
5.00	29	0.430	0.65574
5.03	29	0.350	0.59161
5.15	29	0.550	0.74162
5.50	29	0.530	0.72801
5.55	29	0.750	0.86603
5.73	29	0.790	0.88882
5.78	29	0.760	0.87178
5.95	29	0.860	0.92736
6.05	29	0.830	0.91104
6.10	29	0.780	0.88318
6.25	29	0.880	0.93808
6.42	29	0.925	0.96177
6.62	29	0.925	0.96177
6.70	29	0.990	0.99499
7.00	29	1.000	1.00000
7.27	29	0.895	0.94604
7.45	29	0.883	0.93968
7.80	29	0.800	0.89443
7.97	29	0.755	0.86891
8.35	29	0.695	0.83367
8.45	29	0.685	0.82765
4.98	15	0.175	0.41833
4.98	15	0.185	0.43012
5.05	15	0.160	0.40000
5.32	15	0.210	0.45826
5.32	15	0.220	0.46904
5.47	15	0.260	0.50990
5.90	15	0.350	0.59161
5.95	15	0.320	0.56569
6.00	15	0.380	0.61644
7.05	15	0.320	0.56569
7.10	15	0.260	0.50990
7.09	15	0.310	0.55678
7.50	15	0.280	0.52915
7.52	15	0.260	0.50990
7.60	15	0.280	0.52915

Appendix 2.6 - *Lactococcus curvatus* from Wijtzes et al. (1995)

Data			
pH	Temperature (°C)	Rate	√Rate
7.90	15	0.235	0.48477
7.95	15	0.232	0.48166
8.05	15	0.230	0.47958
8.52	15	0.190	0.43589
8.53	15	0.210	0.45826
8.54	15	0.160	0.40000
8.87	15	0.285	0.53385
8.90	15	0.195	0.44159
8.96	15	0.375	0.61237
5.50	6	0.070	0.26458
6.05	6	0.080	0.28284
6.50	6	0.090	0.30000
7.00	6	0.085	0.29155
7.60	6	0.082	0.28636
7.99	6	0.078	0.27928
8.52	6	0.070	0.26458

Source of Model	Estimates c		pHmin	pHmax	Goodness of Fit Chi^2
Wijtzes '93	72231	b=3.14	3.40	10.76	0.21244
Wijtzes '95	-0.000109	-	4.18	10.00	0.20283
Eqn 2.14	0.121	Q=0.180	4.39	10.40	0.03934
		R=0.0126			

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
1	0	2.8	10	0.996	0	4	**0**	**0**	0	0	0
2	0	3.4	10	0.996	0	4	**0**	**0**	0	0	0
3	0	3.6	10	0.996	0	4	**0**	**0**	0	0	0
4	0	3.8	10	0.996	0	4	**0**	**0**	0	0	0
5	0	3.9	10	0.996	0	4	0	0	0.46	0.27	0.31
6	0	4	10	0.996	4	4	0.40	0.44	0.68	0.48	0.55
7	0	4.4	10	0.996	4	4	0.99	1	0.94	0.94	0.97
8	0	4.6	10	0.996	4	4	1	1	0.96	0.98	0.99
9	0	5.3	10	0.996	4	4	1	1	0.98	1	1
10	0	6.9	10	0.996	4	4	1	1	0.98	1	1
11	0	2.8	15	0.996	0	4	**0**	**0**	0	0	0
12	0	3.4	15	0.996	0	4	**0**	**0**	0	0	0
13	0	3.6	15	0.996	0	4	**0**	**0**	0	0	0
14	0	3.8	15	0.996	0	4	**0**	**0**	0	0	1
15	0	3.9	15	0.996	4	4	0	0	0.77	0.67	0.80
16	0	4	15	0.996	4	4	0.69	0.76	0.90	0.84	0.91
17	0	4.4	15	0.996	4	4	1	1	0.98	0.99	1
18	0	4.6	15	0.996	4	4	1	1	0.99	1	1
19	0	5.3	15	0.996	4	4	1	1	0.99	1	1
20	0	6.9	15	0.996	4	4	1	1	0.99	1	1
21	0	2.8	20	0.996	0	4	**0**	**0**	0	0	0
22	0	3.4	20	0.996	0	4	**0**	**0**	0	0	0
23	0	3.6	20	0.996	3	4	**0**	**0**	0	0	0
24	0	3.8	20	0.996	4	4	**0**	**0**	0	1	1
25	0	3.9	20	0.996	4	4	0	0	0.88	0.81	0.88
26	0	4	20	0.996	4	4	0.83	0.88	0.95	0.92	0.95
27	0	4.4	20	0.996	4	4	1	1	0.99	0.99	1
28	0	4.6	20	0.996	4	4	1	1	0.99	1	1
29	0	5.3	20	0.996	4	4	1	1	1	1	1
30	0	6.9	20	0.996	4	4	1	1	1	1	1
31	0	2.8	25	0.996	0	4	**0**	**0**	0	0	0
32	0	3.4	25	0.996	0	4	**0**	**0**	0	0	0
33	0	3.6	25	0.996	0	4	**0**	**0**	0	0	0
34	0	3.8	25	0.996	0	4	**0**	**0**	0	1	1
35	0	3.9	25	0.996	0	4	0	0	0.92	0.88	0.89
36	0	4	25	0.996	4	4	0.89	0.93	0.97	0.95	0.96
37	0	4.4	25	0.996	4	4	1	1	1	1	1
38	0	4.6	25	0.996	4	4	1	1	1	1	1
39	0	5.3	25	0.996	4	4	1	1	1	1	1
40	0	6.9	25	0.996	4	4	1	1	1	1	1
41	0	2.8	30	0.996	0	4	**0**	**0**	0	0	0
42	0	3.4	30	0.996	0	4	**0**	**0**	0	0	0
43	0	3.6	30	0.996	4	4	**0**	**0**	0	0	0
44	0	3.8	30	0.996	4	4	**0**	**0**	0	1	1
45	0	3.9	30	0.996	4	4	0	0	0.94	0.91	0.88
46	0	4	30	0.996	4	4	0.93	0.95	0.98	0.96	0.95
47	0	4.4	30	0.996	4	4	1	1	1	1	1
48	0	4.6	30	0.996	4	4	1	1	1	1	1
49	0	5.3	30	0.996	4	4	1	1	1	1	1
50	0	6.9	30	0.996	4	4	1	1	1	1	1

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
51	0	2.8	37	0.996	0	4	**0**	**0**	0	0	0
52	0	3.4	37	0.996	0	4	**0**	**0**	0	0	0
53	0	3.6	37	0.996	0	4	**0**	**0**	0	0	0
54	0	3.8	37	0.996	1	4	**0**	**0**	0	1	1
55	0	3.9	37	0.996	1	4	0	0	0.90	0.94	0.82
56	0	4	37	0.996	4	4	0.95	0.94	0.96	0.97	0.93
57	0	4.4	37	0.996	4	4	1	1	0.99	1	1
58	0	4.6	37	0.996	4	4	1	1	1	1	1
59	0	5.3	37	0.996	4	4	1	1	1	1	1
60	0	6.9	37	0.996	4	4	1	1	1	1	1
61	25	3	10	0.996	0	4	**0**	**0**	0	**0**	**0**
62	25	3.9	10	0.996	0	4	**0**	**0**	0	**0**	**0**
63	25	4.1	10	0.996	0	4	0	0	0.01	0	0
64	25	4.2	10	0.996	0	4	0	0	0.04	0	0
65	25	4.3	10	0.996	0	4	0.09	0.05	0.15	0.02	0.02
66	25	4.4	10	0.996	0	4	0.47	0.30	0.35	0.10	0.10
67	25	4.5	10	0.996	4	4	0.81	0.69	0.57	0.32	0.36
68	25	5	10	0.996	4	4	0.99	0.99	0.94	0.98	0.99
69	25	6.6	10	0.996	4	4	1	1	0.98	1	1
70	25	3	15	0.996	0	4	**0**	**0**	0	**0**	**0**
71	25	3.9	15	0.996	0	4	**0**	**0**	0	**0**	**0**
72	25	4.1	15	0.996	0	4	0	0	0.03	0	0
73	25	4.2	15	0.996	0	4	0.01	0.01	0.15	0.02	0.02
74	25	4.3	15	0.996	0	4	0.25	0.18	0.41	0.11	0.14
75	25	4.4	15	0.996	4	4	0.75	0.64	0.68	0.38	0.50
76	25	4.5	15	0.996	4	4	0.94	0.90	0.84	0.72	0.84
77	25	5	15	0.996	4	4	1	1	0.98	1	1
78	25	6.6	15	0.996	4	4	1	1	0.99	1	1
79	25	3	20	0.996	0	4	**0**	**0**	0	**0**	**0**
80	25	3.9	20	0.996	0	4	**0**	**0**	0	**0**	**0**
81	25	4.1	20	0.996	0	4	0	0	0.06	0	0
82	25	4.2	20	0.996	0	4	0.03	0.03	0.27	0.04	0.04
83	25	4.3	20	0.996	0	4	0.41	0.34	0.60	0.20	0.24
84	25	4.4	20	0.996	4	4	0.86	0.81	0.82	0.57	0.66
85	25	4.5	20	0.996	4	4	0.97	0.96	0.92	0.85	0.91
86	25	5	20	0.996	4	4	1	1	0.99	1	1
87	25	6.6	20	0.996	4	4	1	1	1	1	1
88	25	3	25	0.996	0	4	**0**	**0**	0	**0**	**0**
89	25	3.9	25	0.996	0	4	**0**	**0**	0	**0**	**0**
90	25	4.1	25	0.996	0	4	0	0	0.10	0.01	0.01
91	25	4.2	25	0.996	0	4	0.04	0.06	0.38	0.06	0.05
92	25	4.3	25	0.996	0	4	0.55	0.48	0.71	0.29	0.26
93	25	4.4	25	0.996	4	4	0.92	0.88	0.88	0.68	0.69
94	25	4.5	25	0.996	4	4	0.98	0.98	0.95	0.90	0.92
95	25	5	25	0.996	4	4	1	1	1	1	1
96	25	6.6	25	0.996	4	4	1	1	1	1	1
97	25	3	30	0.996	0	4	**0**	**0**	0	**0**	**0**
98	25	3.9	30	0.996	0	4	**0**	**0**	0	**0**	**0**
99	25	4.1	30	0.996	0	4	0	0	0.12	0.01	0
100	25	4.2	30	0.996	0	4	0.07	0.09	0.45	0.08	0.04

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
101	25	4.3	30	0.996	0	4	0.65	0.58	0.77	0.37	0.23
102	25	4.4	30	0.996	4	4	0.94	0.92	0.91	0.75	0.65
103	25	4.5	30	0.996	4	4	0.99	0.98	0.96	0.93	0.90
104	25	5	30	0.996	4	4	1	1	1	1	1
105	25	6.6	30	0.996	4	4	1	1	1	1	1
106	25	3	37	0.996	0	4	**0**	**0**	0	**0**	**0**
107	25	3.9	37	0.996	0	4	**0**	**0**	0	**0**	**0**
108	25	4.1	37	0.996	0	4	0	0	0.07	0.02	0
109	25	4.2	37	0.996	0	4	0.10	0.07	0.31	0.12	0.03
110	25	4.3	37	0.996	0	4	0.75	0.51	0.65	0.46	0.16
111	25	4.4	37	0.996	4	4	0.96	0.89	0.85	0.82	0.55
112	25	4.5	37	0.996	4	4	0.99	0.98	0.93	0.95	0.86
113	25	5	37	0.996	4	4	1	1	0.99	1	1
114	25	6.6	37	0.996	4	4	1	1	1	1	1
115	50	3.2	10	0.996	0	4	**0**	**0**	0	**0**	**0**
116	50	4.1	10	0.996	0	4	**0**	**0**	0	**0**	**0**
117	50	4.4	10	0.996	0	4	**0**	**0**	0	**0**	**0**
118	50	4.5	10	0.996	0	4	0	0	0.03	0	0
119	50	4.6	10	0.996	0	4	0.04	0.04	0.13	0.04	0.04
120	50	4.7	10	0.996	0	4	0.42	0.30	0.36	0.20	0.24
121	50	4.8	10	0.996	0	4	0.82	0.72	0.60	0.54	0.64
122	50	5.1	10	0.996	4	4	0.99	0.98	0.90	0.97	0.99
123	50	6	10	0.996	4	4	1	1	0.97	1	1
124	50	6.8	10	0.996	4	4	1	1	0.98	1	1
125	50	3.2	15	0.996	0	4	**0**	**0**	0	**0**	**0**
126	50	4.1	15	0.996	0	4	**0**	**0**	0	**0**	**0**
127	50	4.4	15	0.996	0	4	**0**	**0**	0	**0**	**0**
128	50	4.5	15	0.996	0	4	0	0.01	0.10	0.02	0.03
129	50	4.6	15	0.996	3	4	0.12	0.15	0.38	0.18	0.27
130	50	4.7	15	0.996	4	4	0.71	0.64	0.69	0.58	0.74
131	50	4.8	15	0.996	4	4	0.94	0.91	0.85	0.87	0.94
132	50	5.1	15	0.996	4	4	1	1	0.97	0.99	1
133	50	6	15	0.996	4	4	1	1	0.99	1	1
134	50	6.8	15	0.996	4	4	1	1	0.99	1	1
135	50	3.2	20	0.996	0	4	**0**	**0**	0	**0**	**0**
136	50	4.1	20	0.996	0	4	**0**	**0**	0	**0**	**0**
137	50	4.4	20	0.996	0	4	**0**	**0**	0	**0**	**0**
138	50	4.5	20	0.996	0	4	0	0.02	0.19	0.05	0.06
139	50	4.6	20	0.996	1	4	0.23	0.30	0.57	0.31	0.42
140	50	4.7	20	0.996	4	4	0.84	0.81	0.83	0.75	0.84
141	50	4.8	20	0.996	4	4	0.97	0.96	0.93	0.93	0.97
142	50	5.1	20	0.996	4	4	1	1	0.99	1	1
143	50	6	20	0.996	4	4	1	1	1	1	1
144	50	6.8	20	0.996	4	4	1	1	1	1	1
145	50	3.2	25	0.996	0	4	**0**	**0**	0	**0**	**0**
146	50	4.1	25	0.996	0	4	**0**	**0**	0	**0**	**0**
147	50	4.4	25	0.996	0	4	**0**	**0**	0	**0**	**0**
148	50	4.5	25	0.996	1	4	0	0.04	0.27	0.07	0.07
149	50	4.6	25	0.996	2	4	0.34	0.44	0.68	0.43	0.44
150	50	4.7	25	0.996	4	4	0.90	0.88	0.89	0.83	0.86

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
151	50	4.8	25	0.996	4	4	0.98	0.98	0.95	0.96	0.97
152	50	5.1	25	0.996	4	4	1	1	0.99	1	1
153	50	6	25	0.996	4	4	1	1	1	1	1
154	50	6.8	25	0.996	4	4	1	1	1	1	1
155	50	3.2	30	0.996	0	4	**0**	**0**	0	**0**	**0**
156	50	4.1	30	0.996	0	4	**0**	**0**	0	**0**	**0**
157	50	4.4	30	0.996	0	4	**0**	**0**	0	**0**	**0**
158	50	4.5	30	0.996	0	4	0	0.05	0.34	0.10	0.06
159	50	4.6	30	0.996	0	4	0.44	0.54	0.74	0.52	0.41
160	50	4.7	30	0.996	4	4	0.93	0.92	0.91	0.87	0.84
161	50	4.8	30	0.996	4	4	0.99	0.99	0.97	0.97	0.97
162	50	5.1	30	0.996	4	4	1	1	0.99	1	1
163	50	6	30	0.996	4	4	1	1	1	1	1
164	50	6.8	30	0.996	4	4	1	1	1	1	1
165	50	3.2	37	0.996	0	4	**0**	**0**	0	**0**	**0**
166	50	4.1	37	0.996	0	4	**0**	**0**	0	**0**	**0**
167	50	4.4	37	0.996	0	4	**0**	**0**	0	**0**	**0**
168	50	4.5	37	0.996	0	4	0	0.04	0.22	0.15	0.04
169	50	4.6	37	0.996	0	4	0.56	0.46	0.62	0.61	0.31
170	50	4.7	37	0.996	4	4	0.96	0.90	0.85	0.91	0.77
171	50	4.8	37	0.996	4	4	0.99	0.98	0.94	0.98	0.95
172	50	5.1	37	0.996	4	4	1	1	0.99	1	1
173	50	6	37	0.996	4	4	1	1	1	1	1
174	50	6.8	37	0.996	4	4	1	1	1	1	1
175	100	4	10	0.996	0	4	**0**	**0**	0	**0**	**0**
176	100	4.4	10	0.996	0	4	**0**	**0**	0	**0**	**0**
177	100	4.6	10	0.996	0	4	**0**	**0**	0	**0**	**0**
178	100	4.7	10	0.996	0	4	**0**	**0**	0	**0**	**0**
179	100	4.9	10	0.996	0	4	0.01	0.02	0.10	0.06	0.08
180	100	5	10	0.996	0	4	0.30	0.25	0.33	0.32	0.42
181	100	5.2	10	0.996	4	4	0.93	0.90	0.76	0.90	0.95
182	100	5.4	10	0.996	4	4	0.99	0.98	0.90	0.98	0.99
183	100	5.7	10	0.996	4	4	1	1	0.95	1	1
184	100	6.2	10	0.996	4	4	1	1	0.97	1	1
185	100	4	15	0.996	0	4	**0**	**0**	0	**0**	**0**
186	100	4.4	15	0.996	0	4	**0**	**0**	0	**0**	**0**
187	100	4.6	15	0.996	0	4	**0**	**0**	0	**0**	**0**
188	100	4.7	15	0.996	0	4	**0**	**0**	0	**0**	**0**
189	100	4.9	15	0.996	0	4	0.03	0.09	0.32	0.26	0.43
190	100	5	15	0.996	4	4	0.59	0.57	0.67	0.73	0.87
191	100	5.2	15	0.996	4	4	0.98	0.97	0.93	0.98	0.99
192	100	5.4	15	0.996	4	4	1	1	0.97	1	1
193	100	5.7	15	0.996	4	4	1	1	0.99	1	1
194	100	6.2	15	0.996	4	4	1	1	0.99	1	1
195	100	4	20	0.996	0	4	**0**	**0**	0	**0**	**0**
196	100	4.4	20	0.996	0	4	**0**	**0**	0	**0**	**0**
197	100	4.6	20	0.996	0	4	**0**	**0**	0	**0**	**0**
198	100	4.7	20	0.996	0	4	**0**	**0**	0	**0**	**0**
199	100	4.9	20	0.996	0	4	0.07	0.19	0.50	0.43	0.59
200	100	5	20	0.996	0	4	0.75	0.76	0.81	0.85	0.93

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
151	50	4.8	25	0.996	4	4	0.98	0.98	0.95	0.96	0.97
152	50	5.1	25	0.996	4	4	1	1	0.99	1	1
153	50	6	25	0.996	4	4	1	1	1	1	1
154	50	6.8	25	0.996	4	4	1	1	1	1	1
155	50	3.2	30	0.996	0	4	**0**	**0**	0	**0**	**0**
156	50	4.1	30	0.996	0	4	**0**	**0**	0	**0**	**0**
157	50	4.4	30	0.996	0	4	**0**	**0**	0	**0**	**0**
158	50	4.5	30	0.996	0	4	0	0.05	0.34	0.10	0.06
159	50	4.6	30	0.996	0	4	0.44	0.54	0.74	0.52	0.41
160	50	4.7	30	0.996	4	4	0.93	0.92	0.91	0.87	0.84
161	50	4.8	30	0.996	4	4	0.99	0.99	0.97	0.97	0.97
162	50	5.1	30	0.996	4	4	1	1	0.99	1	1
163	50	6	30	0.996	4	4	1	1	1	1	1
164	50	6.8	30	0.996	4	4	1	1	1	1	1
165	50	3.2	37	0.996	0	4	**0**	**0**	0	**0**	**0**
166	50	4.1	37	0.996	0	4	**0**	**0**	0	**0**	**0**
167	50	4.4	37	0.996	0	4	**0**	**0**	0	**0**	**0**
168	50	4.5	37	0.996	0	4	0	0.04	0.22	0.15	0.04
169	50	4.6	37	0.996	0	4	0.56	0.46	0.62	0.61	0.31
170	50	4.7	37	0.996	4	4	0.96	0.90	0.85	0.91	0.77
171	50	4.8	37	0.996	4	4	0.99	0.98	0.94	0.98	0.95
172	50	5.1	37	0.996	4	4	1	1	0.99	1	1
173	50	6	37	0.996	4	4	1	1	1	1	1
174	50	6.8	37	0.996	4	4	1	1	1	1	1
175	100	4	10	0.996	0	4	**0**	**0**	0	**0**	**0**
176	100	4.4	10	0.996	0	4	**0**	**0**	0	**0**	**0**
177	100	4.6	10	0.996	0	4	**0**	**0**	0	**0**	**0**
178	100	4.7	10	0.996	0	4	**0**	**0**	0	**0**	**0**
179	100	4.9	10	0.996	0	4	0.01	0.02	0.10	0.06	0.08
180	100	5	10	0.996	0	4	0.30	0.25	0.33	0.32	0.42
181	100	5.2	10	0.996	4	4	0.93	0.90	0.76	0.90	0.95
182	100	5.4	10	0.996	4	4	0.99	0.98	0.90	0.98	0.99
183	100	5.7	10	0.996	4	4	1	1	0.95	1	1
184	100	6.2	10	0.996	4	4	1	1	0.97	1	1
185	100	4	15	0.996	0	4	**0**	**0**	0	**0**	**0**
186	100	4.4	15	0.996	0	4	**0**	**0**	0	**0**	**0**
187	100	4.6	15	0.996	0	4	**0**	**0**	0	**0**	**0**
188	100	4.7	15	0.996	0	4	**0**	**0**	0	**0**	**0**
189	100	4.9	15	0.996	0	4	0.03	0.09	0.32	0.26	0.43
190	100	5	15	0.996	4	4	0.59	0.57	0.67	0.73	0.87
191	100	5.2	15	0.996	4	4	0.98	0.97	0.93	0.98	0.99
192	100	5.4	15	0.996	4	4	1	1	0.97	1	1
193	100	5.7	15	0.996	4	4	1	1	0.99	1	1
194	100	6.2	15	0.996	4	4	1	1	0.99	1	1
195	100	4	20	0.996	0	4	**0**	**0**	0	**0**	**0**
196	100	4.4	20	0.996	0	4	**0**	**0**	0	**0**	**0**
197	100	4.6	20	0.996	0	4	**0**	**0**	0	**0**	**0**
198	100	4.7	20	0.996	0	4	**0**	**0**	0	**0**	**0**
199	100	4.9	20	0.996	0	4	0.07	0.19	0.50	0.43	0.59
200	100	5	20	0.996	0	4	0.75	0.76	0.81	0.85	0.93

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
251	200	4.5	20	0.994	0	4	**0**	**0**	0	**0**	**0**
252	200	4.7	20	0.994	0	4	**0**	**0**	0	**0**	**0**
253	200	4.9	20	0.994	0	4	**0**	**0**	0	**0**	**0**
254	200	5	20	0.994	0	4	**0**	**0**	0	**0**	**0**
255	200	5.1	20	0.994	0	8	**0**	**0**	0.05	0.05	0.09
256	200	5.3	20	0.994	4	4	0.53	0.61	0.74	0.86	0.94
257	200	5.4	20	0.994	4	4	0.92	0.92	0.90	0.97	0.99
258	200	6.1	20	0.994	4	4	1	1	0.99	1	1
259	200	4.5	25	0.994	0	4	**0**	**0**	0	**0**	**0**
260	200	4.7	25	0.994	0	4	**0**	**0**	0	**0**	**0**
261	200	4.9	25	0.994	0	4	**0**	**0**	0	**0**	**0**
262	200	5	25	0.994	0	4	**0**	**0**	0	**0**	**0**
263	200	5.1	25	0.994	0	8	**0**	**0**	0.08	0.08	0.10
264	200	5.3	25	0.994	4	4	0.66	0.74	0.83	0.91	0.94
265	200	5.4	25	0.994	4	4	0.95	0.95	0.93	0.98	0.99
266	200	6.1	25	0.994	4	4	1	1	1	1	1
267	200	4.5	30	0.994	0	4	**0**	**0**	0	**0**	**0**
268	200	4.7	30	0.994	0	4	**0**	**0**	0	**0**	**0**
269	200	4.9	30	0.994	0	4	**0**	**0**	0	**0**	**0**
270	200	5	30	0.994	0	4	**0**	**0**	0	**0**	**0**
271	200	5.1	30	0.994	4	8	**0**	**0**	0.10	0.11	0.09
272	200	5.3	30	0.994	4	4	0.75	0.81	0.87	0.93	0.94
273	200	5.4	30	0.994	4	4	0.97	0.97	0.95	0.99	0.99
274	200	6.1	30	0.994	4	4	1	1	1	1	1
275	200	4.5	37	0.994	0	4	**0**	**0**	0	**0**	**0**
276	200	4.7	37	0.994	0	4	**0**	**0**	0	**0**	**0**
277	200	4.9	37	0.994	0	4	**0**	**0**	0	**0**	**0**
278	200	5	37	0.994	0	4	**0**	**0**	0	**0**	**0**
279	200	5.1	37	0.994	1	8	**0**	**0**	0.06	0.15	0.07
280	200	5.3	37	0.994	4	4	0.83	0.76	0.78	0.95	0.91
281	200	5.4	37	0.994	4	4	0.98	0.96	0.91	0.99	0.98
282	200	6.1	37	0.994	4	4	1	1	0.99	1	1
283	500	4.5	10	0.986	0	4	**0**	**0**	0	**0**	**0**
284	500	5	10	0.986	0	4	**0**	**0**	0	**0**	**0**
285	500	5.3	10	0.986	0	4	**0**	**0**	0	**0**	**0**
286	500	5.4	10	0.986	0	4	**0**	**0**	0	**0**	**0**
287	500	5.5	10	0.986	0	8	**0**	**0**	0	**0**	**0**
288	500	5.6	10	0.986	0	8	0	0	0.01	0	0
289	500	5.8	10	0.986	0	4	0.05	0.04	0.10	0.03	0.01
290	500	5.9	10	0.986	1	4	0.20	0.15	0.19	0.10	0.04
291	500	4.5	15	0.986	0	4	**0**	**0**	0	**0**	**0**
292	500	5	15	0.986	0	4	**0**	**0**	0	**0**	**0**
293	500	5.3	15	0.986	0	4	**0**	**0**	0	**0**	**0**
294	500	5.4	15	0.986	0	4	**0**	**0**	0	**0**	**0**
295	500	5.5	15	0.986	0	8	**0**	**0**	0	**0**	**0**
296	500	5.6	15	0.986	0	8	0	0	0.03	0.01	0
297	500	5.8	15	0.986	0	4	0.16	0.14	0.30	0.16	0.15
298	500	5.9	15	0.986	4	4	0.45	0.42	0.48	0.38	0.39
299	500	4.5	20	0.986	0	4	**0**	**0**	0	**0**	**0**
300	500	5	20	0.986	0	4	**0**	**0**	0	**0**	**0**

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
301	500	5.3	20	0.986	0	4	**0**	**0**	0	**0**	**0**
302	500	5.4	20	0.986	0	4	**0**	**0**	0	**0**	**0**
303	500	5.5	20	0.986	0	8	**0**	**0**	0	**0**	**0**
304	500	5.6	20	0.986	0	8	0	0	0.06	0.01	0.01
305	500	5.8	20	0.986	0	4	0.29	0.28	0.48	0.29	0.33
306	500	5.9	20	0.986	4	4	0.64	0.63	0.67	0.57	0.64
307	500	4.5	25	0.986	0	4	**0**	**0**	0	**0**	**0**
308	500	5	25	0.986	0	4	**0**	**0**	0	**0**	**0**
309	500	5.3	25	0.986	0	4	**0**	**0**	0	**0**	**0**
310	500	5.4	25	0.986	0	4	**0**	**0**	0	**0**	**0**
311	500	5.5	25	0.986	0	8	**0**	**0**	0	**0**	**0**
312	500	5.6	25	0.986	0	8	0	0	0.09	0.02	0.02
313	500	5.8	25	0.986	0	4	0.42	0.41	0.60	0.40	0.41
314	500	5.9	25	0.986	0	4	0.75	0.76	0.77	0.68	0.72
315	500	4.5	30	0.986	0	4	**0**	**0**	0	**0**	**0**
316	500	5	30	0.986	0	4	**0**	**0**	0	**0**	**0**
317	500	5.3	30	0.986	0	4	**0**	**0**	0	**0**	**0**
318	500	5.4	30	0.986	0	4	**0**	**0**	0	**0**	**0**
319	500	5.5	30	0.986	0	8	**0**	**0**	0	**0**	**0**
320	500	5.6	30	0.986	0	8	0	0.01	0.12	0.03	0.02
321	500	5.8	30	0.986	4	4	0.53	0.52	0.67	0.49	0.43
322	500	5.9	30	0.986	4	4	0.82	0.82	0.82	0.76	0.73
323	500	4.5	37	0.986	0	4	**0**	**0**	0	**0**	**0**
324	500	5	37	0.986	0	4	**0**	**0**	0	**0**	**0**
325	500	5.3	37	0.986	0	4	**0**	**0**	0	**0**	**0**
326	500	5.4	37	0.986	0	4	**0**	**0**	0	**0**	**0**
327	500	5.5	37	0.986	0	8	**0**	**0**	0	**0**	**0**
328	500	5.6	37	0.986	0	8	0	0.01	0.07	0.04	0.02
329	500	5.8	37	0.986	0	4	0.64	0.44	0.53	0.59	0.38
330	500	5.9	37	0.986	4	4	0.88	0.78	0.71	0.82	0.69
331	0	4.27	22.06	0.996	1	1	1	1.00	0.99	0.99	1
332	0	4.88	22.18	0.996	1	1	1	1	1	1	1
333	0	5.14	22.02	0.996	1	1	1	1	1	1	1
334	0	5.53	22.48	0.996	1	1	1	1	1	1	1
335	0	5.86	22.1	0.996	1	1	1	1	1	1	1
336	0	6.15	22.12	0.996	1	1	1	1	1	1	1
337	0	6.14	22.06	0.996	1	1	1	1	1	1	1
338	0	6.58	22.04	0.996	1	1	1	1	1	1	1
339	0	6.94	22.12	0.996	1	1	1	1	1	1	1
340	0	7.14	22	0.996	1	1	1	1	1	1	1
341	0	7.44	22.26	0.996	1	1	1	1	1	1	1
342	0	7.6	22.26	0.996	1	1	1	1	1	**1**	**1**
343	0	7.88	22.62	0.996	1	1	1	1	1	**1**	**1**
344	50	4.78	22.12	0.996	1	1	0.97	0.96	0.93	0.93	0.96
345	50	5.02	21.96	0.996	1	1	1	1	0.98	1	1
346	50	5.39	22.44	0.996	1	1	1	1	1	1	1
347	50	5.56	22.08	0.996	1	1	1	1	1	1	1
348	50	6.01	22.1	0.996	1	1	1	1	1	1	1
349	50	6.1	22.38	0.996	1	1	1	1	1	1	1
350	50	6.68	22.02	0.996	1	1	1	1	1	1	1

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
351	50	7.56	22.1	0.996	1	1	1	1	1	**1**	**1**
352	50	7.21	22.02	0.996	1	1	1	1	1	1	1
353	50	7.49	22.26	0.996	1	1	1	1	1	1	1
354	50	7.58	22.18	0.996	1	1	1	1	1	**1**	**1**
355	50	7.78	22.52	0.996	1	1	1	1	1	**1**	**1**
356	100	5.1	22.04	0.996	1	1	0.97	0.97	0.94	0.97	0.99
357	100	5.57	20.94	0.996	1	1	1	1	0.99	1	1
358	100	5.58	21.12	0.996	1	1	1	1	0.99	1	1
359	100	5.59	22.5	0.996	1	1	1	1	0.99	1	1
360	100	5.85	20.94	0.996	1	1	1	1	1	1	1
361	100	6.04	22.1	0.996	1	1	1	1	1	1	1
362	100	6.32	21.02	0.996	1	1	1	1	1	1	1
363	100	6.52	22.16	0.996	1	1	1	1	1	1	1
364	100	6.94	22.1	0.996	1	1	1	1	1	1	1
365	100	7.1	22.04	0.996	1	1	1	1	1	1	1
366	100	7.19	22.16	0.996	1	1	1	1	1	1	1
367	100	7.25	20.94	0.996	1	1	1	1	1	1	1
368	100	7.51	21.96	0.996	1	1	1	1	1	**1**	**1**
369	100	7.61	22.38	0.996	1	1	1	1	1	**1**	**1**
370	100	7.8	22.36	0.996	1	1	1	1	1	**1**	**1**
371	100	8	22.76	0.996	1	1	1	1	1	**1**	**1**
372	200	5.4	22.08	0.994	1	1	0.93	0.94	0.92	0.98	0.99
373	200	5.77	21.23	0.994	1	1	1	1	0.99	1	1
374	200	5.93	22.12	0.994	1	1	1	1	0.99	1	1
375	200	6.25	22.46	0.994	1	1	1	1	1	1	1
376	200	6.32	21.32	0.994	1	1	1	1	1	1	1
377	200	6.96	22.06	0.994	1	1	1	1	1	1	1
378	200	7.1	21.1	0.994	1	1	1	1	1	1	1
379	200	7.28	22.1	0.994	1	1	1	1	1	1	1
380	200	7.45	21.98	0.994	1	1	1	1	1	1	1
381	200	7.53	20.98	0.994	1	1	1	1	1	**1**	**1**
382	200	7.65	22.4	0.994	1	1	1	1	1	**1**	**1**
383	200	5.65	21.84	0.994	1	1	1	1	0.98	1	1
384	200	6	21.94	0.994	1	1	1	1	0.99	1	1
385	200	6.72	21.32	0.994	1	1	1	1	1	1	1
386	200	5.58	21.32	0.994	1	1	0.99	0.99	0.97	1	1
387	200	6.01	21.42	0.994	1	1	1	1	0.99	1	1
388	200	6.69	21.7	0.994	1	1	1	1	1	1	1
389	200	7.28	21.56	0.994	1	1	1	1	1	1	1
390	200	7.86	21.14	0.994	1	1	1	1	1	**1**	**1**
391	200	7.88	22.6	0.994	1	1	1	1	1	**1**	**1**
392	200	7.78	22.26	0.994	1	1	1	1	1	**1**	**1**
393	200	8.14	20.98	0.994	1	1	1	1	1	**1**	**1**
394	200	8.28	21.32	0.994	1	1	1	1	1	**1**	**1**
395	500	6.76	21.38	0.986	1	1	0.98	1	0.95	0.99	1
396	500	7.78	21.3	0.986	1	1	0.99	1	0.96	**1**	**1**
397	500	8.14	21.28	0.986	1	1	0.99	1	0.96	**1**	**1**
398	500	6.86	21.6	0.986	1	1	0.99	1	0.96	0.99	1
399	500	7.75	21.76	0.986	1	1	0.99	1	0.96	**1**	**1**
400	500	8.14	21.88	0.986	1	1	0.99	1	0.96	**1**	**1**

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
401	0	3.23	22	0.996	0	1	**0**	**0**	0	**0**	**0**
402	0	3.69	22	0.996	0	1	**0**	**0**	0	**0**	**0**
403	50	3.12	22	0.996	0	1	**0**	**0**	0	**0**	**0**
404	50	3.63	22	0.996	0	1	**0**	**0**	0	**0**	**0**
405	50	4.29	22	0.996	0	1	**0**	**0**	0	**0**	**0**
406	100	3.16	22	0.996	0	1	**0**	**0**	0	**0**	**0**
407	100	3.64	22	0.996	0	1	**0**	**0**	0	**0**	**0**
408	100	4.24	22	0.996	0	1	**0**	**0**	0	**0**	**0**
409	100	4.78	22	0.996	0	1	**0**	**0**	0.08	0.04	0.06
410	200	3.19	22	0.994	0	1	**0**	**0**	0	**0**	**0**
411	200	3.67	22	0.994	0	1	**0**	**0**	0	**0**	**0**
412	200	4.2	22	0.994	0	1	**0**	**0**	0	**0**	**0**
413	200	4.69	22	0.994	0	1	**0**	**0**	0	**0**	**0**
414	200	4.87	22	0.994	0	1	**0**	**0**	0	**0**	**0**
415	200	5.09	22	0.994	0	1	**0**	**0**	0	**0**	**0**
416	200	3.26	22	0.994	0	1	**0**	**0**	0	**0**	**0**
417	200	3.78	22	0.994	0	1	**0**	**0**	0	**0**	**0**
418	200	4.38	22	0.994	0	1	**0**	**0**	0	**0**	**0**
419	200	4.77	22	0.994	0	1	**0**	**0**	0	**0**	**0**
420	200	5.02	22	0.994	0	1	**0**	**0**	0	**0**	**0**
421	200	5.24	22	0.994	0	1	0.13	0.30	0.60	0.70	0.82
422	200	3.21	22	0.994	0	1	**0**	**0**	0	**0**	**0**
423	200	3.75	22	0.994	0	1	**0**	**0**	0	**0**	**0**
424	200	4.35	22	0.994	0	1	**0**	**0**	0	**0**	**0**
425	200	4.75	22	0.994	0	1	**0**	**0**	0	**0**	**0**
426	200	5.01	22	0.994	0	1	**0**	**0**	0	**0**	**0**
427	200	5.19	22	0.994	0	1	0.01	0.08	0.38	0.42	0.58
428	500	2.71	22	0.986	0	1	**0**	**0**	0	**0**	**0**
429	500	3.46	22	0.986	0	1	**0**	**0**	0	**0**	**0**
430	500	4.04	22	0.986	0	1	**0**	**0**	0	**0**	**0**
431	500	4.45	22	0.986	0	1	**0**	**0**	0	**0**	**0**
432	500	4.62	22	0.986	0	1	**0**	**0**	0	**0**	**0**
433	500	4.75	22	0.986	0	1	**0**	**0**	0	**0**	**0**
434	500	4.9	22	0.986	0	1	**0**	**0**	0	**0**	**0**
435	500	5	22	0.986	0	1	**0**	**0**	0	**0**	**0**
436	500	5.13	22	0.986	0	1	**0**	**0**	0	**0**	**0**
437	500	5.27	22	0.986	0	1	**0**	**0**	0	**0**	**0**
438	500	5.49	22	0.986	0	1	**0**	**0**	0	**0**	**0**
439	500	5.87	22	0.986	0	1	0.61	0.60	0.67	0.54	0.60
440	500	2.74	22	0.986	0	1	**0**	**0**	0	**0**	**0**
441	500	3.47	22	0.986	0	1	**0**	**0**	0	**0**	**0**
442	500	4.04	22	0.986	0	1	**0**	**0**	0	**0**	**0**
443	500	4.6	22	0.986	0	1	**0**	**0**	0	**0**	**0**
444	500	4.74	22	0.986	0	1	**0**	**0**	0	**0**	**0**
445	500	4.9	22	0.986	0	1	**0**	**0**	0	**0**	**0**
446	500	5	22	0.986	0	1	**0**	**0**	0	**0**	**0**
447	500	5.11	22	0.986	0	1	**0**	**0**	0	**0**	**0**
448	500	5.25	22	0.986	0	1	**0**	**0**	0	**0**	**0**
449	500	5.47	22	0.986	0	1	**0**	**0**	0	**0**	**0**
450	500	5.93	22	0.986	0	1	0.76	0.76	0.75	0.69	0.75

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
451	500	4.44	22	0.986	0	1	**0**	**0**	0	**0**	**0**
452	0	4.02	21.08	0.996	1	1	0.92	0.95	0.96	0.94	0.96
453	0	4.07	21.12	0.996	1	1	0.98	0.99	0.97	0.96	0.98
454	0	4.13	21.14	0.996	1	1	0.99	1	0.98	0.97	0.99
455	0	4.39	21.18	0.996	1	1	1	1	0.99	1	1
456	0	4.6	21.20	0.996	1	1	1	1	1	1	1
457	0	5.27	21.26	0.996	1	1	1	1	1	1	1
458	0	6.88	21.26	0.996	1	1	1	1	1	1	1
459	25	4.42	20.88	0.996	1	1	0.91	0.87	0.86	0.66	0.74
460	25	4.52	20.94	0.996	1	1	0.98	0.97	0.94	0.89	0.93
461	25	4.57	20.94	0.996	1	1	0.99	0.98	0.95	0.94	0.97
462	25	5.16	20.98	0.996	1	1	1	1	1	1	1
463	25	6.02	20.96	0.996	1	1	1	1	1	1	1
464	25	6.73	21.00	0.996	1	1	1	1	1	1	1
465	50	4.56	21.20	0.996	1	1	0.06	0.14	0.44	0.18	0.24
466	50	4.6	21.22	0.996	1	1	0.26	0.33	0.60	0.34	0.43
467	50	4.71	21.26	0.996	1	1	0.88	0.86	0.86	0.80	0.87
468	50	5.08	21.30	0.996	1	1	1	1	0.99	1	1
469	50	6.13	21.38	0.996	1	1	1	1	1	1	1
470	100	5	20.88	0.996	1	1	0.77	0.78	0.82	0.86	0.93
471	100	5.2	20.92	0.996	1	1	0.99	0.99	0.97	0.99	1.00
472	100	5.41	20.94	0.996	1	1	1	1	0.99	1	1
473	100	5.74	20.94	0.996	1	1	1	1	1	1	1
474	100	6.16	20.96	0.996	1	1	1	1	1	1	1
475	200	5.2	21.70	0.994	1	1	0.02	0.10	0.42	0.48	0.64
476	200	5.31	21.76	0.994	1	1	0.65	0.71	0.80	0.90	0.95
477	200	5.84	21.82	0.994	1	1	1	1	1	1	1
478	0	2.94	21.00	0.996	0	1	**0**	**0**	**0**	**0**	**0**
479	0	3.48	21.02	0.996	0	1	**0**	**0**	**0**	**0**	**0**
480	0	3.74	21.06	0.996	0	1	**0**	**0**	**0**	**0**	**1**
481	25	3.13	20.84	0.996	0	1	**0**	**0**	0	**0**	**0**
482	25	4.04	20.84	0.996	0	1	0	0	0.02	0	0
483	25	4.23	20.86	0.996	0	1	0.08	0.09	0.39	0.07	0.08
484	25	4.26	20.86	0.996	0	1	0.20	0.18	0.49	0.12	0.13
485	50	3.16	21.02	0.996	0	1	**0**	**0**	0	**0**	**0**
486	50	4.13	21.08	0.996	0	1	**0**	**0**	0	**0**	**0**
487	50	4.34	21.08	0.996	0	1	**0**	**0**	0	**0**	**0**
488	50	4.47	21.10	0.996	0	1	0	0.01	0.12	0.03	0.03
489	100	4.42	20.82	0.996	0	1	**0**	**0**	0	**0**	**0**
490	100	4.6	20.82	0.996	0	1	**0**	**0**	0	**0**	**0**
491	100	4.75	20.84	0.996	0	1	**0**	**0**	0	**0**	**0**
492	100	4.83	20.84	0.996	0	1	0	0.02	0.21	0.13	0.20
493	200	4.45	21.50	0.994	0	1	**0**	**0**	0	**0**	**0**
494	200	4.67	21.52	0.994	0	1	**0**	**0**	0	**0**	**0**
495	200	4.89	21.60	0.994	0	1	**0**	**0**	0	**0**	**0**
496	200	4.94	21.62	0.994	0	1	**0**	**0**	0	**0**	**0**
497	200	5.04	21.66	0.994	0	1	**0**	**0**	0	**0**	**0**
498	200	5.07	21.68	0.994	0	1	**0**	**0**	0	**0**	**0**
499	500	4.49	21.44	0.986	0	1	**0**	**0**	0	**0**	**0**
500	500	4.9	21.48	0.986	0	1	**0**	**0**	0	**0**	**0**

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
501	500	5.31	21.46	0.986	0	1	**0**	**0**	0	**0**	**0**
502	500	5.28	21.46	0.986	0	1	**0**	**0**	0	**0**	**0**
503	500	5.33	21.42	0.986	0	1	**0**	**0**	0	**0**	**0**
504	500	5.41	21.40	0.986	0	1	**0**	**0**	0	**0**	**0**
505	500	5.44	21.36	0.986	0	1	**0**	**0**	0	**0**	**0**
506	500	5.55	21.34	0.986	0	1	0	0	0.02	0	0
507	500	5.62	21.30	0.986	0	1	0	0.01	0.09	0.02	0.02
508	0	4	37	0.985	2	2	0.78	0.70	0.88	0.85	0.76
509	0	4.15	37	0.985	2	2	0.99	0.99	0.95	0.95	0.92
510	0	4.83	37	0.985	2	2	1	1	0.99	1	1
511	0	5.42	37	0.985	2	2	1	1	0.99	1	1
512	0	5.96	37	0.985	2	2	1	1	0.99	1	1
513	0	6.78	37	0.985	2	2	1	1	0.99	1	1
514	0	4	30	0.985	2	2	0.69	0.76	0.93	0.80	0.79
515	0	4.15	30	0.985	2	2	0.99	0.99	0.97	0.93	0.93
516	0	4.83	30	0.985	2	2	1	1	1	1	1
517	0	5.42	30	0.985	2	2	1	1	1	1	1
518	0	5.96	30	0.985	2	2	1	1	1	1	1
519	0	6.78	30	0.985	2	2	1	1	1	1	1
520	0	4	25	0.985	2	2	0.59	0.68	0.91	0.73	0.77
521	0	4.15	25	0.985	2	2	0.98	0.99	0.96	0.90	0.93
522	0	4.83	25	0.985	2	2	1	1	0.99	1	1
523	0	5.42	25	0.985	2	2	1	1	1	1	1
524	0	5.96	25	0.985	2	2	1	1	1	1	1
525	0	6.78	25	0.985	2	2	1	1	1	1	1
526	0	4	20	0.985	0	2	0.46	0.54	0.86	0.62	0.69
527	0	4.15	20	0.985	2	2	0.96	0.97	0.94	0.85	0.90
528	0	4.83	20	0.985	2	2	1	1	0.99	1	1
529	0	5.42	20	0.985	2	2	1	1	0.99	1	1
530	0	5.96	20	0.985	2	2	1	1	0.99	1	1
531	0	6.78	20	0.985	2	2	1	1	0.99	1	1
532	0	4	10	0.985	0	2	0.10	0.11	0.42	0.12	0.05
533	0	4.15	10	0.985	0	2	0.79	0.80	0.66	0.32	0.16
534	0	4.83	10	0.985	2	2	0.99	0.99	0.91	0.95	0.94
535	0	5.42	10	0.985	2	2	0.99	1	0.94	0.99	0.99
536	0	5.96	10	0.985	2	2	0.99	1	0.94	1	1
537	0	6.78	10	0.985	2	2	0.99	1	0.94	1	1
538	0	4.11	37	0.975	2	2	0.90	0.84	0.82	0.64	0.63
539	0	4.6	37	0.975	2	2	1	0.99	0.96	0.97	0.98
540	0	5.09	37	0.975	2	2	1	1	0.98	1	1
541	0	5.65	37	0.975	2	2	1	1	0.98	1	1
542	0	6.18	37	0.975	2	2	1	1	0.98	1	1
543	0	6.86	37	0.975	2	2	1	1	0.98	1	1
544	0	4.11	30	0.975	2	2	0.85	0.87	0.90	0.54	0.59
545	0	4.6	30	0.975	2	2	0.99	1	0.98	0.96	0.98
546	0	5.09	30	0.975	2	2	1	1	0.99	0.99	1
547	0	5.65	30	0.975	2	2	1	1	0.99	1	1
548	0	6.18	30	0.975	2	2	1	1	0.99	1	1
549	0	6.86	30	0.975	2	2	1	1	0.99	1	1
550	0	4.11	25	0.975	2	2	0.78	0.82	0.86	0.45	0.51

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
551	0	4.6	25	0.975	2	2	0.99	0.99	0.97	0.95	0.97
552	0	5.09	25	0.975	2	2	1	1	0.98	0.99	1
553	0	5.65	25	0.975	2	2	1	1	0.98	1	1
554	0	6.18	25	0.975	2	2	1	1	0.99	1	1
555	0	6.86	25	0.975	2	2	1	1	0.99	1	1
556	0	4.11	20	0.975	0	2	0.68	0.72	0.79	0.34	0.33
557	0	4.6	20	0.975	2	2	0.98	0.99	0.95	0.91	0.94
558	0	5.09	20	0.975	2	2	0.99	1	0.97	0.99	0.99
559	0	5.65	20	0.975	2	2	0.99	1	0.97	1	1
560	0	6.18	20	0.975	2	2	0.99	1	0.98	1	1
561	0	6.86	20	0.975	2	2	0.99	1	0.98	1	1
562	0	4.11	10	0.975	0	2	0.23	0.21	0.31	0.04	0
563	0	4.6	10	0.975	0	2	0.90	0.90	0.70	0.48	0.10
564	0	5.09	10	0.975	0	2	0.95	0.95	0.79	0.87	0.55
565	0	5.65	10	0.975	2	2	0.96	0.97	0.82	0.97	0.90
566	0	6.18	10	0.975	2	2	0.96	0.97	0.83	0.99	0.97
567	0	6.86	10	0.975	2	2	0.96	0.99	0.83	0.99	0.99
568	0	4.15	37	0.965	2	2	0.58	0.39	0.55	0.14	0.22
569	0	4.63	37	0.965	2	2	0.96	0.93	0.84	0.75	0.89
570	0	5.12	37	0.965	2	2	0.98	0.97	0.89	0.96	0.99
571	0	5.66	37	0.965	2	2	0.98	0.97	0.91	0.99	1
572	0	6.14	37	0.965	2	2	0.98	0.98	0.91	1	1
573	0	6.87	37	0.965	2	2	0.98	0.99	0.91	1	1
574	0	4.15	30	0.965	1	2	0.46	0.46	0.69	0.10	0.14
575	0	4.63	30	0.965	2	2	0.94	0.95	0.91	0.67	0.82
576	0	5.12	30	0.965	2	2	0.97	0.97	0.94	0.94	0.98
577	0	5.66	30	0.965	2	2	0.97	0.98	0.95	0.98	1
578	0	6.14	30	0.965	2	2	0.97	0.98	0.95	0.99	1
579	0	6.87	30	0.965	2	2	0.97	0.99	0.95	1	1
580	0	4.15	25	0.965	0	2	0.36	0.36	0.62	0.07	0.08
581	0	4.63	25	0.965	1	2	0.91	0.92	0.88	0.59	0.70
582	0	5.12	25	0.965	2	2	0.95	0.96	0.92	0.91	0.96
583	0	5.66	25	0.965	2	2	0.96	0.97	0.93	0.98	0.99
584	0	6.14	25	0.965	2	2	0.96	0.98	0.93	0.99	1
585	0	6.87	25	0.965	2	2	0.96	0.99	0.93	1	1
586	0	4.15	20	0.965	0	2	0.24	0.24	0.50	0.05	0.02
587	0	4.63	20	0.965	0	2	0.85	0.87	0.81	0.47	0.41
588	0	5.12	20	0.965	0	2	0.91	0.93	0.87	0.86	0.88
589	0	5.66	20	0.965	2	2	0.93	0.95	0.89	0.96	0.98
590	0	6.14	20	0.965	2	2	0.93	0.96	0.89	0.99	0.99
591	0	6.87	20	0.965	2	2	0.93	0.98	0.89	0.99	1
592	0	4.15	10	0.965	0	2	0.04	0.03	0.11	0	0
593	0	4.63	10	0.965	0	2	0.43	0.41	0.34	0.07	0
594	0	5.12	10	0.965	0	2	0.60	0.59	0.45	0.35	0.01
595	0	5.66	10	0.965	2	2	0.65	0.65	0.49	0.70	0.07
596	0	6.14	10	0.965	2	2	0.66	0.69	0.50	0.85	0.19
597	0	6.87	10	0.965	2	2	0.66	0.84	0.50	0.93	0.45
598	0	4.17	37	0.955	0	2	0.05	0.02	0.13	0.01	0.01
599	0	4.66	37	0.955	0	2	0.44	0.24	0.38	0.08	0.29
600	0	5.2	37	0.955	0	2	0.60	0.39	0.49	0.42	0.83

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
601	0	5.8	37	0.955	2	2	0.64	0.45	0.53	0.77	0.97
602	0	6.23	37	0.955	2	2	0.65	0.50	0.53	0.87	0.99
603	0	6.84	37	0.955	2	2	0.66	0.67	0.53	0.93	1
604	0	4.17	30	0.955	0	2	0.03	0.02	0.22	0	0
605	0	4.66	30	0.955	0	2	0.33	0.30	0.53	0.06	0.12
606	0	5.2	30	0.955	0	2	0.48	0.46	0.64	0.33	0.62
607	0	5.8	30	0.955	2	2	0.53	0.53	0.67	0.69	0.92
608	0	6.23	30	0.955	2	2	0.54	0.57	0.67	0.82	0.97
609	0	6.84	30	0.955	2	2	0.54	0.74	0.68	0.91	0.99
610	0	4.17	25	0.955	0	2	0.02	0.02	0.17	0	0
611	0	4.66	25	0.955	0	2	0.24	0.22	0.45	0.04	0.04
612	0	5.2	25	0.955	0	2	0.38	0.36	0.57	0.26	0.32
613	0	5.8	25	0.955	2	2	0.42	0.43	0.60	0.61	0.76
614	0	6.23	25	0.955	2	2	0.43	0.47	0.60	0.76	0.89
615	0	6.84	25	0.955	2	2	0.43	0.65	0.61	0.87	0.96
616	0	4.17	20	0.955	0	2	0.01	0.01	0.11	0	0
617	0	4.66	20	0.955	0	2	0.16	0.13	0.33	0.03	0.01
618	0	5.2	20	0.955	0	2	0.26	0.24	0.44	0.18	0.07
619	0	5.8	20	0.955	0	2	0.30	0.29	0.47	0.49	0.33
620	0	6.23	20	0.955	2	2	0.30	0.33	0.48	0.67	0.57
621	0	6.84	20	0.955	2	2	0.31	0.50	0.48	0.81	0.79
622	0	4.17	10	0.955	0	2	0	0	0.01	0	0
623	0	4.66	10	0.955	0	2	0.03	0.02	0.06	0	0
624	0	5.2	10	0.955	0	2	0.05	0.03	0.09	0.02	0
625	0	5.8	10	0.955	0	2	0.06	0.04	0.10	0.07	0
626	0	6.23	10	0.955	0	2	0.06	0.05	0.10	0.14	0
627	0	6.84	10	0.955	0	2	0.06	0.10	0.10	0.26	0
628	0	4.28	8	0.945	0	1	**0**	**0**	0	0	0
629	0	4.48	8	0.945	0	1	**0**	**0**	0	0	0
630	0	4.74	8	0.945	0	1	**0**	**0**	0	0	0
631	0	5.01	8	0.945	0	1	**0**	**0**	0	0	0
632	0	5.36	8	0.945	0	1	**0**	**0**	0	0	0
633	0	5.74	8	0.945	0	1	**0**	**0**	0	0	0
634	0	6.11	8	0.945	0	1	**0**	**0**	0	0	0
635	0	6.83	8	0.945	0	1	**0**	**0**	0	0	0
636	0	4.28	12	0.945	0	1	**0**	**0**	0	0	0
637	0	4.48	12	0.945	0	1	**0**	**0**	0	0	0
638	0	4.74	12	0.945	0	1	**0**	**0**	0	0	0
639	0	5.01	12	0.945	0	1	**0**	**0**	0	0	0
640	0	5.36	12	0.945	0	1	**0**	**0**	0	0	0
641	0	4.28	17	0.945	0	1	**0**	**0**	0	0	0
642	0	4.48	17	0.945	0	1	**0**	**0**	0.01	0	0
643	0	4.74	17	0.945	0	1	**0**	**0**	0.01	0	0
644	0	5.01	17	0.945	0	1	**0**	**0**	0.01	0	0
645	0	5.36	17	0.945	0	1	**0**	**0**	0.01	0	0
646	0	4.28	21	0.945	0	1	**0**	**0**	0.01	0	0
647	0	4.48	21	0.945	0	1	**0**	**0**	0.01	0	0
648	0	4.74	21	0.945	0	1	**0**	**0**	0.02	0	0
649	0	5.01	21	0.945	0	1	**0**	**0**	0.02	0	0
650	0	5.36	21	0.945	0	1	**0**	**0**	0.02	0	0

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
651	0	4.28	24	0.945	0	1	**0**	**0**	0.01	0	0
652	0	4.48	24	0.945	0	1	**0**	**0**	0.01	0	0
653	0	4.74	24	0.945	0	1	**0**	**0**	0.02	0	0
654	0	5.01	24	0.945	0	1	**0**	**0**	0.03	0	0
655	0	5.36	24	0.945	0	1	**0**	**0**	0.03	0	0
656	0	4.28	27	0.945	0	1	**0**	**0**	0.01	0	0
657	0	4.48	27	0.945	0	1	**0**	**0**	0.02	0	0
658	0	4.74	27	0.945	0	1	**0**	**0**	0.03	0	0
659	0	5.01	27	0.945	0	1	**0**	**0**	0.03	0	0
660	0	5.36	27	0.945	0	1	**0**	**0**	0.04	0	0
661	0	4.28	32	0.945	0	1	**0**	**0**	0.01	0	0
662	0	4.48	32	0.945	0	1	**0**	**0**	0.02	0	0
663	0	4.74	32	0.945	0	1	**0**	**0**	0.03	0	0
664	0	5.01	32	0.945	0	1	**0**	**0**	0.04	0	0
665	0	5.36	32	0.945	0	1	**0**	**0**	0.05	0	0
666	0	4.28	36	0.945	0	1	**0**	**0**	0.01	0	0
667	0	4.48	36	0.945	0	1	**0**	**0**	0.01	0	0
668	0	4.74	36	0.945	0	1	**0**	**0**	0.02	0	0
669	0	5.01	36	0.945	0	1	**0**	**0**	0.03	0	0
670	0	5.36	36	0.945	0	1	**0**	**0**	0.03	0	0.01
671	0	5.42	8	0.955	0	1	**0.02**	**0.01**	0.03	0	0
672	0	5.9	8	0.955	0	1	**0.03**	**0.02**	0.03	0	0
673	0	6.19	8	0.955	0	1	**0.03**	**0.02**	0.03	0	0
674	0	6.58	8	0.955	0	1	**0.03**	**0.03**	0.03	0	0
675	0	7.02	8	0.955	0	1	**0.03**	**0.06**	0.03	0.01	0
676	0	5.42	12	0.955	0	1	**0.09**	**0.07**	0.17	0.08	0
677	0	5.9	12	0.955	0	1	**0.10**	**0.08**	0.18	0.20	0
678	0	6.19	12	0.955	0	1	**0.10**	**0.09**	0.18	0.29	0
679	0	6.58	12	0.955	0	1	**0.10**	**0.12**	0.18	0.41	0.01
680	0	7.02	12	0.955	0	1	**0.10**	**0.25**	0.18	0.51	0.01
681	0	5.42	17	0.955	0	1	**0.20**	**0.18**	0.36	0.21	0.03
682	0	5.9	17	0.955	0	1	**0.22**	**0.21**	0.38	0.44	0.12
683	0	6.19	17	0.955	0	1	**0.22**	**0.23**	0.38	0.56	0.20
684	0	6.58	17	0.955	0	1	**0.22**	**0.29**	0.38	0.68	0.34
685	0	7.02	17	0.955	0	1	**0.23**	**0.51**	0.38	0.77	0.49
686	0	5.42	21	0.955	1	1	**0.30**	**0.29**	0.49	0.31	0.20
687	0	5.9	21	0.955	1	1	**0.32**	**0.33**	0.51	0.56	0.50
688	0	6.19	21	0.955	1	1	**0.33**	**0.35**	0.51	0.68	0.66
689	0	6.58	21	0.955	1	1	**0.33**	**0.43**	0.51	0.78	0.80
690	0	7.02	21	0.955	1	1	**0.33**	**0.65**	0.51	0.85	0.88
691	0	5.42	24	0.955	1	1	**0.38**	**0.37**	0.56	0.37	0.43
692	0	5.9	24	0.955	1	1	**0.40**	**0.41**	0.58	0.63	0.75
693	0	6.19	24	0.955	1	1	**0.40**	**0.44**	0.58	0.74	0.85
694	0	6.58	24	0.955	1	1	**0.41**	**0.52**	0.59	0.82	0.92
695	0	7.02	24	0.955	1	1	**0.41**	**0.73**	0.59	0.88	0.96
696	0	5.42	27	0.955	1	1	**0.45**	**0.44**	0.62	0.43	0.64
697	0	5.9	27	0.955	1	1	**0.47**	**0.48**	0.64	0.69	0.88
698	0	6.19	27	0.955	1	1	**0.47**	**0.51**	0.64	0.78	0.93
699	0	6.58	27	0.955	1	1	**0.48**	**0.59**	0.64	0.86	0.97
700	0	7.02	27	0.955	1	1	**0.48**	**0.79**	0.64	0.90	0.98

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
701	0	5.42	32	0.955	1	1	**0.54**	**0.51**	0.66	0.51	0.84
702	0	5.9	32	0.955	1	1	**0.57**	**0.55**	0.68	0.75	0.95
703	0	6.19	32	0.955	1	1	**0.57**	**0.59**	0.68	0.83	0.97
704	0	6.58	32	0.955	1	1	**0.58**	**0.66**	0.68	0.89	0.99
705	0	7.02	32	0.955	1	1	**0.58**	**0.83**	0.68	0.93	0.99
706	0	5.42	36	0.955	1	1	**0.61**	**0.46**	0.57	0.57	0.90
707	0	5.9	36	0.955	1	1	**0.63**	**0.51**	0.59	0.79	0.97
708	0	6.19	36	0.955	1	1	**0.64**	**0.54**	0.60	0.86	0.99
709	0	6.58	36	0.955	1	1	**0.64**	**0.62**	0.60	0.91	0.99
710	0	7.02	36	0.955	1	1	**0.64**	**0.80**	0.60	0.94	1
711	0	5.42	8	0.965	0	1	**0.43**	**0.39**	0.22	0.02	0
712	0	5.8	8	0.965	0	1	**0.45**	**0.43**	0.23	0.04	0
713	0	6.12	8	0.965	0	1	**0.46**	**0.46**	0.23	0.08	0.01
714	0	6.52	8	0.965	0	1	**0.46**	**0.53**	0.23	0.12	0.02
715	0	6.87	8	0.965	0	1	**0.46**	**0.67**	0.23	0.17	0.03
716	0	5.42	12	0.965	0	1	**0.75**	**0.77**	0.64	0.77	0.23
717	0	5.8	12	0.965	0	1	**0.77**	**0.79**	0.66	0.89	0.48
718	0	6.12	12	0.965	0	1	**0.77**	**0.81**	0.66	0.93	0.67
719	0	6.52	12	0.965	0	1	**0.78**	**0.85**	0.66	0.96	0.81
720	0	6.87	12	0.965	0	1	**0.78**	**0.91**	0.66	0.97	0.88
721	0	5.42	17	0.965	0	1	**0.89**	**0.91**	0.83	0.91	0.88
722	0	5.8	17	0.965	1	1	**0.90**	**0.92**	0.84	0.96	0.96
723	0	6.12	17	0.965	1	1	**0.90**	**0.93**	0.85	0.98	0.98
724	0	6.52	17	0.965	1	1	**0.90**	**0.95**	0.85	0.99	0.99
725	0	6.87	17	0.965	1	1	**0.90**	**0.97**	0.85	0.99	0.99
726	0	5.42	21	0.965	1	1	**0.93**	**0.95**	0.90	0.94	0.97
727	0	5.8	21	0.965	1	1	**0.94**	**0.96**	0.90	0.98	0.99
728	0	6.12	21	0.965	1	1	**0.94**	**0.96**	0.90	0.99	0.99
729	0	6.52	21	0.965	1	1	**0.94**	**0.97**	0.90	0.99	1
730	0	6.87	21	0.965	1	1	**0.94**	**0.98**	0.90	0.99	1
731	0	4.27	24	0.965	0	1	**0.63**	**0.66**	0.72	0.15	0.15
732	0	5.42	24	0.965	1	1	**0.95**	**0.96**	0.92	0.96	0.98
733	0	5.8	24	0.965	1	1	**0.95**	**0.97**	0.92	0.98	0.99
734	0	6.12	24	0.965	1	1	**0.96**	**0.97**	0.93	0.99	1
735	0	6.87	24	0.965	1	1	**0.96**	**0.99**	0.93	1	1
736	0	4.27	27	0.965	0	1	**0.70**	**0.72**	0.77	0.18	0.23
737	0	4.36	27	0.965	0	1	**0.81**	**0.83**	0.82	0.28	0.36
738	0	4.69	27	0.965	1	1	**0.93**	**0.94**	0.90	0.69	0.82
739	0	4.89	27	0.965	1	1	**0.95**	**0.96**	0.92	0.84	0.92
740	0	5.42	27	0.965	1	1	**0.96**	**0.97**	0.94	0.97	0.99
741	0	4.27	32	0.965	0	1	**0.77**	**0.78**	0.80	0.23	0.34
742	0	4.36	32	0.965	1	1	**0.86**	**0.87**	0.84	0.35	0.50
743	0	4.69	32	0.965	1	1	**0.95**	**0.96**	0.92	0.76	0.89
744	0	4.89	32	0.965	1	1	**0.96**	**0.97**	0.93	0.88	0.96
745	0	5.42	32	0.965	1	1	**0.97**	**0.98**	0.95	0.98	0.99
746	0	4.27	36	0.965	0	1	**0.82**	**0.74**	0.73	0.27	0.41
747	0	4.36	36	0.965	0	1	**0.89**	**0.84**	0.79	0.40	0.57
748	0	4.69	36	0.965	1	1	**0.96**	**0.95**	0.88	0.79	0.91
749	0	4.89	36	0.965	1	1	**0.97**	**0.96**	0.90	0.90	0.97
750	0	5.42	36	0.965	1	1	**0.98**	**0.98**	0.92	0.98	1

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
751	0	5.23	8	0.975	0	1	**0.89**	**0.90**	0.56	0.14	0.11
752	0	5.75	8	0.975	0	1	**0.91**	**0.92**	0.58	0.37	0.40
753	0	6.15	8	0.975	0	1	**0.91**	**0.93**	0.59	0.55	0.64
754	0	6.5	8	0.975	0	1	**0.91**	**0.94**	0.59	0.66	0.78
755	0	6.98	8	0.975	0	1	**0.91**	**0.98**	0.59	0.76	0.88
756	0	5.23	12	0.975	1	1	**0.97**	**0.98**	0.89	0.96	0.93
757	0	5.75	12	0.975	1	1	**0.98**	**0.98**	0.90	0.99	0.99
758	0	6.15	12	0.975	1	1	**0.98**	**0.99**	0.90	1	0.99
759	0	6.5	12	0.975	1	1	**0.98**	**0.99**	0.90	1	1
760	0	6.98	12	0.975	1	1	**0.98**	**1**	0.90	1	1
761	0	4.26	17	0.975	0	1	**0.89**	**0.91**	0.84	0.50	0.44
762	0	5.23	17	0.975	1	1	**0.99**	**0.99**	0.96	0.99	0.99
763	0	5.75	17	0.975	1	1	**0.99**	**0.99**	0.96	1	1
764	0	6.15	17	0.975	1	1	**0.99**	**1**	0.96	1	1
765	0	6.98	17	0.975	1	1	**0.99**	**1**	0.96	1	1
766	0	4.26	21	0.975	1	1	**0.93**	**0.95**	0.90	0.63	0.67
767	0	5.23	21	0.975	1	1	**0.99**	**1**	0.97	0.99	1
768	0	5.75	21	0.975	1	1	**0.99**	**1**	0.98	1	1
769	0	6.15	21	0.975	1	1	**0.99**	**1**	0.98	1	1
770	0	6.98	21	0.975	1	1	**0.99**	**1**	0.98	1	1
771	0	4.26	24	0.975	1	1	**0.95**	**0.96**	0.92	0.69	0.76
772	0	4.35	24	0.975	1	1	**0.97**	**0.98**	0.94	0.80	0.86
773	0	4.61	24	0.975	1	1	**0.99**	**0.99**	0.97	0.94	0.97
774	0	5	24	0.975	1	1	**0.99**	**1**	0.98	0.99	1
775	0	5.23	24	0.975	1	1	**1**	**1**	0.98	0.99	1
776	0	4.26	27	0.975	1	1	**0.96**	**0.97**	0.94	0.74	0.81
777	0	4.35	27	0.975	1	1	**0.98**	**0.99**	0.95	0.84	0.89
778	0	4.61	27	0.975	1	1	**0.99**	**1**	0.97	0.96	0.98
779	0	5	27	0.975	1	1	**1**	**1**	0.98	0.99	1
780	0	5.23	27	0.975	1	1	**1**	**1**	0.99	1	1
781	0	4.26	32	0.975	1	1	**0.97**	**0.98**	0.95	0.80	0.84
782	0	4.35	32	0.975	1	1	**0.99**	**0.99**	0.96	0.88	0.91
783	0	4.61	32	0.975	1	1	**0.99**	**1**	0.98	0.97	0.98
784	0	5	32	0.975	1	1	**1**	**1**	0.99	0.99	1
785	0	5.23	32	0.975	1	1	**1**	**1**	0.99	1	1
786	0	4.26	36	0.975	1	1	**0.98**	**0.98**	0.93	0.83	0.85
787	0	4.35	36	0.975	1	1	**0.99**	**0.99**	0.95	0.90	0.92
788	0	4.61	36	0.975	1	1	**1**	**1**	0.97	0.97	0.98
789	0	5	36	0.975	1	1	**1**	**1**	0.98	0.99	1
790	0	5.23	36	0.975	1	1	**1**	**1**	0.98	1	1
791	0	4.28	8	0.985	0	1	**0.86**	**0.86**	0.51	0.02	0.05
792	0	4.48	8	0.985	1	1	**0.95**	**0.95**	0.66	0.06	0.16
793	0	4.74	8	0.985	1	1	**0.97**	**0.98**	0.75	0.17	0.46
794	0	5.01	8	0.985	1	1	**0.98**	**0.98**	0.79	0.38	0.76
795	0	5.36	8	0.985	1	1	**0.98**	**0.99**	0.82	0.65	0.93
796	0	5.74	8	0.985	1	1	**0.99**	**0.99**	0.83	0.82	0.98
797	0	6.11	8	0.985	1	1	**0.99**	**0.99**	0.83	0.90	0.99
798	0	6.83	8	0.985	1	1	**0.99**	**1**	0.83	0.96	1
799	0	4.28	12	0.985	0	1	**0.96**	**0.97**	0.87	0.75	0.69
800	0	4.48	12	0.985	1	1	**0.99**	**0.99**	0.93	0.91	0.90

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
801	0	4.74	12	0.985	1	1	**0.99**	**1**	0.95	0.97	0.98
802	0	5.01	12	0.985	1	1	**1**	**1**	0.96	0.99	0.99
803	0	5.36	12	0.985	1	1	**1**	**1**	0.97	1	1
804	0	4.28	17	0.985	1	1	**0.99**	**0.99**	0.95	0.90	0.94
805	0	4.48	17	0.985	1	1	**0.99**	**1**	0.97	0.97	0.98
806	0	4.74	17	0.985	1	1	**1**	**1**	0.98	0.99	1
807	0	5.01	17	0.985	1	1	**1**	**1**	0.99	1	1
808	0	5.36	17	0.985	1	1	**1**	**1**	0.99	1	1
809	0	4.28	21	0.985	1	1	**0.99**	**0.99**	0.97	0.94	0.97
810	0	4.48	21	0.985	1	1	**1**	**1**	0.98	0.98	0.99
811	0	4.74	21	0.985	1	1	**1**	**1**	0.99	0.99	1
812	0	5.01	21	0.985	1	1	**1**	**1**	0.99	1	1
813	0	5.36	21	0.985	1	1	**1**	**1**	0.99	1	1
814	0	4.28	24	0.985	1	1	**0.99**	**1**	0.98	0.95	0.97
815	0	4.48	24	0.985	1	1	**1**	**1**	0.99	0.99	0.99
816	0	4.74	24	0.985	1	1	**1**	**1**	0.99	1.00	1.00
817	0	5.01	24	0.985	1	1	**1**	**1**	0.99	1.00	1.00
818	0	5.36	24	0.985	1	1	**1**	**1**	0.99	1.00	1.00
819	0	4.28	27	0.985	1	1	**1**	**1**	0.98	0.96	0.98
820	0	4.48	27	0.985	1	1	**1**	**1**	0.99	0.99	0.99
821	0	4.74	27	0.985	1	1	**1**	**1**	0.99	1.00	1.00
822	0	5.01	27	0.985	1	1	**1**	**1**	1.00	1.00	1.00
823	0	5.36	27	0.985	1	1	**1**	**1**	1.00	1.00	1.00
824	0	4.28	32	0.985	1	1	**1**	**1**	0.99	0.97	0.97
825	0	4.48	32	0.985	1	1	**1**	**1**	0.99	0.99	0.99
826	0	4.74	32	0.985	1	1	**1**	**1**	0.99	1.00	1.00
827	0	5.01	32	0.985	1	1	**1**	**1**	1.00	1.00	1.00
828	0	5.36	32	0.985	1	1	**1**	**1**	1.00	1.00	1.00
829	0	4.28	36	0.985	1	1	**1**	**1**	0.98	0.98	0.97
830	0	4.48	36	0.985	1	1	**1**	**1**	0.99	0.99	0.99
831	0	4.74	36	0.985	1	1	**1**	**1**	0.99	1.00	1.00
832	0	5.01	36	0.985	1	1	**1**	**1**	0.99	1.00	1.00
833	0	5.36	36	0.985	1	1	**1**	**1**	0.99	1.00	1.00
834	0	10.21	35	0.996	0	1	**1**	**0**	0	**1**	**1**
835	0	10.13	35	0.996	0	1	**1**	**0**	0	**1**	**1**
836	0	10.02	34	0.996	0	1	**1**	**0**	0	**1**	**1**
837	0	9.87	34	0.996	0	1	**1**	**0**	0	**1**	**1**
838	0	9.79	34	0.996	0	1	**1**	**0**	0	**1**	**1**
839	0	9.62	34	0.996	0	1	**1**	**0**	0	**1**	**1**
840	0	9.47	34	0.996	0	1	**1**	**0**	0	**1**	**1**
841	0	9.29	34	0.996	1	1	**1**	**0**	0.97	**1**	**1**
842	0	9.14	35	0.996	1	1	**1**	**0**	0.99	**1**	**1**
843	0	8.98	35	0.996	1	1	**1**	**1**	1	**1**	**1**
844	0	8.88	35	0.996	1	1	**1**	**1**	1	**1**	**1**
845	0	8.64	35	0.996	1	1	**1**	**1**	1	**1**	**1**
846	0	8.49	35	0.996	1	1	**1**	**1**	1	**1**	**1**
847	0	8.36	35	0.996	1	1	**1**	**1**	1	**1**	**1**
848	0	8.1	35	0.996	1	1	**1**	**1**	1	**1**	**1**
849	0	7.92	34	0.996	1	1	**1**	**1**	1	**1**	**1**
850	0	7.82	34	0.996	1	1	**1**	**1**	1	**1**	**1**

Bold indicates observations not used to create this model

Appendix 3.1 - Growth/no growth observations for the total 871 datapoints with predictions for all five models (Eqns 3.6-3.10)

Conditions for growth/no growth observations							Predicted Probabilities				
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6	Eqn 3.7	Eqn 3.8	Eqn 3.9	Eqn 3.10
851	0	7.71	34	0.996	1	1	**1**	**1**	1	**1**	**1**
852	0	7.58	34	0.996	1	1	**1**	**1**	1	**1**	**1**
853	0	10.3	35	0.996	0	1	**1**	**0**	0	**1**	**1**
854	0	10.6	35	0.996	0	1	**1**	**0**	0	**1**	**1**
855	0	9.93	34	0.996	0	1	**1**	**0**	0	**1**	**1**
856	0	9.85	34	0.996	0	1	**1**	**0**	0	**1**	**1**
857	0	9.73	34	0.996	0	1	**1**	**0**	0	**1**	**1**
858	0	9.59	34	0.996	0	1	**1**	**0**	0	**1**	**1**
859	0	9.45	34	0.996	0	1	**1**	**0**	0	**1**	**1**
860	0	9.26	34	0.996	1	1	**1**	**0**	0.98	**1**	**1**
861	0	9.14	35	0.996	1	1	**1**	**0**	0.99	**1**	**1**
862	0	8.98	35	0.996	1	1	**1**	**1**	1	**1**	**1**
863	0	8.88	35	0.996	1	1	**1**	**1**	1	**1**	**1**
864	0	8.64	35	0.996	1	1	**1**	**1**	1	**1**	**1**
865	0	8.49	35	0.996	1	1	**1**	**1**	1	**1**	**1**
866	0	8.36	35	0.996	1	1	**1**	**1**	1	**1**	**1**
867	0	8.1	35	0.996	1	1	**1**	**1**	1	**1**	**1**
868	0	7.92	34	0.996	1	1	**1**	**1**	1	**1**	**1**
869	0	7.82	34	0.996	1	1	**1**	**1**	1	**1**	**1**
870	0	7.67	34	0.996	1	1	**1**	**1**	1	**1**	**1**
871	0	7.57	34	0.996	1	1	**1**	**1**	1	**1**	**1**

Bold indicates observations not used to create this model

Appendix 3.2 - Growth/no growth observations underpredicted by Eqn 3.6 of the total 871 datapoints.

Conditions for growth/no growth observations							Predicted Probabilities
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6
15	0	3.9	15	0.996	4	4	0
23	**0**	**3.6**	**20**	**0.996**	**3**	**4**	**0**
24	**0**	**3.8**	**20**	**0.996**	**4**	**4**	**0**
25	0	3.9	20	0.996	4	4	0
43	**0**	**3.6**	**30**	**0.996**	**4**	**4**	**0**
44	**0**	**3.8**	**30**	**0.996**	**4**	**4**	**0**
45	0	3.9	30	0.996	4	4	0
247	**200**	**5.1**	**15**	**0.994**	**4**	**8**	**0**
271	**200**	**5.1**	**30**	**0.994**	**4**	**8**	**0**
475	200	5.2	21.70	0.994	1	1	0.02
465	50	4.56	21.20	0.996	1	1	0.06
129	50	4.6	15	0.996	3	4	0.12
240	200	5.3	10	0.994	3	4	0.13
466	50	4.6	21.22	0.996	1	1	0.26
620	0	6.23	20	0.955	2	2	0.30
686	**0**	**5.42**	**21**	**0.955**	**1**	**1**	**0.30**
621	0	6.84	20	0.955	2	2	0.31
687	**0**	**5.9**	**21**	**0.955**	**1**	**1**	**0.32**
688	**0**	**6.19**	**21**	**0.955**	**1**	**1**	**0.33**
689	**0**	**6.58**	**21**	**0.955**	**1**	**1**	**0.33**
690	**0**	**7.02**	**21**	**0.955**	**1**	**1**	**0.33**
149	50	4.6	25	0.996	2	4	0.34
248	200	5.3	15	0.994	4	4	0.35
691	**0**	**5.42**	**24**	**0.955**	**1**	**1**	**0.38**
692	**0**	**5.9**	**24**	**0.955**	**1**	**1**	**0.40**
6	0	4	10	0.996	4	4	0.40
693	**0**	**6.19**	**24**	**0.955**	**1**	**1**	**0.40**
694	**0**	**6.58**	**24**	**0.955**	**1**	**1**	**0.41**
695	**0**	**7.02**	**24**	**0.955**	**1**	**1**	**0.41**
613	0	5.8	25	0.955	2	2	0.42
614	0	6.23	25	0.955	2	2	0.43
615	0	6.84	25	0.955	2	2	0.43
696	**0**	**5.42**	**27**	**0.955**	**1**	**1**	**0.45**
298	500	5.9	15	0.986	4	4	0.45
574	0	4.15	30	0.965	1	2	0.46
697	**0**	**5.9**	**27**	**0.955**	**1**	**1**	**0.47**
698	**0**	**6.19**	**27**	**0.955**	**1**	**1**	**0.47**
699	**0**	**6.58**	**27**	**0.955**	**1**	**1**	**0.48**
700	**0**	**7.02**	**27**	**0.955**	**1**	**1**	**0.48**

Bold indicates observations not used to create this model

Appendix 3.2 - Growth/no growth observations overpredicted by Eqn 3.6 of the total 871 datapoints.

Conditions for growth/no growth observations							Predicted Probabilities
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.6
92	25	4.3	25	0.996	0	4	0.55
169	50	4.6	37	0.996	0	4	0.56
594	0	5.12	10	0.965	0	2	0.60
600	0	5.2	37	0.955	0	2	0.60
439	500	5.87	22	0.986	0	1	0.61
731	**0**	**4.27**	**24**	**0.965**	**0**	**1**	**0.63**
329	500	5.8	37	0.986	0	4	0.64
101	25	4.3	30	0.996	0	4	0.65
556	0	4.11	20	0.975	0	2	0.68
736	**0**	**4.27**	**27**	**0.965**	**0**	**1**	**0.70**
110	25	4.3	37	0.996	0	4	0.75
314	500	5.9	25	0.986	0	4	0.75
716	**0**	**5.42**	**12**	**0.965**	**0**	**1**	**0.75**
200	100	5	20	0.996	0	4	0.75
450	500	5.93	22	0.986	0	1	0.76
717	**0**	**5.8**	**12**	**0.965**	**0**	**1**	**0.77**
741	**0**	**4.27**	**32**	**0.965**	**0**	**1**	**0.77**
718	**0**	**6.12**	**12**	**0.965**	**0**	**1**	**0.77**
719	**0**	**6.52**	**12**	**0.965**	**0**	**1**	**0.78**
720	**0**	**6.87**	**12**	**0.965**	**0**	**1**	**0.78**
533	0	4.15	10	0.985	0	2	0.79
737	**0**	**4.36**	**27**	**0.965**	**0**	**1**	**0.81**
121	50	4.8	10	0.996	0	4	0.82
746	**0**	**4.27**	**36**	**0.965**	**0**	**1**	**0.82**
587	0	4.63	20	0.965	0	2	0.85
791	**0**	**4.28**	**8**	**0.985**	**0**	**1**	**0.86**
761	**0**	**4.26**	**17**	**0.975**	**0**	**1**	**0.89**
721	**0**	**5.42**	**17**	**0.965**	**0**	**1**	**0.89**
747	**0**	**4.36**	**36**	**0.965**	**0**	**1**	**0.89**
751	**0**	**5.23**	**8**	**0.975**	**0**	**1**	**0.89**
563	0	4.6	10	0.975	0	2	0.90
752	**0**	**5.75**	**8**	**0.975**	**0**	**1**	**0.91**
753	**0**	**6.15**	**8**	**0.975**	**0**	**1**	**0.91**
754	**0**	**6.5**	**8**	**0.975**	**0**	**1**	**0.91**
755	**0**	**6.98**	**8**	**0.975**	**0**	**1**	**0.91**
588	0	5.12	20	0.965	0	2	0.91
564	0	5.09	10	0.975	0	2	0.95
799	**0**	**4.28**	**12**	**0.985**	**0**	**1**	**0.96**
859	**0**	**9.45**	**34**	**0.996**	**0**	**1**	**1.00**
840	**0**	**9.47**	**34**	**0.996**	**0**	**1**	**1.00**
858	**0**	**9.59**	**34**	**0.996**	**0**	**1**	**1.00**
839	**0**	**9.62**	**34**	**0.996**	**0**	**1**	**1.00**
857	**0**	**9.73**	**34**	**0.996**	**0**	**1**	**1.00**
838	**0**	**9.79**	**34**	**0.996**	**0**	**1**	**1.00**
856	**0**	**9.85**	**34**	**0.996**	**0**	**1**	**1.00**
837	**0**	**9.87**	**34**	**0.996**	**0**	**1**	**1.00**
855	**0**	**9.93**	**34**	**0.996**	**0**	**1**	**1.00**
836	**0**	**10.02**	**34**	**0.996**	**0**	**1**	**1.00**
835	**0**	**10.13**	**35**	**0.996**	**0**	**1**	**1.00**
834	**0**	**10.21**	**35**	**0.996**	**0**	**1**	**1.00**
853	**0**	**10.3**	**35**	**0.996**	**0**	**1**	**1.00**
854	**0**	**10.6**	**35**	**0.996**	**0**	**1**	**1.00**

Bold indicates observations not used to create this model

Appendix 3.2 - Growth/no growth observations underpredicted by Eqn 3.7 of the total 871 datapoints.

Conditions for growth/no growth observations							Predicted Probabilities
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.7
23	**0**	**3.6**	**20**	**0.996**	**3**	**4**	**0**
24	**0**	**3.8**	**20**	**0.996**	**4**	**4**	**0**
43	**0**	**3.6**	**30**	**0.996**	**4**	**4**	**0**
44	**0**	**3.8**	**30**	**0.996**	**4**	**4**	**0**
15	0	3.9	15	0.996	4	4	0
25	0	3.9	20	0.996	4	4	0
45	0	3.9	30	0.996	4	4	0
247	**200**	**5.1**	**15**	**0.994**	**4**	**8**	**0**
271	**200**	**5.1**	**30**	**0.994**	**4**	**8**	**0**
475	200	5.2	21.70	0.994	1	1	0.10
465	50	4.56	21.20	0.996	1	1	0.14
240	200	5.3	10	0.994	3	4	0.14
129	50	4.6	15	0.996	3	4	0.15
686	**0**	**5.42**	**21**	**0.955**	**1**	**1**	**0.30**
466	50	4.6	21.22	0.996	1	1	0.33
687	**0**	**5.9**	**21**	**0.955**	**1**	**1**	**0.34**
620	0	6.23	20	0.955	2	2	0.34
688	**0**	**6.19**	**21**	**0.955**	**1**	**1**	**0.37**
691	**0**	**5.42**	**24**	**0.955**	**1**	**1**	**0.38**
568	0	4.15	37	0.965	2	2	0.39
6	0	4	10	0.996	4	4	0.40
248	200	5.3	15	0.994	4	4	0.40
298	500	5.9	15	0.986	4	4	0.43
692	**0**	**5.9**	**24**	**0.955**	**1**	**1**	**0.43**
149	50	4.6	25	0.996	2	4	0.43
613	0	5.8	25	0.955	2	2	0.44
689	**0**	**6.58**	**21**	**0.955**	**1**	**1**	**0.45**
696	**0**	**5.42**	**27**	**0.955**	**1**	**1**	**0.46**
574	0	4.15	30	0.965	1	2	0.46
693	**0**	**6.19**	**24**	**0.955**	**1**	**1**	**0.46**
601	0	5.8	37	0.955	2	2	0.47
706	**0**	**5.42**	**36**	**0.955**	**1**	**1**	**0.48**
614	0	6.23	25	0.955	2	2	0.49
697	**0**	**5.9**	**27**	**0.955**	**1**	**1**	**0.50**

Bold indicates observations not used to create this model

Appendix 3.2 - Growth/no growth observations overpredicted by Eqn 3.7 of the total 871 datapoints.

	Conditions for growth/no growth observations						Predicted Probabilities
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.7
110	25	4.3	37	0.996	0	4	0.50
685	**0**	**7.02**	**17**	**0.955**	**0**	**1**	**0.52**
159	50	4.6	30	0.996	0	4	0.53
714	**0**	**6.52**	**8**	**0.965**	**0**	**1**	**0.54**
101	25	4.3	30	0.996	0	4	0.58
594	0	5.12	10	0.965	0	2	0.60
439	500	5.87	22	0.986	0	1	0.60
731	**0**	**4.27**	**24**	**0.965**	**0**	**1**	**0.66**
715	**0**	**6.87**	**8**	**0.965**	**0**	**1**	**0.67**
556	0	4.11	20	0.975	0	2	0.70
121	50	4.8	10	0.996	0	4	0.72
736	**0**	**4.27**	**27**	**0.965**	**0**	**1**	**0.72**
746	**0**	**4.27**	**36**	**0.965**	**0**	**1**	**0.74**
200	100	5	20	0.996	0	4	0.76
314	500	5.9	25	0.986	0	4	0.76
450	500	5.93	22	0.986	0	1	0.77
716	**0**	**5.42**	**12**	**0.965**	**0**	**1**	**0.78**
741	**0**	**4.27**	**32**	**0.965**	**0**	**1**	**0.78**
533	0	4.15	10	0.985	0	2	0.79
717	**0**	**5.8**	**12**	**0.965**	**0**	**1**	**0.80**
718	**0**	**6.12**	**12**	**0.965**	**0**	**1**	**0.82**
737	**0**	**4.36**	**27**	**0.965**	**0**	**1**	**0.83**
747	**0**	**4.36**	**36**	**0.965**	**0**	**1**	**0.85**
719	**0**	**6.52**	**12**	**0.965**	**0**	**1**	**0.86**
791	**0**	**4.28**	**8**	**0.985**	**0**	**1**	**0.86**
587	0	4.63	20	0.965	0	2	0.87
751	**0**	**5.23**	**8**	**0.975**	**0**	**1**	**0.90**
563	0	4.6	10	0.975	0	2	0.91
761	**0**	**4.26**	**17**	**0.975**	**0**	**1**	**0.91**
721	**0**	**5.42**	**17**	**0.965**	**0**	**1**	**0.91**
720	**0**	**6.87**	**12**	**0.965**	**0**	**1**	**0.91**
752	**0**	**5.75**	**8**	**0.975**	**0**	**1**	**0.92**
753	**0**	**6.15**	**8**	**0.975**	**0**	**1**	**0.93**
588	0	5.12	20	0.965	0	2	0.93
754	**0**	**6.5**	**8**	**0.975**	**0**	**1**	**0.94**
564	0	5.09	10	0.975	0	2	0.96
799	**0**	**4.28**	**12**	**0.985**	**0**	**1**	**0.97**
755	**0**	**6.98**	**8**	**0.975**	**0**	**1**	**0.98**
841	**0**	**9.29**	**34**	**0.996**	**1**	**1**	**1.00**
842	**0**	**9.14**	**35**	**0.996**	**1**	**1**	**1.00**
860	**0**	**9.26**	**34**	**0.996**	**1**	**1**	**1.00**
861	**0**	**9.14**	**35**	**0.996**	**1**	**1**	**1.00**

Bold indicates observations not used to create this model

Appendix 3.2 - Growth/no growth observations underpredicted by Eqn 3.8 of the total 871 datapoints.

Conditions for growth/no growth observations							Predicted Probabilities
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.8
23	0	3.6	20	0.996	3	4	0
24	0	3.8	20	0.996	4	4	0
43	0	3.6	30	0.996	4	4	0
44	0	3.8	30	0.996	4	4	0
247	200	5.1	15	0.994	4	8	0.02
271	200	5.1	30	0.994	4	8	0.10
240	200	5.3	10	0.994	3	4	0.26
129	50	4.6	15	0.996	3	4	0.38
475	200	5.2	21.70	0.994	1	1	0.42
465	50	4.56	21.20	0.996	1	1	0.44
620	0	6.23	20	0.955	2	2	0.48
621	0	6.84	20	0.955	2	2	0.48
298	500	5.9	15	0.986	4	4	0.48
595	0	5.66	10	0.965	2	2	0.49
686	0	5.42	21	0.955	1	1	0.49

Bold indicates observations not used to create this model

Appendix 3.2 - Growth/no growth observations overpredicted by Eqn 3.8 of the total 871 datapoints.

Conditions for growth/no growth observations							Predicted Probabilities
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.8
586	0	4.15	20	0.965	0	2	0.50
791	0	4.28	8	0.985	0	1	0.51
605	0	4.66	30	0.955	0	2	0.53
329	500	5.8	37	0.986	0	4	0.53
229	100	4.9	37	0.996	0	4	0.55
751	0	5.23	8	0.975	0	1	0.56
612	0	5.2	25	0.955	0	2	0.57
139	50	4.6	20	0.996	1	4	0.57
752	0	5.75	8	0.975	0	1	0.58
753	0	6.15	8	0.975	0	1	0.59
755	0	6.98	8	0.975	0	1	0.59
754	0	6.5	8	0.975	0	1	0.59
121	50	4.8	10	0.996	0	4	0.60
83	25	4.3	20	0.996	0	4	0.60
421	200	5.24	22	0.994	0	1	0.60
313	500	5.8	25	0.986	0	4	0.60
169	50	4.6	37	0.996	0	4	0.62
209	100	4.9	25	0.996	1	4	0.62
580	0	4.15	25	0.965	0	2	0.62
606	0	5.2	30	0.955	0	2	0.64
716	0	5.42	12	0.965	0	1	0.64
110	25	4.3	37	0.996	0	4	0.65
717	0	5.8	12	0.965	0	1	0.66
533	0	4.15	10	0.985	0	2	0.66
718	0	6.12	12	0.965	0	1	0.66
719	0	6.52	12	0.965	0	1	0.66
720	0	6.87	12	0.965	0	1	0.66
439	500	5.87	22	0.986	0	1	0.67
219	100	4.9	30	0.996	0	4	0.69
563	0	4.6	10	0.975	0	2	0.70
92	25	4.3	25	0.996	0	4	0.71
731	0	4.27	24	0.965	0	1	0.72
746	0	4.27	36	0.965	0	1	0.73
159	50	4.6	30	0.996	0	4	0.74
450	500	5.93	22	0.986	0	1	0.75
314	500	5.9	25	0.986	0	4	0.77
736	0	4.27	27	0.965	0	1	0.77
101	25	4.3	30	0.996	0	4	0.77
747	0	4.36	36	0.965	0	1	0.79
564	0	5.09	10	0.975	0	2	0.79
556	0	4.11	20	0.975	0	2	0.79
741	0	4.27	32	0.965	0	1	0.80
200	100	5	20	0.996	0	4	0.81
587	0	4.63	20	0.965	0	2	0.81
737	0	4.36	27	0.965	0	1	0.82
721	0	5.42	17	0.965	0	1	0.83
761	0	4.26	17	0.975	0	1	0.84
526	0	4	20	0.985	0	2	0.86
588	0	5.12	20	0.965	0	2	0.87
799	0	4.28	12	0.985	0	1	0.87
55	0	3.9	37	0.996	1	4	0.90
35	0	3.9	25	0.996	0	4	0.92

Bold indicates observations not used to create this model

Appendix 3.2 - Growth/no growth observations underpredicted by Eqn 3.9 of the total 871 datapoints.

Conditions for growth/no growth observations							Predicted Probabilities
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.9
247	200	5.1	15	0.994	4	8	0.02
792	0	4.48	8	0.985	1	1	0.06
574	0	4.15	30	0.965	1	2	0.10
271	200	5.1	30	0.994	4	8	0.11
23	0	3.6	20	0.996	3	4	0.13
568	0	4.15	37	0.965	2	2	0.14
793	0	4.74	8	0.985	1	1	0.17
129	50	4.6	15	0.996	3	4	0.18
465	50	4.56	21.20	0.996	1	1	0.18
43	0	3.6	30	0.996	4	4	0.26
686	0	5.42	21	0.955	1	1	0.31
67	25	4.5	10	0.996	4	4	0.32
240	200	5.3	10	0.994	3	4	0.34
466	50	4.6	21.22	0.996	1	1	0.34
742	0	4.36	32	0.965	1	1	0.35
691	0	5.42	24	0.955	1	1	0.37
75	25	4.4	15	0.996	4	4	0.38
794	0	5.01	8	0.985	1	1	0.38
298	500	5.9	15	0.986	4	4	0.38
149	50	4.6	25	0.996	2	4	0.43
696	0	5.42	27	0.955	1	1	0.43
550	0	4.11	25	0.975	2	2	0.45
475	200	5.2	21.70	0.994	1	1	0.48
6	0	4	10	0.996	4	4	0.48
321	500	5.8	30	0.986	4	4	0.49

Bold indicates observations not used to create this model

Appendix 3.2 - Growth/no growth observations overpredicted by Eqn 3.9 of the total 871 datapoints.

Conditions for growth/no growth observations							Predicted Probabilities
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.9
761	0	4.26	17	0.975	0	1	0.50
680	0	7.02	12	0.955	0	1	0.51
159	50	4.6	30	0.996	0	4	0.52
121	50	4.8	10	0.996	0	4	0.54
439	500	5.87	22	0.986	0	1	0.54
753	0	6.15	8	0.975	0	1	0.55
209	100	4.9	25	0.996	1	4	0.55
683	0	6.19	17	0.955	0	1	0.56
329	500	5.8	37	0.986	0	4	0.59
169	50	4.6	37	0.996	0	4	0.61
526	0	4	20	0.985	0	2	0.62
219	100	4.9	30	0.996	0	4	0.64
754	0	6.5	8	0.975	0	1	0.66
684	0	6.58	17	0.955	0	1	0.68
314	500	5.9	25	0.986	0	4	0.68
450	500	5.93	22	0.986	0	1	0.69
421	200	5.24	22	0.994	0	1	0.70
34	0	3.8	25	0.996	0	4	0.72
229	100	4.9	37	0.996	0	4	0.72
799	0	4.28	12	0.985	0	1	0.75
755	0	6.98	8	0.975	0	1	0.76
685	0	7.02	17	0.955	0	1	0.77
716	0	5.42	12	0.965	0	1	0.77
54	0	3.8	37	0.996	1	4	0.85
200	100	5	20	0.996	0	4	0.85
588	0	5.12	20	0.965	0	2	0.86
564	0	5.09	10	0.975	0	2	0.87
35	0	3.9	25	0.996	0	4	0.88
717	0	5.8	12	0.965	0	1	0.89
721	0	5.42	17	0.965	0	1	0.91
718	0	6.12	12	0.965	0	1	0.93
55	0	3.9	37	0.996	1	4	0.94
719	0	6.52	12	0.965	0	1	0.96
720	0	6.87	12	0.965	0	1	0.97
859	**0**	**9.45**	**34**	**0.996**	**0**	**1**	**1.00**
840	**0**	**9.47**	**34**	**0.996**	**0**	**1**	**1.00**
858	**0**	**9.59**	**34**	**0.996**	**0**	**1**	**1.00**
839	**0**	**9.62**	**34**	**0.996**	**0**	**1**	**1.00**
857	**0**	**9.73**	**34**	**0.996**	**0**	**1**	**1.00**
838	**0**	**9.79**	**34**	**0.996**	**0**	**1**	**1.00**
856	**0**	**9.85**	**34**	**0.996**	**0**	**1**	**1.00**
837	**0**	**9.87**	**34**	**0.996**	**0**	**1**	**1.00**
855	**0**	**9.93**	**34**	**0.996**	**0**	**1**	**1.00**
836	**0**	**10.02**	**34**	**0.996**	**0**	**1**	**1.00**
835	**0**	**10.13**	**35**	**0.996**	**0**	**1**	**1.00**
834	**0**	**10.21**	**35**	**0.996**	**0**	**1**	**1.00**
853	**0**	**10.3**	**35**	**0.996**	**0**	**1**	**1.00**
854	**0**	**10.6**	**35**	**0.996**	**0**	**1**	**1.00**

Bold indicates observations not used to create this model

Appendix 3.2 - Growth/no growth observations underpredicted by Eqn 3.10 of the total 871 datapoints.

Conditions for growth/no growth observations							Predicted Probabilities
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.10
247	200	5.1	15	0.994	4	8	0.05
595	0	5.66	10	0.965	2	2	0.07
271	200	5.1	30	0.994	4	8	0.09
574	0	4.15	30	0.965	1	2	0.14
43	0	3.6	30	0.996	4	4	0.15
792	0	4.48	8	0.985	1	1	0.16
23	0	3.6	20	0.996	3	4	0.16
596	0	6.14	10	0.965	2	2	0.19
686	0	5.42	21	0.955	1	1	0.20
568	0	4.15	37	0.965	2	2	0.22
465	50	4.56	21.20	0.996	1	1	0.24
129	50	4.6	15	0.996	3	4	0.27
67	25	4.5	10	0.996	4	4	0.36
298	500	5.9	15	0.986	4	4	0.39
240	200	5.3	10	0.994	3	4	0.41
321	500	5.8	30	0.986	4	4	0.43
691	0	5.42	24	0.955	1	1	0.43
466	50	4.6	21.22	0.996	1	1	0.43
149	50	4.6	25	0.996	2	4	0.44
597	0	6.87	10	0.965	2	2	0.45
793	0	4.74	8	0.985	1	1	0.46

Bold indicates observations not used to create this model

Appendix 3.2 - Growth/no growth observations overpredicted by Eqn 3.10 of the total 871 datapoints.

Conditions for growth/no growth observations							Predicted Probabilities
OBS	[LAC]	pH	T (°C)	Aw	Growth	Total	Eqn 3.10
564	0	5.09	10	0.975	0	2	0.55
14	0	3.8	15	0.996	0	4	0.56
480	0	3.74	21.06	0.996	0	1	0.57
747	0	4.36	36	0.965	0	1	0.57
427	200	5.19	22	0.994	0	1	0.58
219	100	4.9	30	0.996	0	4	0.58
199	100	4.9	20	0.996	0	4	0.59
439	500	5.87	22	0.986	0	1	0.60
54	0	3.8	37	0.996	1	4	0.61
606	0	5.2	30	0.955	0	2	0.62
209	100	4.9	25	0.996	1	4	0.62
121	50	4.8	10	0.996	0	4	0.64
753	0	6.15	8	0.975	0	1	0.64
718	0	6.12	12	0.965	0	1	0.67
799	0	4.28	12	0.985	0	1	0.69
526	0	4	20	0.985	0	2	0.69
314	500	5.9	25	0.986	0	4	0.72
34	0	3.8	25	0.996	0	4	0.74
450	500	5.93	22	0.986	0	1	0.75
754	0	6.5	8	0.975	0	1	0.78
719	0	6.52	12	0.965	0	1	0.81
421	200	5.24	22	0.994	0	1	0.82
55	0	3.9	37	0.996	1	4	0.82
600	0	5.2	37	0.955	0	2	0.83
755	0	6.98	8	0.975	0	1	0.88
720	0	6.87	12	0.965	0	1	0.88
721	0	5.42	17	0.965	0	1	0.88
588	0	5.12	20	0.965	0	2	0.88
35	0	3.9	25	0.996	0	4	0.89
200	100	5	20	0.996	0	4	0.93
859	**0**	**9.45**	**34**	**0.996**	**0**	**1**	**1.00**
840	**0**	**9.47**	**34**	**0.996**	**0**	**1**	**1.00**
858	**0**	**9.59**	**34**	**0.996**	**0**	**1**	**1.00**
839	**0**	**9.62**	**34**	**0.996**	**0**	**1**	**1.00**
857	**0**	**9.73**	**34**	**0.996**	**0**	**1**	**1.00**
838	**0**	**9.79**	**34**	**0.996**	**0**	**1**	**1.00**
856	**0**	**9.85**	**34**	**0.996**	**0**	**1**	**1.00**
837	**0**	**9.87**	**34**	**0.996**	**0**	**1**	**1.00**
855	**0**	**9.93**	**34**	**0.996**	**0**	**1**	**1.00**
836	**0**	**10.02**	**34**	**0.996**	**0**	**1**	**1.00**
835	**0**	**10.13**	**35**	**0.996**	**0**	**1**	**1.00**
834	**0**	**10.21**	**35**	**0.996**	**0**	**1**	**1.00**
853	**0**	**10.3**	**35**	**0.996**	**0**	**1**	**1.00**
854	**0**	**10.6**	**35**	**0.996**	**0**	**1**	**1.00**

Bold indicates observations not used to create this model

Appendix 4.1 - Internal pH Calibration using *Listeria monocytogenes*

pH 7.0, Amplification = 2X

G=glucose added, V - valinomycin added, N - nigericin added, S - cells filtered out, supernatent only.

Date	25/9/1997		
Conditions	pH 6		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	174	720	4.14
2	179	717	4.01
3	182	717	3.94
4	179	710	3.97
5	181	701	3.87
6	181	695	3.84
7	179	692	3.87
8	184	690	3.75
9	179	689	3.85
G-10	159	577	3.63
11	162	572	3.53
12	160	565	3.53
13	160	568	3.55
14	160	568	3.55
15	165	563	3.41
16	161	556	3.45
17	162	557	3.44
18	162	552	3.41
19	160	551	3.44
20	161	548	3.40
21	162	549	3.39
22	162	542	3.35
23	162	539	3.33
24	162	543	3.35
25	161	542	3.37
26	162	541	3.34
27	162	536	3.31
28	162	534	3.30
29	160	530	3.31
V-30	149	480	3.22
31	150	473	3.15
32	150	465	3.10
33	149	458	3.07
34	146	452	3.10
N-35	138	196	1.42
36	136	194	1.43
37	139	195	1.40
38	137	197	1.44
39	138	195	1.41
40	136	194	1.43
41	140	197	1.41
42	137	197	1.44
43	139	197	1.42
44	140	198	1.41
S-45	19	40	
46	19	41	
47	19	40	
48	20	41	
49	19	40	
50	20	40	
	zero=36	zero=12	

Date	25/9/1997		
Conditions	pH 7.07		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	239	1052	4.40
2	213	1006	4.72
3	204	897	4.40
4	205	972	4.74
5	212	966	4.56
6	211	1006	4.77
7	229	1077	4.70
8	233	1057	4.54
9	231	1066	4.61
G-10	217	974	4.49
11	214	976	4.56
12	212	970	4.58
13	215	961	4.47
14	215	957	4.45
15	214	962	4.50
16	213	966	4.54
17	215	955	4.44
18	217	954	4.40
19	214	948	4.43
20	212	946	4.46
21	212	937	4.42
22	213	938	4.40
23	213	934	4.38
24	212	940	4.43
25	213	935	4.39
26	211	935	4.43
27	212	937	4.42
28	213	936	4.39
29	215	941	4.38
V-30	216	936	4.33
31	216	927	4.29
32	213	926	4.35
33	214	934	4.36
34	215	935	4.35
N-35	210	785	3.74
36	210	777	3.70
37	210	780	3.71
38	210	783	3.73
39	209	784	3.75
40	210	788	3.75
41	210	783	3.73
42	212	785	3.70
43	211	787	3.73
44	211	783	3.71
S-45	31	114	
46	31	116	
	zero=32	zero=12	

Date	25/9/1997		
Conditions	pH 6.5		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	221	968	4.38
2	218	978	4.49
3	221	967	4.38
4	219	946	4.32
5	219	946	4.32
6	216	938	4.34
7	220	933	4.24
8	217	912	4.20
9	225	909	4.04
10	216	903	4.18
11	216	898	4.16
12	217	900	4.15
13	216	886	4.10
14	218	881	4.04
15	215	879	4.09
G-16	210	838	3.99
17	213	834	3.92
18	213	831	3.90
19	213	827	3.88
20	211	822	3.90
21	214	821	3.84
22	213	821	3.85
23	217	810	3.73
24	213	804	3.77
25	213	798	3.75
26	214	800	3.74
27	215	794	3.69
28	214	788	3.68
29	217	781	3.60
30	212	780	3.68
31	215	773	3.60
32	216	773	3.58
33	216	776	3.59
34	216	770	3.56
35	215	763	3.55
V-36	215	744	3.46
37	214	742	3.47
38	214	734	3.43
39	214	726	3.39
N-40	204	411	2.01
41	206	420	2.04
42	206	417	2.02
43	206	419	2.03
44	205	423	2.06
45	204	424	2.08
46	212	425	2.00
47	205	427	2.08
48	208	432	2.08
49	206	428	2.08
S-50	27	95	
51	28	95	
	zero=36	zero=6	

Appendix 4.1 - Internal pH Calibration using *Listeria monocytogenes*

pH 7.0, Amplification = 2X

G=glucose added, V - valinomycin added, N - nigericin added, S - cells filtered out, supernatent only.

Date		25/9/1997	
Conditions		pH 5	
Time	Fluorescence at		Ratio
(a.u.)	440nM	500mM	I/I
1	180	650	3.61
2	180	650	3.61
3	185	591	3.19
4	183	568	3.10
5	185	545	2.95
6	184	525	2.85
7	182	506	2.78
8	188	501	2.66
9	188	496	2.64
10	191	472	2.47
11	189	447	2.37
12	185	429	2.32
13	188	415	2.21
14	185	413	2.23
15	188	403	2.14
G-16	160	281	1.76
17	154	271	1.76
18	153	269	1.76
19	153	263	1.72
20	157	256	1.63
21	156	252	1.62
22	155	249	1.61
23	158	245	1.55
24	156	240	1.54
25	157	232	1.48
26	156	230	1.47
27	157	223	1.42
28	158	219	1.39
29	157	214	1.36
30	156	208	1.33
31	158	203	1.28
32	155	185	1.19
33	155	176	1.14
34	156	169	1.08
35	157	167	1.06
36	154	155	1.01
V-37	155	158	1.02
38	154	156	1.01
39	155	157	1.01
40	156	156	1.00
N-41	151	133	0.88
42	148	128	0.86
43	149	129	0.87
44	148	127	0.86
S-48	28	18	
49	28	19	
	zero=43	zero=14	

Date		26/9/97	
Conditions		pH 7.07	
Time	Fluorescence at		Ratio
(a.u.)	440nM	500mM	I/I
1	312	1088	3.49
2	266	1088	4.09
3	224	1058	4.72
4	276	1088	3.94
5	289	1088	3.76
6	127.4	597.2	4.69
7	131.4	597.2	4.54
8	130.4	587.2	4.50
9	129.4	573.2	4.43
10	125.4	562.2	4.48
G-11	122.4	548.2	4.48
12	121.4	550.2	4.53
13	121.4	543.2	4.47
14	121.4	544.2	4.48
15	122.4	544.2	4.45
16	124.4	540.2	4.34
17	123.4	540.2	4.38
18	123.4	542.2	4.39
19	123.4	535.2	4.34
V-25	122.4	523.2	4.27
26	122.4	525.2	4.29
27	121.4	527.2	4.34
28	120.4	524.2	4.35
29	121.4	530.2	4.37
N-30	118.4	457.2	3.86
31	120.4	458.2	3.81
32	119.4	456.2	3.82
33	120.4	459.2	3.81
34	120.4	457.2	3.80
35	119.4	455.2	3.81
S-36	15.4	66.2	
37	14.4	67.2	
	zero=29	zero=12	
	zero=11.6	zero=4.8	

Date		26/9/97	
Conditions		pH 9	
Time	Fluorescence at		Ratio
(a.u.)	440nM	500mM	I/I
1	133	635	4.77
2	127	658	5.18
3	126	663	5.26
4	127	686	5.40
5	133	832	6.26
6	131	842	6.43
7	131	852	6.50
G-8	127	862	6.79
9	126	864	6.86
10	126	865	6.87
11	127	877	6.91
V-12	126	869	6.90
13	124	883	7.12
14	125	895	7.16
15	126	900	7.14
16	125	898	7.18
N-17	126	834	6.62
18	126	825	6.55
19	127	829	6.53
20	128	832	6.50
21	127	836	6.58
22	128	845	6.60
23	128	846	6.61
24	126	851	6.75
25	127	857	6.75
26	129	860	6.67
27	128	863	6.74
28	129	868	6.73
S-29	20	115	
30	21	114	
	zero=14	zero=6	

Appendix 4.1- Internal pH Calibration using *Listeria monocytogenes*

pH 7.0, Amplification = 5X G=glucose added, V - valinomycin added, N - nigericin added, S - cells filtered out, supernatent only

Date	25/9/1997		
Conditions	pH 4		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	158	502	3.18
2	155	414	2.67
3	146	347	2.38
4	143	306	2.14
5	139	277	1.99
6	141	252	1.79
7	139	232	1.67
8	139	215	1.55
9	137	204	1.49
10	135	194	1.44
11	137	185	1.35
12	135	173	1.28
13	137	168	1.23
G-14	137	150	1.09
16	141	149	1.06
17	137	141	1.03
18	135	139	1.03
19	133	134	1.01
20	134	133	0.99
21	133	132	0.99
22	132	126	0.95
V-23	129	113	0.88
24	129	109	0.84
25	128	106	0.83
26	128	102	0.80
N-27	121	87	0.72
28	123	87	0.71
29	122	88	0.72
30	120	87	0.73
31	123	88	0.72
S-32	9	1	
33	11	0	
34	10	1	
zero=39	zero=13		

Date	25/9/1997		
Conditions	pH 7.5		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	166	722	4.35
2	160	716	4.48
3	159	734	4.62
4	166	748	4.51
5	166	753	4.54
6	167	766	4.59
7	166	754	4.54
8	163	744	4.56
G-9	163	746	4.58
10	163	740	4.54
11	161	747	4.64
12	163	745	4.57
13	161	743	4.61
14	166	746	4.49
V-15	162	748	4.62
16	162	750	4.63
17	160	751	4.69
18	162	749	4.62
N-19	158	712	4.51
20	161	715	4.44
21	160	716	4.48
22	159	724	4.55
23	159	724	4.55
24	160	727	4.54
25	160	737	4.61
26	157	738	4.70
S-27	28	122	
28	23	124	
zero=49	zero=9		

Date	25/9/1997		
Conditions	pH 8		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	161	752	4.67
2	163	756	4.64
3	158	748	4.73
4	156	746	4.78
5	157	740	4.71
6	153	746	4.88
7	153	748	4.89
8	153	764	4.99
9	153	769	5.03
10	152	777	5.11
11	153	785	5.13
12	156	787	5.04
G-13	154	798	5.18
14	155	800	5.16
15	155	806	5.20
16	153	807	5.27
17	153	810	5.29
18	151	807	5.34
19	153	811	5.30
V-20	154	808	5 25
21	151	810	5.36
22	152	818	5.38
23	152	818	5.38
N-24	149	848	5.69
25	150	857	5.71
26	148	861	5 82
27	149	862	5.79
28	148	871	5.89
29	150	873	5.82
S-30	25	123	
31	25	123	
zero=36	zero=12		

Date	25/9/1997		
Conditions	pH 9		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	174	891	5.12
2	173	906	5.24
3	175	937	5.35
4	177	1023	5.78
G-5	177	1079	6.10
6	180	1085	6.03
7	178	1088	6.11
8	180	1088	6.04
9	180	1088	6.04
V-10	162	1041	6.43
11	164	1055	6.43
12	165	1062	6.44
13	164	1068	6.51
N-14	156	1049	6.72
15	157	1050	6.69
16	157	1064	6.78
17	158	1066	6.75
18	158	1078	6.82
19	160	1081	6.76
S-20	29	213	
21	28	212	
zero=34	zero=12		

Date	25/9/1997		
Conditions	pH 10		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	178	969	5.44
2	177	987	5.58
3	176	1045	5.94
4	175	1067	6.10
G-5	182	1088	5.98
6	171	1088	6.36
7	173	1088	6.29
8	172	1088	6.33
9	174	1088	6.25
V-10	165	1088	6.59
11	160	1088	6.80
12	164	1088	6.63
13	165	1088	6.59
N-14	163	1088	6.67
15	165	1088	6.59
16	167	1159	6.94
17	169	1156	6.84
S-19	31	491	
20	31	491	
zero=34	zero=12		

Appendix 4.2 - Internal pH Raw Data for *E.coli*

pH 7 0, Amplification = 5X G=glucose added, V - valinomycin added, N - nigericin added, S - cells filtered out, supernatent only

A2	29/9/97		
Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	7	20	2 86
2	7	19	2.71
3	7	20	2 86
4-G	7	20	2 86
5	8	20	2.50
6	8	20	2 50
7	7	19	2.71
8	8	20	2 50
9	8	20	2 50
10	8	20	2 50
11	7	19	2 71
12	8	19	2.38
13	8	20	2 50
14	7	19	2.71
15-V	7	20	2 86
16	7	19	2 71
17	8	20	2.50
18	8	20	2 50
19	8	20	2.50
20-N	9	21	2.33
21	8	21	2.63
22	9	21	2.33
23	9	21	2 33
24	9	21	2 33
25-S	12	5	
26	12	4	
27	13	4	
28	13	5	
29	13	4	
	zero=25	zero=4	

A4	29/9/97		
Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	37	35	0 95
2	39	36	0 92
3	39	33	0 85
4	40	38	0.95
5	40	34	0 85
6	38	36	0 95
7-G	37	36	0.97
8	38	34	0 89
9	38	36	0 95
10	38	39	1 03
11	40	45	1.13
12	39	37	0 95
13	38	36	0.95
14	39	37	0.95
15	39	36	0 92
16	39	35	0.90
17	39	35	0.90
18-V	38	34	0 89
19	40	41	1 03
20	38	37	0.97
21	39	35	0.90
22	41	37	0.90
23-N	39	38	0 97
24	40	38	0 95
25	41	34	0 83
26	42	38	0 90
27	40	41	1 03
28	42	38	0.90
29	39	37	0.95
30-S	35	18	
31	33	16	
32	36	18	
33	33	16	
34	35	16	
	zero=26	zero=12	

B3	30/9/97		
Time (a u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	7	12	1.71
2	7	11	1 57
3	7	11	1 57
4	7	11	1.57
5	7	11	1 57
6	7	11	1.57
7-V	7	12	1 71
8	7	12	1 71
9	7	12	1.71
10	7	12	1.71
11	7	12	1 71
12-N	8	13	1.63
13	8	12	1 50
14	8	12	1 50
15	8	12	1 50
16	8	12	1 50
17-S	4	3	
18	4	2	
19	4	2	
20	4	2	
21	4	2	
	zero=14	zero=7	

B6	30/9/97		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	10	10	1.00
2	10	10	1 00
3	10	10	1.00
4	10	10	1 00
5	10	10	1.00
6-V	10	11	1.10
7	10	10	1 00
8	10	10	1 00
9	10	10	1.00
10	10	11	1 10
11-N	11	12	1 09
12	11	11	1.00
13	11	11	1.00
14	11	11	1 00
15	11	11	1.00
16-S	5	2	
17	5	2	
18	5	2	
19	5	2	
20	5	2	
	zero=13	zero=2	

B1	30/9/97		
Time (a u)	Fluorescence at 440nM	500mM	Ratio I/I
1	14	17	1.21
2	15	18	1.20
3	15	17	1 13
4	14	17	1.21
5	14	18	1 29
6-N	15	18	1.20
7	15	19	1.27
8	16	19	1.19
9	16	18	1.13
10	15	18	1.20
11-S	8	3	
12	7	4	
13	7	3	
14	7	3	
15	7	2	
	zero=17	zero=4	

B2	30/9/97		
Time (a u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	13	12	0.92
2	13	12	0 92
3	13	11	0 85
4	14	12	0.86
5	13	12	0.92
6-N	14	12	0 86
7	14	13	0 93
8	13	12	0 92
9	14	13	0 93
10	14	13	0.93
11-S	8	0	
12	6	0	
13	6	-1	
14	6	0	
15	6	0	
	zero=18	zero=9	

B4	30/9/97		
Time (a u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	7	9	1.29
2	8	9	1.13
3	8	10	1 25
4	8	9	1.13
5	8	9	1.13
6-N	8	11	1 38
7	9	9	1 00
8	9	9	1.00
9	9	10	1 11
10	9	10	1 11
11-S	5	4	
12	5	3	
13	4	2	
14	4	2	
15	5	2	
	zero=18	zero=8	

B5	30/9/97		
Time (a u)	Fluorescence at 440nM	500mM	Ratio I/I
1	18	28	1 56
2	19	27	1.42
3	18	27	1.5
4	20	28	1 4
5	20	28	1.4
6-N	21	29	1.38
7	21	28	1.33
8	21	29	1.38
9	22	30	1 36
10	21	30	1.43
11-S	17	11	
12	17	9	
13	17	9	
14	16	10	
15	17	9	
	zero=19	zero=11	

B7	30/9/97		
Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	20	30	1 50
2	19	28	1.47
3	18	29	1 61
4	19	29	1 53
5	19	29	1.53
6-N	21	31	1 48
7	21	32	1 52
8	21	32	1 52
9	22	32	1.45
10	21	31	1.48
11-S	10	6	
12	10	6	
13	10	6	
14	10	6	
15	10	6	
	zero=17	zero=5	

B8	30/9/97		
Time (a u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	17	20	1 18
2	18	19	1 06
3	18	19	1 06
4	18	19	1 06
5	19	19	1.00
6-N	19	21	1 11
7	19	21	1 11
8	19	21	1.11
9	19	21	1.11
10	20	20	1.00
11-S	14	6	
12	15	5	
13	15	6	
14	16	6	
15	16	6	
	zero=19	zero=10	

Appendix 4.2 - Internal pH Raw Data for *E.coli*

pH 7 0, Amplification = 5X G=glucose added, V - valinomycin added, N - nigericin added, S - cells filtered out, supernatent only

C1	6/10/97		
Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	13	33	2 54
2	13	35	2.69
3	13	35	2 69
4	13	35	2 69
5	13	35	2 69
6	13	34	2 62
7	13	36	2.77
8-G	14	36	2 57
9	14	36	2 57
10	15	36	2 40
11	15	35	2 33
12	15	37	2 47
13	15	36	2.40
14	15	36	2 40
15-V	17	38	2 24
16	17	39	2 29
17	17	40	2 35
18	18	38	2 11
19	17	38	2 24
20-N	19	37	1 95
21	18	38	2 11
22	19	37	1.95
23	19	39	2 05
24	19	38	2 00
25-S	12	10	
26	13	10	
27	12	9	
28	13	9	
29	13	9	
	zero=15	zero=5	

C2	6/10/97		
Time (a u)	Fluorescence at 440nM	500mM	Ratio I/I
1	32	65	2 03
2	24	64	2 67
3	23	69	3 00
4	27	70	2 59
5	23	65	2 83
6	26	74	2 85
7	26	67	2 58
8	24	66	2 75
9	26	67	2 58
10-G	26	69	2 65
11	26	67	2 58
12	26	67	2.58
13	27	68	2 52
14	27	69	2 56
15	27	69	2 56
16	27	68	2 52
17	27	69	2 56
18	28	74	2 64
19	29	70	2.41
20	29	71	2 45
21	29	69	2 38
22	30	71	2 37
23	30	71	2.37
24	30	70	2 33
25	30	72	2 40
26	30	71	2 37
27	32	70	2.19
28	31	71	2 29
29	32	72	2.25
30-V	33	75	2.27
31	34	74	2 18
32	35	75	2 14
33	36	74	2 06
34	35	74	2.11
35-N	35	71	2.03
36	36	71	1.97
37	36	71	1 97
38	36	70	1.94
39	37	70	1 89
40-S	21	12	
41	20	10	
42	21	10	
43	21	11	
44	20	11	
	zero=15	zero=5	

C3	6/10/97		
Time (a u)	Fluorescence at 440nM	500mM	Ratio I/I
1	15	34	2 27
2	15	34	2 27
3	16	34	2 13
4	14	34	2 43
5	16	35	2 19
6	16	36	2 25
7	16	35	2 19
8-G	16	36	2 25
9	15	35	2.33
10	16	35	2 19
11	16	34	2.13
12	17	33	1 94
13	17	34	2 00
14	17	34	2 00
15-V	18	34	1.89
16	18	36	2 00
17	18	36	2.00
18	18	35	1 94
19	20	37	1.85
20-N	19	37	1 95
21	19	38	2 00
22	20	37	1 85
23	19	37	1.95
24	21	37	1.76
25-S	16	20	
26	15	20	
27	14	20	
28	14	20	
29	15	20	
	zero=15	zero=6	

D1	25/9/1997		
Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	131	779	5 95
2	128	789	6 16
3	128	792	6.19
4	126	791	6 28
5	127	821	6 46
6	130	829	6 38
7	128	799	6 24
8	128	795	6 21
9	129	806	6 25
10-G	132	851	6 45
11	133	857	6 44
12	134	864	6 45
13	135	858	6 36
14	134	863	6.44
15	134	873	6 51
16	136	862	6 34
17	136	865	6 36
18	135	870	6 44
19	135	877	6 50
20	137	865	6 31
21	135	868	6 43
22	136	868	6.38
23	135	868	6.43
24	137	872	6 36
25	137	871	6 36
26	135	869	6 44
27	137	877	6 40
28	137	877	6 40
29	138	877	6 36
30-V	139	883	6.35
31	139	874	6 29
32	139	884	6 36
33	139	883	6 35
34	139	882	6.35
35-N	139	891	6 41
36	142	897	6 32
37	142	890	6 27
38	141	894	6 34
39	142	892	6 28
40	141	897	6 36
41	144	901	6 26
42	143	898	6 28
43	142	898	6 32
44	145	899	6.20
45-S	77	583	
46	76	582	
47	76	578	
48	76	578	
49	74	577	
50	74	575	
	zero=21	zero=7	

D2	25/9/1997		
Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	175	729	4 17
2	166	723	4 36
3	173	741	4 28
4	170	830	4 88
5	171	737	4 31
6	168	736	4 38
7	166	740	4 46
8	170	735	4 32
9	169	757	4 48
10-G	170	744	4 38
11	168	748	4 45
12	168	756	4.50
13	171	757	4 43
14	168	761	4 53
15	168	759	4 52
16	170	757	4 45
17	171	754	4 41
18	170	768	4 52
19	171	760	4 44
20	170	773	4 55
21-V	176	770	4 38
22	173	772	4 46
23	174	782	4 49
24	175	777	4 44
25	177	789	4 46
26-N	176	791	4 49
27	178	789	4 43
28	175	788	4 50
29	173	791	4.57
30	173	792	4 58
31-S	63	311	
32	64	309	
33	63	305	
34	62	305	
35	63	307	
	zero=22	zero=6	

D3	25/9/1997		
Time (a u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	28	127	4.54
2	28	117	4 18
3	27	132	4 89
4	29	141	4 86
5	28	132	4 71
6	28	131	4 68
7	28	133	4 75
8	27	130	4 81
9	28	135	4 82
10	28	138	4 93
11-G	28	138	4 93
12	29	142	4 90
13	28	143	5.11
14	29	141	4 86
15	28	142	5 07
16	29	142	4 90
17	30	142	4 73
18	29	146	5 03
19	30	140	4 67
20	30	146	4 87
21	30	143	4 77
22	30	145	4 83
23-V	30	146	4 87
24	30	146	4 87
25	30	147	4.90
26	32	145	4 53
27-N	32	139	4 34
28	32	139	4 34
29	32	140	4 38
30	32	138	4 31
31-S	12	32	
32	12	29	
33	11	31	
34	12	31	
35	12	30	
	zero=23	zero=7	

D4	25/9/1997		
Time (a u)	Fluorescence at 440nM	500mM	Ratio I/I
1	103	437	4 24
2	102	444	4 35
3	102	430	4 22
4	103	446	4 33
5	104	446	4 29
6	104	442	4 25
7	102	444	4 35
8	104	455	4 38
9	103	447	4.34
10	102	443	4 34
11-G	102	449	4 40
12	104	449	4 32
13	101	446	4 42
14	103	448	4.35
15	102	444	4 35
16	103	450	4 37
17	101	451	4 47
18	103	454	4 41
19	104	451	4 34
20	104	451	4.34
21	104	451	4 34
22	103	456	4 43
23-V	104	457	4 39
24	105	461	4 39
25	106	463	4 37
26	105	465	4.43
27-N	104	459	4 41
28	107	459	4 29
29	107	462	4 32
30	107	459	4 29
31-S	10	38	
32	10	37	
33	10	37	
34	10	37	
35	10	38	
	zero=23	zero=9	

Appendix 4.2 - Internal pH Raw Data for *E.coli*

pH 7 0, Amplification = 5X G=glucose added, V - valinomycin added, N - nigericin added, S - cells filtered out, supernatent only

E1	14/10/97		
Time (a u)	Fluorescence at 440nM	500mM	Ratio I/I
1	128	489	3 82
2	124	506	4 08
3	120	503	4.19
4	121	512	4 23
5	121	494	4 08
6	113	498	4 41
7	121	507	4 19
8	117	506	4.32
9	120	512	4 27
10-G	121	494	4 08
11	119	501	4 21
12	121	507	4 19
13	123	506	4.11
14	121	499	4 12
15	121	501	4 14
16	120	498	4 15
17	124	512	4 13
18	126	541	4 29
19	124	523	4.22
20	126	532	4 22
21	129	519	4 02
22	127	524	4 13
23	125	527	4 22
24	132	543	4.11
25	130	525	4.04
26	125	534	4 27
27	127	529	4 17
28	128	533	4.16
29	138	550	3 99
30-V	131	531	4 05
31	132	530	4 02
32	132	534	4 05
33	133	532	4 00
34	132	532	4.03
35-N	137	616	4 50
36	147	594	4 04
37	142	573	4 04
38	142	580	4.08
39	147	614	4 18
40	152	615	4 05
41	152	604	3 97
42	148	596	4 03
43	149	596	4 00
44	145	561	3 87
45-S	36	63	
46	37	64	
47	36	63	
48	36	65	
49	35	63	
50	35	64	
	zero=40	zero=12	

E2	14/10/97		
Time (a u)	Fluorescence at 440nM	500mM	Ratio I/I
1	231	1007	4 36
2	197	824	4 18
3	215	878	4 08
4	198	888	4 48
5	200	1041	5.21
6	187	822	4 40
7	207	858	4.14
8	200	854	4 27
9	227	859	3.78
10-G	187	824	4.41
11	187	883	4 72
12	192	855	4 45
13	179	789	4.41
14	185	840	4 54
15	192	815	4 24
16	173	819	4.73
17	187	792	4 24
18	178	770	4 33
19	187	802	4 29
20	170	770	4.53
21-V	172	783	4 55
22	178	785	4 41
23	174	788	4 53
24	181	811	4 48
25	186	833	4.48
26-N	187	756	4 04
27	177	777	4 39
28	181	784	4 33
29	175	769	4 39
30	176	779	4.43
31-S	52	261	
32	52	265	
33	52	260	
34	51	261	
35	51	260	
	zero=38	zero=5	

E3	14/10/97		
Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	242	962	3 98
2	231	916	3 97
3	217	883	4 07
4	266	1000	3 76
5	238	894	3 76
6	234	913	3 90
7	224	839	3 75
8	219	872	3.98
9	245	915	3.73
10	246	880	3 58
11-G	224	857	3 83
12	228	822	3 61
13	227	841	3 70
14	238	941	3.95
15	234	814	3 48
16	232	931	4 01
17	243	876	3 60
18	233	907	3 89
19	241	808	3 35
20	213	845	3 97
21	222	847	3 82
22	236	892	3.78
23-V	219	818	3 74
24	226	834	3 69
25	230	836	3 63
26	230	832	3 62
27-N	269	1079	4 01
28	266	950	3 57
29	261	864	3 31
30	249	905	3.63
30	234	837	3.58
30	237	828	3 49
31-S	81	170	
32	79	174	
33	77	170	
34	79	167	
35	78	173	
	zero=39	zero=5	

E4	14/10/97		
Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	134	519	3 87
2	130	485	3.73
3	124	524	4 23
4	133	506	3 80
5	129	514	3.98
6	131	518	3 95
7	132	523	3 96
8	126	497	3 94
9	124	497	4 01
10	127	508	4 00
11-G	126	513	4 07
12	127	508	4 00
13	130	504	3 88
14	131	539	4.11
15	133	525	3.95
16	129	524	4 06
17	130	522	4 02
18	131	521	3 98
19	129	518	4 02
20-G	127	515	4 06
21	130	555	4.27
22	130	508	3 91
23	129	516	4 00
24	132	530	4 02
25	131	514	3.92
26-V	131	524	4 00
27	132	529	4.01
28	132	524	3 97
29	133	527	3 96
30-N	136	536	3 94
31	138	534	3 87
32	133	538	4 05
33	136	537	3 95
34-S	31	61	
35	27	59	
36	28	53	
37	25	46	
38	22	46	
	zero=42	zero=13	

E5	14/10/97		
Time (a u)	Fluorescence at 440nM	500mM	Ratio I/I
1	119	477	4 01
2	116	551	4 75
3	128	589	4 60
4	137	539	3 93
5	128	540	4 22
6	126	521	4.13
7	123	504	4 10
8	119	498	4 18
9	124	579	4 67
10-G	132	501	3 80
11	122	520	4 26
12	132	542	4 11
13	131	533	4 07
14	130	556	4 28
15	127	533	4 20
16	128	550	4 30
17	130	545	4.19
18	128	538	4 20
19	133	523	3 93
20	135	545	4 04
21	133	569	4 28
22	137	577	4 21
23	132	549	4 16
24	127	545	4 29
25	126	550	4.37
26	137	538	3 93
27	140	525	3 75
28	126	536	4 25
29	129	566	4.39
30-V	129	546	4 23
31	129	604	4.68
32	137	583	4.26
33	140	543	3 88
34	125	592	4 74
35	128	553	4 32
36	127	598	4 71
37	134	619	4.62
38	137	576	4 20
39-N	143	588	4 11
40	141	590	4 18
41	142	602	4.24
42	147	593	4 03
43	146	609	4 17
44	149	618	4 15
45-S	25	59	
46	30	60	
47	27	57	
48	25	53	
49	23	54	
50	23	54	
	zero=41	zero=11	

E6	14/10/97		
Time (a u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	231	894	3 87
2	212	830	3 92
3	205	853	4 16
4	207	868	4.19
5	222	853	3.84
6	216	856	3 96
7	214	837	3 91
8	219	875	4 00
9	223	861	3 86
10-G	213	845	3 97
11	214	827	3.86
12	216	845	3 91
13	217	846	3.90
14	216	851	3 94
15	211	817	3 87
16	227	872	3 84
17	217	811	3 74
18	211	874	4.14
19	223	890	3 99
20	224	901	4.02
21-V	211	810	3 84
22	206	807	3 92
23	213	821	3 85
24	217	837	3 86
25	216	827	3 83
26-N	208	784	3 77
27	205	788	3.84
28	208	795	3 82
29	209	799	3 82
30	208	803	3 86
31-S	33	63	
32	33	64	
33	32	64	
34	33	64	
35	32	64	
	zero=41	zero=10	

Appendix 4.2 - Internal pH Raw Data for *E.coli*

pH 7 0, Amplification = 5X

G=glucose added, V - valinomycin added, N - nigericin added, S - cells filtered out, supernatent only, 2X - amplification changed to 2X

E7	14/10/97		
Time (a u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	171	534	3.12
2	156	578	3.71
3	150	551	3.67
4	153	575	3.76
5	157	585	3 73
6	162	582	3.59
7	157	572	3.64
8	148	543	3 67
9	148	559	3.78
10	155	571	3.68
11-G	155	558	3 60
12	151	562	3 72
13	152	589	3.88
14	156	565	3.62
15	154	566	3.68
16	152	559	3.68
17	152	572	3.76
18	155	563	3.63
19	152	566	3 72
20	156	566	3.63
21	156	561	3.60
22	157	575	3.66
23-V	154	567	3 68
24	158	578	3 66
25	157	567	3.61
26	156	568	3 64
27-N	158	575	3 64
28	159	573	3.60
29	159	574	3.61
30	157	582	3.71
30	162	587	3.62
30	162	595	3 67
31-S	35	59	
32	35	60	
33	34	59	
34	34	59	
35	35	59	
	zero=41	zero=8	

E8	14/10/97		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	304	1017	3 35
2	292	1091	3 74
3	300	1091	3.64
4	298	1091	3.66
5	261	1081	4.14
6	276	1091	3 95
7 - 2X	113	457	4 06
8	107	432	4 06
9	109	447	4.12
10	110	416	3 80
11-G	111	431	3 90
12	108	440	4 09
13	109	442	4 07
14	109	437	4 03
15	110	443	4.05
16	114	468	4 12
17	112	419	3 76
18	122	471	3 88
19	114	448	3 95
20-G	109	430	3.96
21	113	437	3 88
22	111	439	3 97
23	105	426	4 08
24	108	453	4 21
25	107	430	4 04
26-V	112	442	3 96
27	119	514	4 34
28	132	463	3.52
29	115	450	3.93
30-N	110	433	3 95
31	109	441	4.06
32	110	444	4.05
33	111	443	4.01
34-S	16	41	
35	18	44	
36	18	40	
37	17	38	
38	15	37	
5X	zero=41	zero=9	
2X	zero=16	zero=4	

E9	14/10/97		
Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	83	325	3.92
2	82	321	3.91
3	78	310	3 97
4	78	324	4.15
5	81	325	4 01
6	81	322	3.98
7	93	330	3 55
8	84	318	3 79
9	82	320	3.90
10-S	17	33	
11	16	34	
12	18	36	
13	17		
14	16		
15	16		
	zero=53	zero=14	

E10	14/10/97		
Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	150	545	3.63
2	144	472	3.28
3	116	409	3.53
4	104	395	3 80
5	98	370	3 78
6	100	422	4 22
7	113	453	4 01
8	122	459	3.76
9	119	469	3.94
10-S	8	18	
11	6	18	
12	7		
13	9		
14	8		
	zero=56	zero=17	

E11	14/10/97		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	98	376	3 84
2	95	378	3.98
3	95	327	3 44
4	76	275	3 62
5	71	275	3.87
6	74	299	4 04
7	82	310	3.78
8	79	299	3 78
9	81	310	3 83
10	84	318	3.79
11-S	8	11	
12	3	10	
13	3	11	
14	5	10	
15	3	10	
16	4	11	
	zero=54	zero=11	

E12	14/10/97		
Time (a u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	155	593	3.83
2	157	580	3.69
3	160	547	3 42
4	94	379	4 03
5	123	437	3 55
6	111	401	3.61
7	115	450	3.91
8	126	427	3 39
9	111	403	3.63
10	112	426	3.80
11-S	5	13	
12	5	13	
13	4	14	
14	4		
15	4		
16	4		
	zero=54	zero=14	

Appendix 4.2 - Internal pH Raw Data for *E.coli*

pH 7 0, Amplification = 5X

G=glucose added, V - valinomycin added, N - nigericin added, S - cells filtered out, supernatent only, 2X - amplification changed to 2X

F1	22/10/97		
Time	Fluorescence at		Ratio
(a u)	440nM	500mM	I/I
1	208	694	3.34
2	185	664	3.59
3	201	710	3 53
4	207	724	3.50
5	208	716	3.44
6	202	678	3 36
7	184	634	3.45
8	189	665	3 52
9	194	691	3 56
10-G	195	692	3.55
11	196	698	3.56
12	196	697	3 56
13	198	701	3.54
14	201	702	3.49
15	201	697	3 47
16	201	704	3 50
17	202	710	3 51
18	204	708	3.47
30-V	202	706	3.50
31	202	715	3.54
32	204	712	3.49
33	202	716	3 54
34	206	712	3.46
35-N	203	713	3 51
36	204	711	3 49
37	206	721	3.50
38	207	717	3.46
39	207	719	3 47
40	207	720	3 48
45-S	48	107	
46	46	108	
47	47	107	
48	45	106	
49	46	106	
50	47	108	
	zero=64	zero=16	

F2	22/10/97		
Time	Fluorescence at		Ratio
(a u.)	440nM	500mM	I/I
1	186	639	3 44
2	175	619	3 54
3	176	645	3 66
4	187	664	3 55
5	189	663	3 51
6	185	654	3 54
7	182	642	3.53
8	179	633	3 54
9	177	627	3.54
10-G	184	655	3 56
11	184	655	3.56
12	184	656	3 57
13	184	657	3 57
14	186	663	3 56
15	188	659	3 51
16	189	659	3 49
17	190	663	3.49
18	190	664	3.49
19	191	666	3 49
20	192	666	3 47
21-V	191	670	3 51
22	191	670	3 51
23	191	673	3.52
24	193	677	3.51
25	196	680	3.47
26-N	194	676	3 48
27	196	675	3 44
28	193	678	3.51
29	195	675	3.46
30	197	682	3 46
31-S	57	121	
32	59	115	
33	54	104	
34	44	99	
35	44	92	
	zero=60	zero=17	

F3	22/10/97		
Time	Fluorescence at		Ratio
(a.u)	440nM	500mM	I/I
1	291	1085	3 73
2	288	1085	3.77
3 - 2X	116	435	3 75
4	118	437	3 70
5	116	440	3 79
6	117	437	3 74
7	116	438	3.78
8	115	438	3.81
9	115	440	3.83
10	117	444	3 79
11-G	114	451	3.96
12	115	453	3.94
13	116	453	3 91
14	118	456	3 86
15	118	457	3.87
16	118	455	3.86
17	118	460	3 90
18	120	461	3 84
19	119	463	3.89
20	119	462	3.88
21	120	462	3.85
22	120	463	3 86
23-V	120	462	3 85
24	120	463	3 86
25	120	462	3 85
26	121	468	3.87
27-N	121	467	3.86
28	121	466	3 85
29	122	468	3.84
30	124	470	3.79
30	123	469	3.81
30	124	471	3.80
31-S	24	66	
32	24		
33	25		
34	24		
35	25		
5X	zero=60	zero=15	
2X	zero=24	zero=6	

F4	22/10/97		
Time	Fluorescence at		Ratio
(a u)	440nM	500mM	I/I
1 - 2X	102	352	3 45
2 - 5X	270	882	3.26
3	253	903	3 57
4	258	907	3 51
5	255	897	3.52
6	255	922	3.61
7	265	955	3.60
8	277	1016	3.67
9	280	982	3.51
10	276	978	3.54
11-G	268	955	3.56
12	265	950	3.58
13	268	958	3 57
14	269	970	3 60
15	270	969	3 59
16	273	970	3 55
26-V	275	972	3 53
27	279	980	3.51
28	274	982	3 58
29	276	982	3.56
30-N	278	990	3.56
31	279	1000	3 58
32	283	1000	3 53
33	280	998	3.56
34-S	59	137	
35	61		
36	59		
37	59		
38	56		
5X	zero=60	zero=17 5	
2X	zero=24	zero=7	

F5	22/10/97		
Time	Fluorescence at		Ratio
(a u.)	440nM	500mM	I/I
1	196	691	3.53
2	192	710	3.70
3	190	708	3.73
4	192	732	3 81
5	192	707	3 68
6	188	683	3.63
7	186	687	3 69
8	184	699	3 80
9	194	748	3 86
10-G	189	703	3.72
11	189	710	3 76
12	188	701	3.73
13	188	703	3.74
14	189	705	3 73
15	190	710	3.74
16	190	713	3 75
17	197	712	3.61
18	190	723	3 81
19	193	725	3.76
20	193	722	3.74
21-V	194	715	3 69
22	193	720	3.73
23	194	735	3.79
24	194	721	3.72
25	197	719	3 65
26-N	200	749	3.75
27	200	760	3.80
28	200	762	3.81
29	202	760	3.76
30	201	765	3.81
31-S	35	92	
32	36		
33	36		
34	36		
35	36		
	zero=61	zero=16	

F5 pH 5	22/10/97		
Time	Fluorescence at		Ratio
(a.u)	440nM	500mM	I/I
1	190	256	1 35
2	177	252	1 42
3	189	257	1 36
4	185	236	1.28
5	174	226	1 30
11-G	175	222	1.27
12	173	225	1.30
13	174	219	1.26
14	171	212	1.24
15	169	214	1 27
16-G	165	209	1 27
17	167	206	1.23
18	168	209	1.24
19	173	204	1.18
20	167	202	1 21
21	169	202	1.20
22	172	210	1.22
23-V	172	212	1.23
24	180	205	1.14
25	172	203	1 18
26	174	203	1.17
27-N	168	193	1.15
28	172	195	1.13
29	176	198	1.13
30	173	188	1.09
30	174	190	1.09
30	175	191	1.09
31-S	78	159	
32	96		
33	66		
34	43		
35	31		
35	23		
35	23		
35	22		
35	22		
35	22		
	zero=71	zero=31	

Appendix 4.2 - Internal pH Raw Data for *E.coli*

pH 7.0, Amplification = 5X except where 2X = amplification changed to 2X
G=glucose added, V - valinomycin added, N - nigericin added, S - cells filtered out, supernatent only,

F6	23/10/97		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	234	892	3.81
2	245	882	3.6
3	240	858	3.58
4	237	847	3.57
5	238	865	3.63
6	244	884	3.62
7	247	896	3.63
8	250	903	3.61
9	251	901	3.59
10	248	899	3.63
11-G	246	897	3.65
12	245	904	3.69
13	249	909	3.65
14	249	906	3.64
15	250	913	3.65
16	250	915	3.66
17-V	255	927	3.64
18	255	927	3.64
19	259	930	3.59
20	260	931	3.58
21	264	946	3.58
22	263	937	3.56
23	269	950	3.53
24-N	270	949	3.51
25	271	949	3.50
26	268	954	3.56
27	269	952	3.54
28-S	76	148	
29	76	152	
30	75		
31	75		
32	74		
	zero=59	zero=15	

F7 2X	23/10/97		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	113	376	3.33
2	90	337	3.74
3	98	371	3.79
4	110	438	3.98
5	128	481	3.76
6	132	491	3.72
7	128	480	3.75
8	123	460	3.74
9	115	433	3.77
10-G	119	443	3.72
11	120	440	3.67
12	120	444	3.7
13	120	448	3.73
14	122	447	3.66
15	123	452	3.67
16	123	452	3.67
17	124	452	3.65
18	125	454	3.63
19	126	456	3.62
20	126	467	3.71
21-V	125	456	3.65
22	126	458	3.63
23	126	459	3.64
24	127	459	3.61
25	127	462	3.64
26-N	129	461	3.57
27	129	463	3.59
28	131	466	3.56
29	135	471	3.49
30	133	469	3.53
31-S	35	70	
32	36		
33	35		
34	35		
35	35		
	zero=22	zero=6	

F8	23/10/97		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	187	650	3.48
2	179	648	3.62
3	184	652	3.54
4	186	670	3.6
5	185	646	3.49
6	184	657	3.57
7	184	704	3.83
8	198	670	3.38
9	185	648	3.5
10	190	654	3.44
11-G	192	656	3.42
12	191	657	3.44
13	192	666	3.47
14	195	659	3.38
15	194	659	3.40
16	194	663	3.42
17	198	667	3.37
18	196	670	3.42
19	199	670	3.37
20	201	667	3.32
21	200	676	3.38
22	204	679	3.33
23-V	206	685	3.33
24	207	684	3.30
25	208	689	3.31
26	210	690	3.29
27-N	213	703	3.30
28	213	699	3.28
29	213	707	3.32
30	215	708	3.29
31	219	711	3.25
32	218	713	3.27
33-S	78	129	
34	79		
35	81		
36	83		
37	85		
	zero=57	zero=15	

Appendix 4.2 - Internal pH Raw Data for *E.coli*

pH 7.0, Amplification = 5X G=glucose added, V - valinomycin added, N - nigericin added, S - cells filtered out, supernatent only

G1	30/10/97		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	66	155	2.35
2	64	154	2.41
3	64	157	2.45
4	65	154	2.37
5	65	153	2.35
6-G	66	153	2.32
7	67	157	2.34
8	67	164	2.45
9	69	154	2.23
10	69	157	2.28
11-V	72	156	2.17
12	75	157	2.09
13	75	154	2.05
14	76	155	2.04
15	75	159	2.12
16-N	78	158	2.03
17	81	163	2.01
18	81	157	1.94
19	81	158	1.95
20	82	159	1.94
21	88	171	1.94
22-S	45	25	
23	43	26	
24	43	23	
25	41		
26	41		
	zero=58	zero=15	

G1	30/10/97		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	129	108	0.84
2	125	107	0.86
3	124	108	0.87
4	127	105	0.83
5	127	107	0.84
6-G	125	107	0.86
7	125	121	0.97
8	131	106	0.81
9	126	106	0.84
10	126	109	0.87
11-V	130	109	0.84
12	132	108	0.82
13	131	110	0.84
14	129	110	0.85
15	133	112	0.84
16-N	133	112	0.84
17	136	112	0.82
18	135	113	0.84
19	137	114	0.83
20	137	114	0.83
21-S	125	33	
22	126	32	
23	125	33	
24	119		
25	118		
	zero=57	zero=14	

G1	30/10/97		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	24	53	2.21
2	22	56	2.55
3	25	59	2.36
4	26	59	2.27
5	26	59	2.27
6-G	25	56	2.24
7	25	57	2.28
8	28	57	2.04
9	26	57	2.19
10	26	59	2.27
11	26	56	2.15
12-V	26	59	2.27
13	31	78	2.52
14	29	74	2.55
15	27	57	2.11
16-N	29	60	2.07
17	28	60	2.14
18	29	59	2.03
19	29	58	2.00
20	29	59	2.03
21	31	60	1.94
22-S	20	9	
23	20		
24	19		
25	19		
26	20		
	zero=59	zero=16	

G1	30/10/97		
Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	162	130	0.80
2	143	132	0.92
3	150	132	0.88
4	159	140	0.88
5	162	134	0.83
6-G	150	134	0.89
7	156	148	0.95
8	153	134	0.88
9	156	133	0.85
10	152	134	0.88
11	155	134	0.86
12-V	158	135	0.85
13	159	140	0.88
14	158	135	0.85
15	159	133	0.84
16-N	160	138	0.86
17	163	138	0.85
18	162	136	0.84
19	162	139	0.86
20-S	152	43	
21	150		
22	149		
23	146		
24	137		
	zero=60	zero=16	

Appendix 4.2 - Internal pH Raw Data for *E.coli*

pH 7.0, Amplification = 5X G=glucose added, V - valinomycin added, N - nigericin added, S - cells filtered out, supernatent only, 300µl cells

H1	7/11/97		
Time	Fluorescence at		Ratio
(a.u.)	440nM	500mM	I/I
1	157	109	0.69
2	156	109	0.70
3	159	109	0.69
4	160	109	0.68
5	158	109	0.69
6 - GG	155	109	0.70
7	154	109	0.71
8	155	110	0.71
9	158	109	0.69
10 - V	160	109	0.68
11	164	107	0.65
12	164	111	0.68
13	168	111	0.66
14 - N	165	111	0.67
15	169	111	0.66
16	167	120	0.72
17	169	114	0.67
18	152	44	0.29
19 - S	148	39	
20	139	25	
2 min	138	25	
22	137	26	
5 min	138	24	
	zero=60	zero=20	

H2	7/11/97		
Time	Fluorescence at		Ratio
(a.u.)	440nM	500mM	I/I
1	115	446	3.88
2	111	455	4.10
3	111	458	4.13
4	113	458	4.05
5	111	454	4.09
6 - GG	115	447	3.89
7	108	442	4.09
8	110	443	4.03
9	106	443	4.18
10	107	443	4.14
11	109	446	4.09
12 - V	110	445	4.05
13	110	447	4.06
14	109	446	4.09
15	109	452	4.15
16 - N	110	448	4.07
17	111	450	4.05
18	109	446	4.09
19	110	447	4.06
20	110	448	4.07
21 - S	39	83	
22	20	33	
2 min	19	30	
3 min	20	32	
4 min	20	31	
5 min	20	32	
	zero=59	zero=14	

H3	7/11/97		
Time	Fluorescence at		Ratio
(a.u.)	440nM	500mM	I/I
1	147	99	0.67
2	144	95	0.66
3	145	97	0.67
4	149	98	0.66
5	147	99	0.67
6 - G	146	98	0.67
7	143	95	0.66
8	145	96	0.66
9	147	96	0.65
10 - V	148	97	0.66
11	149	96	0.64
12	152	98	0.64
13	153	99	0.65
14 - N	156	97	0.62
15	156	100	0.64
16	156	98	0.63
17	157	99	0.63
18	130	26	0.20
19 - S	128	29	
20	121	15	
2 min	121	15	
22	122	15	
5 min	123	16	
	zero=61	zero=20	

H4	7/11/97		
Time	Fluorescence at		Ratio
(a.u.)	440nM	500mM	I/I
1	188	785	4.18
2	186	795	4.27
3	188	786	4.18
4	185	791	4.28
5	185	795	4.30
6 - G	178	835	4.69
7	177	836	4.72
8	177	838	4.73
9	176	833	4.73
10	179	828	4.63
11	179	833	4.65
12 - V	181	832	4.60
13	180	832	4.62
14	179	833	4.65
15	180	839	4.66
16 - N	180	832	4.62
17	181	839	4.64
18	181	831	4.59
19	180	831	4.62
20 - S	29	56	
21	30	55	
2 min	23	50	
3 min	23	50	
	zero=60	zero=20	

Appendix 4.2 - Internal pH Raw Data for *E.coli*

pH 7.0, Amplification = 5X G=glucose added, V - valinomycin added, N - nigericin added, S - cells filtered out, supernatent only

H8	7/11/97		
Time	Fluorescence at		Ratio
(a.u.)	440nM	500mM	I/I
1	92	357	3.88
2	93	362	3.89
3	94	365	3.88
4	95	364	3.83
5	95	363	3.82
6 - G	93	360	3.87
7	93	364	3.91
8	94	363	3.86
9 - G	90	356	3.96
10	90	356	3.96
11	90	359	3.99
12	94	359	3.82
13 - V	92	360	3.91
14	93	357	3.84
15	93	359	3.86
16	93	363	3.90
17 - N	94	365	3.88
18	94	365	3.88
19	95	367	3.86
20	94	367	3.90
21 - S	21	28	
22	22	28	
	zero=68	zero=28	

H5	7/11/97		
Time	Fluorescence at		Ratio
(a.u.)	440nM	500mM	I/I
1	141	116	0.82
2	136	115	0.85
3	138	116	0.84
4	141	116	0.82
5	138	118	0.86
6 - S	95	19	
7	95	21	
8	92	19	
2 min	94	19	
-	93	19	
5 min	93	18	
	zero=60	zero=17	

H8	7/11/97		
Time	Fluorescence at		Ratio
(a.u.)	440nM	500mM	I/I
1	110	362	3.29
2	107	360	3.36
3	104	355	3.41
4	109	367	3.37
5	109	365	3.35
6 - G	103	357	3.47
7	106	358	3.38
8	104	363	3.49
9	105	361	3.44
10 - V	106	369	3.48
11	109	368	3.38
12	107	375	3.50
13	109	375	3.44
14 - N	109	380	3.49
15	110	384	3.49
16	110	379	3.45
17	110	386	3.51
18 - S	36	68	
19	28	57	
2 min	26	58	
-	27	58	
5 min	27	66	
-	26	56	
	zero=60	zero=11	

H6	7/11/97		
Time	Fluorescence at		Ratio
(a.u.)	440nM	500mM	I/I
1	131	163	1.24
2	135	171	1.27
3	133	163	1.23
4	131	155	1.18
5	134	162	1.21
6 - G	136	173	1.27
7	127	152	1.20
8	131	162	1.24
9	128	165	1.29
10	128	160	1.25
11	134	155	1.16
12 - V	134	165	1.23
13	139	172	1.24
14	139	162	1.17
15	135	155	1.15
16 - N	140	169	1.21
17	137	166	1.21
18	140	161	1.15
19	143	159	1.11
20 - S	111	47	
21	120	70	
2 min	96	31	
-	97	32	
5 min	97	32	
	zero=60	zero=15	

H7	7/11/97		
Time	Fluorescence at		Ratio
(a.u.)	440nM	500mM	I/I
1	27	30	1.11
2	27	31	1.15
3	27	31	1.15
4	28	29	1.04
5	27	30	1.11
6 - G	30	31	1.03
7	28	29	1.04
8	27	32	1.19
9	31	32	1.03
10	31	30	0.97
11-V	34	32	0.94
12	35	30	0.86
13	33	30	0.91
14	38	34	0.89
15	35	31	0.89
16-N	39	34	0.87
17	40	33	0.83
18	39	32	0.82
19	40	32	0.80
20	41	33	0.80
21	42	33	0.79
22-S	43	10	
23	41	10	
24	39	10	
25	39	10	
26	41	10	
	zero=65	zero=21	

Appendix 4.2 - Internal pH Raw Data for *E.coli*

pH 7 0, Amplification = 5X G=glucose added, V - valinomycin added, N - nigericin added, S - cells filtered out, supernatent only

I1 14/11/97

Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	56	49	0 88
2	51	49	0.96
3	83	71	0.86
4	80	71	0.89
5	81	71	0 88
6 - S	48	3	
7	47	4	
8	47	3	
	zero=57	zero=15	

I2 Fri 7/11/97

Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	55	127	2 31
2	54	121	2.24
3	53	123	2 32
4	53	123	2.32
5	53	121	2.28
6 - G	61	134	2 20
7	62	134	2.16
8	61	132	2.16
9	61	131	2.15
10	61	132	2.16
11	62	131	2.11
12	62	134	2 16
13 - V	63	135	2 14
14	65	134	2.06
15	64	135	2 11
16	64	135	2.11
17 - N	66	135	2 05
18	68	135	1.99
19	67	132	1.97
20	69	134	
21-S	35	10	
22	34	10	
	zero=47	zero=11	

I3 pH 6 14/11/97

Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	114	327	2 87
2	105	294	2 80
3	103	279	2 71
4	106	271	2 56
5	106	261	2.46
6 - G	112	267	2 38
7	110	264	2 40
8	107	261	2 44
9	107	263	2 46
10	108	256	2 37
11	108	257	2 38
12 - G	112	254	2 27
13	112	254	2 27
14	112	257	2 29
15	113	258	2 28
16	113	253	2.24
17 - V	112	254	2 27
18	111	255	2.30
19	113	254	2.25
20	114	258	2.26
21 - N	114	250	2 19
22	114	247	2 17
23	115	244	2.12
24	113	247	2 19
25	115	245	2.13
26 - S	22	31	
27	18	20	
28	13	19	
3 min	12	19	
30	12	18	
31	12	18	
	zero=47	zero=11	

I3 pH 7 14/11/97

Time (a u)	Fluorescence at 440nM	500mM	Ratio I/I
1	113	520	4 60
2	113	522	4 62
3	114	533	4 68
4	110	532	4.84
5	112	532	4.75
6 - GG	118	547	4 64
7	117	534	4.56
8	115	535	4.65
9	114	526	4 61
10	114	531	4 66
11	117	536	4 58
12 - V	114	533	4.68
13	119	535	4 50
14	116	533	4.59
15	117	534	4 56
16 - N	116	535	4.61
17	116	528	4 55
18	115	534	4.64
19	117	532	4 55
20	116	533	4 59
21 - S	39	69	
22	43	45	
23	21	23	
3min	15	24	
4min	16	24	
5min	15	23	
	zero=59	zero=16	

I4 14/11/97

Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	82	367	4.48
2	80	353	4.41
3	78	377	4.83
4	83	385	4 64
5	84	373	4 44
6 - G	82	364	4.44
7	82	365	4 45
8	86	367	4.27
9	85	367	4.32
10	86	362	4 21
11	85	366	4 31
12 - V	84	368	4.38
13	82	367	4.48
14	85	366	4.31
15	87	367	4 22
16 - N	82	366	4.46
17	83	368	4.43
18	84	365	4 35
19	84	367	4 37
20 - S	30	78	
3 min	12	24	
22	13	23	
23	13	23	
	zero=59	zero=16	

I5 14/11/97

Time (a u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	170	755	4 44
2	160	747	4 67
3	163	754	4.63
4	159	742	4 67
5	160	732	4 58
6 - G	160	726	4.54
7	160	734	4.59
8	159	737	4 64
9	164	742	4 52
10	160	730	4 56
11	160	731	4.57
12 - G	161	734	4.56
13	163	741	4 55
14	165	740	4 48
15 - V	159	740	4.65
16	160	737	4.61
17	163	741	4.55
18	161	740	4 60
19 - N	160	748	4 68
20	163	736	4.52
21	159	741	4.66
22	160	746	4.66
23 - S	90	147	
24	32	63	
2 mın	20	60	
26	20	61	
5 min	20	60	
	zero=59	zero=15	

I6 14/11/97

Time (a u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	205	905	4 41
2	193	911	4 72
3	190	892	4 69
4	190	880	4.63
5	188	876	4.66
6 - G	191	878	4 60
7	193	874	4 53
8	187	866	4 63
9	188	877	4.66
10	194	883	4.55
11 - G	197	871	4.42
12	193	875	4.53
13	196	876	4.47
14	197	879	4.46
15	196	876	4 47
16	195	876	4.49
17 - G	198	879	4.44
18	200	878	4 39
19	199	876	4.40
20	202	881	4.36
21	200	886	4.43
22	200	888	4.44
23	201	883	4 39
24	198	885	4.47
25 - V	200	884	4.42
26	198	886	4 47
27	201	885	4 40
28	201	909	4.52
29	206	893	4.33
30	199	887	4.46
31	199	892	4 48
32 - N	210	892	4.25
33	201	905	4 50
34	198	892	4.51
35	197	885	4.49
36 - N	200	893	4.47
37	195	900	4.62
38	203	889	4.38
39 - S	38	88	
40	26	73	
41	28	75	
2 min	26	74	
43	27	76	
5 min	26	75	
	zero=60	zero=17	

Appendix 4.2 - Internal pH Raw Data for *E.coli*

pH 7.0, Amplification = 1X G=glucose added, V - valinomycin added, N - nigericin added, S - cells filtered out, supernatent only

J1 17/11/97

Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	18	49	2 72
2	18	47	2 61
3	19	50	2 63
4	24	65	2 71
5	19	48	2 53
6 - G	20	49	2 45
7	20	63	3.15
8	20	52	2 60
9	21	57	2 71
10	21	54	2 57
11	21	53	2 52
12 - G	22	49	2.23
13	22	51	2 32
14	22	51	2 32
15	23	55	2 39
16	23	53	2 30
17	22	50	2 27
18 - V	23	51	2 22
19	23	51	2 22
20	25	52	2 08
21	24	51	2 13
22	24	59	2 46
23	24	51	2 13
24 - N	25	54	2.16
25	26	55	2.12
26	27	63	2 33
27	26	55	2.12
28	26	52	2 00
29	26	52	2 00
30 - S	18	31	
31	18	32	
32	18	30	
33	18	31	
34	18	32	
35	18	31	
	zero=7	zero=3	

J7 17/11/97

Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	50	103	2 06
2	40	98	2 45
3	40	101	2 53
4	40	103	2 58
5	41	103	2 51
6 - S	25	60	
7	27	63	
8	26	59	
9	26	73	
	zero=13	zero=13	

J2 17/11/97

Time (a.u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	156	607	3.89
2	146	538	3 68
3	135	555	4.11
4	149	588	3 95
5	153	596	3.90
6	152	593	3.90
7	151	585	3 87
8	148	576	3 89
9	145	566	3 90
10 - G	144	571	3 97
11	147	574	3 90
12	145	574	3 96
13	146	574	3.93
14	147	583	3 97
15	147	578	3 93
16	149	579	3.89
17	147	580	3.95
18	149	585	3.93
19	150	583	3 89
20 -G	149	577	3 87
21	150	578	3 85
22	150	579	3 86
23	151	580	3 84
24	151	579	3 83
25	152	584	3 84
26	152	583	3 84
27	152	584	3 84
28	152	588	3 87
29	154	592	3 84
30 - V	154	588	3 82
31	153	592	3 87
32	155	594	3 83
33	155	595	3 84
34	156	599	3.84
35 - N	155	594	3.83
36	156	595	3 81
37	156	596	3.82
38	157	598	3 81
39	158	600	3 80
40	158	604	3 82
41	158	603	3.82
42	160	601	3 76
43	158	601	3 80
44	159	600	3 77
45 -S	24	68	
46	25	68	
47	24	69	
48	23	69	
49	23	69	
50	23	69	
	zero=12	zero=4	

J3 17/11/97

Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	209	789	3 78
2	201	811	4 03
3	205	833	4 06
4	205	841	4 10
5	204	832	4 08
6	201	824	4.10
7	199	830	4.17
8	200	842	4.21
9	202	840	4 16
10 - G	195	851	4 36
11	196	846	4 32
12	197	842	4 27
13	197	845	4 29
14	198	840	4.24
15	197	840	4 26
16	197	843	4 28
17	197	841	4.27
18	198	841	4.25
19	199	842	4 23
20	199	846	4 25
21	199	844	4 24
22 - G	198	842	4 25
23	198	841	4 25
24	199	840	4.22
25	197	838	4.25
26	199	841	4 23
27	199	847	4 26
28	199	844	4 24
29	199	847	4 26
30 - V	199	852	4.28
31	200	852	4 26
32	199	853	4 29
33	200	853	4.27
34	201	856	4.26
35 - N	199	857	4.31
36	200	859	4 30
37	201	859	4 27
38	201	856	4.26
39	201	855	4.25
40 - N	201	857	4.26
41	201	859	4.27
42	203	862	4.25
43	204	860	4 22
44	202	857	4 24
45 - S	39	172	
46	42	176	
47	41	170	
48	37	169	
49	37	169	
	zero=13	zero=5	

J4 17/11/97

Time (a u)	Fluorescence at 440nM	500mM	Ratio I/I
1	52	183	3 52
2	49	188	3 84
3	52	191	3 67
4	52	193	3 71
5	51	186	3 65
6	50	184	3.68
7	50	181	3.62
8	50	183	3 66
9	50	183	3.66
10 - G	51	191	3 75
11	52	192	3 69
12	51	192	3 76
13	52	191	3 67
14	52	191	3.67
15	52	192	3 69
16	52	190	3 65
17	52	191	3.67
18	53	191	3 60
19	53	191	3 60
20 - V	58	194	3 34
21	59	193	3.27
22	60	193	3 22
23	60	193	3 22
24	60	193	3 22
25	60	193	3 22
26 - N	62	190	3.06
27	64	190	2 97
28	63	190	3 02
29	63	191	3 03
30	62	190	3 06
31	64	189	2.95
32 - S	30	43	
33	29	36	
34	30	37	
35	30	40	
36	29	36	
37	29	37	
	zero=11	zero=7	

J5 17/11/97

Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	171	695	4.06
2	153	478	3.12
3	101	424	4.20
4	107	480	4 49
5	130	558	4 29
6	139	562	4 04
7	138	574	4 16
8	142	594	4 18
9	140	554	3 96
10 - G	130	551	4 24
11	130	551	4 24
12	130	549	4 22
13	128	549	4 29
14	129	547	4 24
15	129	546	4 23
16	128	543	4 24
17	128	546	4.27
18	128	543	4 24
19	128	545	4.26
20	128	544	4.25
21	129	545	4 22
22	129	545	4.22
23	128	544	4 25
24	128	545	4 26
25	128	544	4 25
26	129	541	4.19
27	128	544	4 25
28	129	545	4 22
29	130	546	4 20
30 - V	127	543	4 28
31	126	540	4 29
32	128	541	4.23
33	129	541	4 19
34	129	542	4 20
35 - N	128	543	4.24
36	128	542	4 23
37	128	542	4.23
38	129	546	4 23
39	130	543	4 18
40	129	543	4.21
41	129	539	4.18
42	128	541	4.23
43	129	540	4 19
44	129	543	4.21
45 -S	16	60	
46	16	60	
47	16	61	
48	15	61	
49	15	61	
50	15	62	
	zero=12	zero=5	

Appendix 4.2 - Internal pH Raw Data for *E.coli*

pH 7.0, Amplification = 5X G=glucose added, V - valinomycin added, N - nigericin added, S - cells filtered out, supernatent only

J6	17/11/97		
Time (a u)	Fluorescence at 440nM	500mM	Ratio I/I
1	184	544	2 96
2	162	620	3 83
3	165	643	3 90
4	167	675	4 04
5	175	701	4 01
6	175	715	4 09
7	177	676	3 82
8	153	639	4 18
9	153	601	3 93
10 - G	155	659	4.25
11	156	651	4 17
12	156	657	4 21
13	156	651	4 17
14	157	649	4 13
15	156	649	4.16
16	157	651	4 15
17	156	647	4.15
18	158	649	4 11
19	157	648	4 13
20	157	654	4 17
21	158	650	4 11
22 - G	155	640	4.13
23	155	638	4 12
24	155	638	4.12
25	156	640	4 10
26	157	640	4 08
27	155	639	4.12
28	155	639	4 12
29	156	640	4 10
30 - V	156	646	4 14
31	157	648	4.13
32	157	645	4 11
33	157	647	4 12
34	157	650	4.14
35 - N	156	649	4.16
36	160	649	4 06
37	158	649	4 11
38	158	647	4.09
39	158	649	4 11
40	158	651	4 12
41	158	650	4 11
42	159	652	4 10
43	158	653	4 13
44	159	651	4 09
45 - S	29	107	
46	29	108	
47	29	107	
48	28	108	
49	29	107	
	zero=13	zero=5	

J8	17/11/97		
Time (a u)	Fluorescence at 440nM	500mM	Ratio I/I
1	105	399	3 80
2	100	388	3 88
3	96	384	4 00
4	96	386	4 02
5	97	388	4 00
6	97	387	3.99
7	97	386	3 98
8	96	383	3.99
9	96	377	3 93
10 - G*	96	377	3 93
11	94	378	4.02
12	94	377	4 01
13	95	375	3 95
14	95	380	4 00
15	96	376	3 92
16	94	373	3 97
17	94	374	3.98
18	93	373	4 01
19	94	372	3 96
20 - G*	94	367	3 90
21	93	367	3 95
22	94	368	3.91
23	94	369	3 93
24	93	367	3 95
25	93	367	3 95
26	95	366	3.85
27	94	367	3 90
28	94	365	3 88
29	94	365	3 88
30 - V**	94	373	3 97
31	95	375	3 95
32	95	377	3 97
33	95	376	3 96
34	96	375	3 91
35 - N**	97	381	3 93
36	97	381	3 93
37	97	380	3 92
38	97	380	3 92
39	97	381	3 93
40	98	382	3 90
41	97	380	3.92
42	98	381	3 89
43	99	381	3 85
44	98	382	3 90
45 - S	31	28	
46	8	24	
47	8	24	
48	8	25	
49	9	25	
50	8	24	
	zero=13	zero=6	

J9	17/11/97		
Time (a u)	Fluorescence at 440nM	500mM	Ratio I/I
1	174	615	3.53
2	175	629	3.59
3	171	624	3 65
4	172	644	3 74
5	174	651	3 74
6	174	648	3.72
7	172	648	3 77
8	172	648	3 77
9	172	651	3.78
10 - G**	170	656	3.86
11	167	654	3 92
12	168	654	3.89
13	168	661	3 93
14	170	646	3 80
15	168	646	3 85
16	168	647	3 85
17	168	641	3 82
18	168	639	3 80
19	168	642	3 82
20	168	642	3 82
21	168	641	3 82
22 - V**	166	650	3 92
23	167	651	3 90
24	168	652	3 88
25	168	651	3 88
26	168	651	3 88
27 - N**	167	661	3.96
28	167	657	3 93
29	168	657	3.91
30	168	658	3 92
31	168	659	3 92
32	168	661	3 93
33 - S	45	47	
34	17	46	
35	16	47	
36	16	47	
	zero=13	zero=5	

* indicates the addition of 50ul
** indicates the addition of 100ul

J11	17/11/97		
Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	88	344	3 91
2	85	318	3 74
3	79	322	4 08
4	79	326	4.13
5	81	326	4 02
6	80	319	3 99
7	77	307	3 99
8	76	305	4 01
9	77	308	4 00
10 - G*	78	310	3 97
11	78	308	3 95
12	77	309	4 01
13	77	306	3.97
14	77	310	4.03
15	77	303	3 94
16	76	303	3 99
17	79	305	3.86
18	77	305	3 96
19	77	302	3 92
20 - G*	77	298	3 87
21	77	297	3 86
22	77	297	3 86
23	77	297	3.86
24	77	297	3.86
25	77	295	3 83
26	77	297	3 86
27	77	297	3 86
28	76	295	3 88
29	77	295	3.83
30 - V**	78	302	3 87
31	79	304	3 85
32	79	304	3 85
33	80	303	3 79
34	80	308	3 85
35 - N**	81	310	3 83
36	80	310	3 88
37	81	313	3.86
38	82	313	3 82
39	81	312	3 85
40	82	311	3.79
41- S	23	38	
42	6	12	
43	6	12	
44	6	12	
45	6	12	
46	6	12	
	zero=12	zero=5	

J12	17/11/97		
Time (a u.)	Fluorescence at 440nM	500mM	Ratio I/I
1	156	535	3 43
2	153	540	3.53
3	152	538	3.54
4	151	543	3 60
5	152	546	3 59
6	151	545	3 61
7	151	547	3.62
8	150	542	3 61
9	150	544	3 63
10 - G*	146	533	3 65
11	146	531	3.64
12	146	531	3 64
13	146	532	3 64
14	146	532	3 64
15	147	535	3 64
16	146	529	3 62
17	146	529	3 62
18	146	528	3 62
19	145	529	3 65
20 - V**	146	546	3 74
21	146	544	3 73
22	146	543	3 72
23	146	543	3 72
24	146	542	3 71
25 - N**	146	560	3 84
26	147	556	3.78
27	147	558	3 80
28	147	558	3 80
29	148	561	3.79
30	148	563	3 80
31 - S	74	33	
32	14	25	
33	14	26	
34	13	25	
35	13	25	
36	14	26	
	zero=12	zero=4	

J10	17/11/97		
Time (a.u)	Fluorescence at 440nM	500mM	Ratio I/I
1	62	268	4 32
2	56	253	4 52
3	59	273	4 63
4	59	270	4 58
5	58	255	4.40
6 - S	19	62	
7	19	61	
	zero=11	zero=7	

Appendix 5.1 - Derivation of New D type Dissociated Acid Term

The Henderson-Hasselbalch Equation states

$$\frac{[A^-]}{[HA]} = 10^{pH-pKa} \qquad (1)$$

where [A$^-$] is the concentration of dissociated acid, [HA] is the concentration of undissociated acid and the pKa is a known value for each acid under a given set of conditions.

By definition $[HA] = [AC] - [A^-]$ (2)
where [AC] is the total concentration of acid.

Therefore substituting (2) in (1)

$$\frac{[A^-]}{[AC] - [A^-]} = 10^{pH-pKa} \qquad (3)$$

Cross multiplying gives

$$[A^-] = 10^{pH-pKa}([AC] - [A^-]) \qquad (4)$$

Multiplying out gives

$$[A^-] = [AC]10^{pH-pKa} - [A^-]10^{pH-pKa} \qquad (5)$$

Taking the [A$^-$] over to the LHS

$$[A^-] + [A^-]10^{pH-pKa} = [AC]10^{pH-pKa} \qquad (6)$$

Taking [A$^-$] outside a bracket

$$[A^-](1 + 10^{pH-pKa}) = [AC]10^{pH-pKa} \qquad (7)$$

Multiplying both sides by $(1 + 10^{pH-pKa})$ gives the Dissociated Acid concentration:

$$[A^-] = \frac{[AC]10^{pH-pKa}}{(1 + 10^{pH-pKa})} \qquad (8)$$

Growth rate graph versus [Dissociated] follows a pattern of $10^{-[\text{dissociated}]}$

$$rate = p10^{-d\left(\frac{[AC]10^{pH-pKa}}{(1+10^{pH-pKa})}\right)} \quad (9)$$

Take [AC] outside the bracket

$$rate = p10^{-d[AC]\left(\frac{10^{pH-pka}}{1+10^{pH-pka}}\right)} \quad (10)$$

Divide through by numerator

$$rate = p10^{-d[AC]\left(\frac{1}{\frac{1}{10^{pH-pka}}+1}\right)} \quad (11)$$

Change sign on exponent terms to bring them to the numerator/denominator of whole fraction

$$rate = p10^{-d[AC]\left(\frac{1}{10^{pka-pH}+1}\right)} \quad (12)$$

Multiply through by -q[AC]

$$rate = p10^{\left(\frac{-d[AC]}{10^{pka-pH}+1}\right)} \quad (13)$$

When added to the rest of the model: p becomes the other factors in the model and d becomes D.

$$\sqrt{k} = C(T - T_{\min})\sqrt{a_w - a_{w\min}}\sqrt{1-10^{pH_{\min}-pH}}\sqrt{1-\frac{[AC]}{[U_{\min}]\left[1+10^{pH-pKa}\right]}}\sqrt{10^{\left(\frac{-D\ [AC]}{10^{pka-pH}+1}\right)}} \quad (14)$$

Appendix 5.2 - Derivation of New pH_{max} Term

The following pH_{max} term was derived in the same way as the suboptimal pH term as described in Presser *et al.* (1997). The observed pH response of microorganisms consists of a plateau of constant growth rates over a range near the optimal pH and decline in the growth rate as pH increases until no growth is observed. The growth rate response is directly proportional to the hydrogen ion concentration $[H^+]$ in the sub-optimal pH range. Therefore assume that the growth rate is also proportional to the amount by which $[H^+]$ is above the minimum value which prohibits growth in the superoptimal region at high pH. Therefore

$$rate = c \cdot \left(\left[H^+ \right] - \left[H^+_{\min} \right] \right) \tag{1}$$

where c is a constant of proportionality, and $\left[H^+_{\min} \right]$ is the theoretical minimum $[H^+]$ beyond which no growth is possible, and which is assumed to be constant for a given strain of bacteria.

Let pH_{max} be the pH corresponding to $\left[H^+_{\min} \right]$.

Since, by definition, pH = -log10$[H^+]$, Equation 1 can be rewritten as:

$$rate = c \cdot \left(10^{-pH} - 10^{-pH_{\max}} \right) \tag{2}$$

$$rate = \left(c \cdot 10^{-pH} \right) \frac{\left(10^{-pH} - 10^{-pH_{\max}} \right)}{10^{-pH}} \tag{3}$$

Let $b = c \cdot 10^{-pH}$

Substituting for c and rearranging gives:

$$rate = b \cdot \left(1 - \frac{10^{-pH_{\max}}}{10^{-pH}}\right) \tag{4}$$

$$rate = b \cdot \left(1 - \frac{10^{pH}}{10^{pH_{\max}}}\right) \tag{5}$$

$$rate = b \cdot \left(1 - 10^{pH - pH_{\max}}\right) \tag{6}$$

This is the pH term used in the model, and embodies our original hypotheses regarding the qualitative features of the response.

Appendix 5.3 - Z Test for Significance of Parameter Estimate Differences

Approximate Z Test involves the comparison of the estimates (Y_1 and Y_2) of a parameter, such as pHmin, by using their Standard Errors (S.E.) to determine if the difference between the estimates is significant.

The Z value given by the equation below is compared to predetermined values for each combination of level of significance (p=0.01, 0.05, 0.001).

$$Z = \frac{Y_1 - Y_2}{\sqrt{(S.E._{Y_1})^2 + (S.E._{Y_2})^2}}$$

Appendix 5.4 - Extra sum of squares principle

This principle can be used to test whether the addition of an extra term to create a new model improves the fit, as measured by the Sum of Squares (SS), over and above the difference in the degrees of freedom (d.f.) caused by adding another term.

$$X = \frac{SS_1 - SS_2}{d.f._1 - d.f._2}$$

The X value (mean square) given by the equation above (where 1 indicates values of the first simpler model and 2 indicates values of the newer complex model) is used to calculate the value below which gives the level of significance of the improvement i.e. 1 or 5%.

$$Ratio(\alpha) = \frac{X}{M.S.E._2}$$

where the second mean square $M.S.E._2 = SS_2/d.f._2$. This gives an approximate F-test which is exact for some linear models.