Effects of Temperature and Water Activity on *Escherichia coli* in Relation to Beef Carcasses

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DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

M.A. Salter

24 / 9 /1998

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Abstract

To understand and be able to predict the behaviour of pathogenic *Escherichia coli* in foods would greatly assist decision-making in the food industry in relation to handling practices, processes and formulation. The Australian red meat industry has a particular interest in the behaviour of *E. coli* on carcasses, on which temperature and water activity are the most important factors affecting bacterial growth. This subject is the topic of this thesis.

Nine Shiga toxin-producing *E. coli* (STEC) strains, including serotypes of particular public health significance (O157:H7 and O111) were obtained from two public hospitals. Predictive models are generated for STEC growth rates with respect to temperature and water activity and are not found to be significantly different to those for non-pathogenic *E. coli*. The performance of a model for non-pathogenic *E. coli* compares favourably with published models when compared to literature data. An investigation is also made of the growth of *E. coli* O157:H7 in the range 44-45.5°C . Growth is found to be sufficient in this range to enable detection using traditional incubation temperatures of 44-45.5°C.

The probability of growth of an STEC strain at the growth/no growth interface is modelled with varying combinations of temperature and water activity. Data fitted to a 'generalised non-linear regression model' gave an approximate concordance rate of 96.5%. The results suggest that even at the growth/no growth interface, bacterial growth is predictable. While earlier 'kinetic models' focused on growth under favourable environmental conditions, this data provides useful information about the probability of growth of a pathogenic microorganism at the boundary of growth and no growth.

Carcass surface temperature is easily monitored but previously, water activity at the surface could not be measured. Two methods of water activity measurement are assessed: electrical conductivity and direct water activity measurement following excision of tissue using a skin-grafting scalpel. The latter method allows fast and

accurate measurement of the water activity of carcass surface tissue. Monitoring water activity of carcass surfaces in two Australian abattoirs during chilling with concomitant collection of temperature data allows more accurate prediction of microbial growth and the ability to modify current chilling regimes to improve microbial status of beef carcasses.

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Contents

Common Abbreviations Used In This Thesis

a_w	water activity
CCP	critical control point (in HACCP)
CFU	colony forming unit
DFD	'dark, firm dry' (in relation to red meat)
EHEC	enterohaemorrhagic *Escherichia coli*
GT	generation time
HACCP	Hazard Analysis Critical Control Point system
HC	haemorrhagic colitis
HUS	haemolytic uraemic syndrome
MPN	most probable number
SAT	surface adipose tissue
SGS	skin-grafting scalpel
STEC	Shiga toxin-producing *Escherichia coli*
Stx	Shiga toxin
T	temperature
T_{max}	super-optimal temperature of a bacterium at which growth rate is predicted to be zero
T_{min}	sub-optimal temperature of a bacterium at which growth rate is predicted to be zero
T_{opt}	optimal growth temperature of a bacterium
TGI	temperature gradient incubator
TTI	time-temperature indicator

Publications

To date, the following publications have resulted fully, or in part, from work contained in this thesis:

Salter, M.A, Ross, T. and McMeekin, T.A (1998) Applicability of a model for non-pathogenic *Escherichia coli* for predicting the growth of pathogenic *Escherichia coli. Journal of Applied Microbiology,* (In Press).

McMeekin, T.A., Brown, J., Krist, K., Miles, D., Neumeyer, K., Nichols, D.S., Olley, J., Presser, K., Ratkowsky, D.A., Ross, T., Salter, M. and Soontranon, S. (1997) Quantitative microbiology: a basis for food safety. *Emerging Infectious Diseases,* **3:** 541-549.

Chapter 1. Literature Review

1.1 Introduction

Humanity has relied on domestic animals as a source of food and power throughout recorded history, and only until relatively recently in most societies has the former requirement been able to be performed by other means. In all societies, dietary requirements dictate that food intake is derived from both plant and animal groups. History has shown that societies consuming only one of these food groups have not advanced to the same extent as societies consuming both food groups. Societies dependent solely on livestock are forced into lives of nomadism, while those dependent on only plants have been deprived of optimal amounts of some essential nutrients.

Until several hundred years ago, improvement of livestock for human consumption only came about by an increase in total numbers of animals. The first successful selective breeding programme occurred in Britain about two hundred years ago. One hundred years later, the discoveries of Gregor Mendel relating to the genetics of peas lead to rapid development in the improvement of animal breeds by genetic means (Johansson, 1962).

Control of the hygienic quality of large-scale meat operations began in the nineteenth century, where the main objectives were to avoid the use of diseased animals or those considered unfit for human consumption, and generally to promote good hygiene within abattoirs. This strategy required the skill of a veterinarian to recognise disease in live animals and carcasses, and the construction of appropriately designed abattoirs with proper toilet facilities, water supply, waste removal and carcass storage areas. Along with programs designed to eliminate disease such as tuberculosis from large herds, the abattoir system at the time greatly reduced the incidence of traditional disease as well as restricting product spoilage (Gill, 1995). Today, despite advances in hygiene and abattoir design, spoilage of carcasses is still an inevitable

process (Bell, 1986) and the elimination of traditional pathogens has made way for the emergence of new disease-causing agents, mainly enteric pathogens. The economic impact of foodborne disease as a whole was estimated to have cost the US $8.4 billion in 1989 (Bawcom *et al.*, 1995), while for spoilage, estimates for the loss of product in Australia range up to 20% in a food industry thought to be worth $24 billion (McMeekin *et al.*, 1993). Therefore, reducing the extent of bacterial contamination and proliferation and thereby increasing the shelf life and lowering the risk of pathogenic contamination makes sense both in terms of economics and human health.

1.2 Beef production

1.2.1 The structure of meat

Meat can be defined as the edible part of the skeletal muscle of an animal that was healthy at the time of slaughter (Lambert *et al.*, 1991). It comprises four major components: water (~75%), protein (~19%), lipid (~2.5%) and carbohydrate and other minor components such as vitamins, enzymes, pigments and flavour compounds (~2.3%) (Lawrie, 1975).

When considered as potential substrates for microbial growth, the components of meat can be divided into three categories. The first group, proteins and fats, must be degraded before being utilised by bacteria. The second group comprises low molecular weight, soluble nitrogenous compounds (*eg.* creatine and nucleotides) which are generated from precursor molecules during *rigor* (the phenomenon which occurs in muscle when ATP is exhausted and Ca^{2+} cannot be transported back into the sarcoplasmic reticulum, an irreversible process which causes muscle to lose nearly all of its stretch characteristics (Bendall, 1973)). The third group is derived from muscle glycogen during the onset of *rigor,* and mainly comprises lactic acid. When insufficient lactic acid is produced in muscle due to a depletion of glycogen (*eg.* due to exercise or stress before slaughter), muscle pH remains relatively high resulting in tissue commonly referred to as 'dark, firm, dry' (DFD) (Gill, 1983). The composition

of lean muscle tissue varies slightly from species to species, but the amount of protein is relatively constant at 20 - 22%. The concentration of water can vary widely (*eg.* mackerel, 64%; beef, 71 - 73%; cod, 81%), as can fat (cod, 0.7%; beef, 5 - 8%; mackerel, 14%) (Lambert *et al.*, 1991).

1.2.2 The beef slaughtering process

Slaughtering and processing of beef is only allowed in facilities complying with certain regulations, but international hygienic requirements are quite often inconsistent.

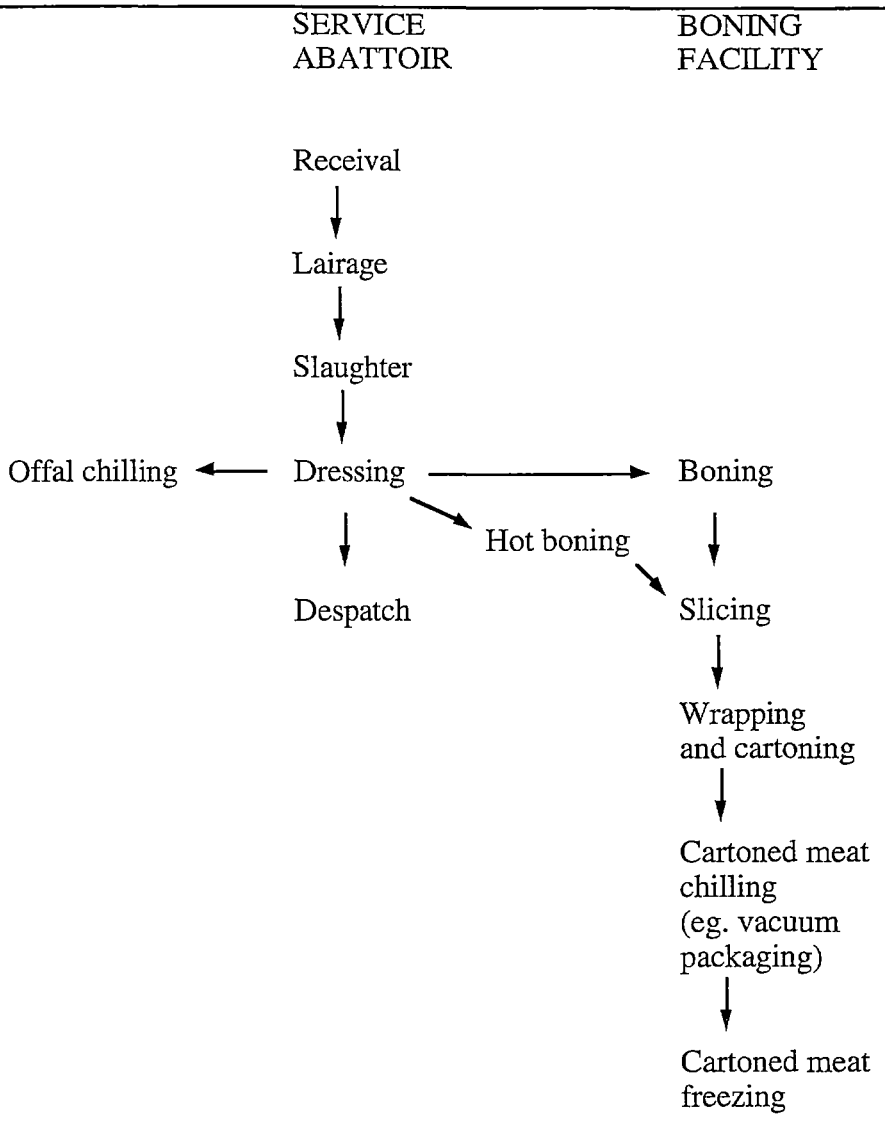
Before animals are slaughtered, they must be given an optimum resting period. The amount of rest will depend on factors such as prevailing climate, distance travelled, method of transport and general health. Exhaustion can result in poor exsanguination (bleeding), increased entry of bacteria into the bloodstream from the gut and DFD meat.

The process by which beef cattle are slaughtered and converted to meat is outlined in Figure 1.1.

The method of stunning used must not be cruel to the animal, nor should it cause excessive damage to the carcass. Stunning is usually carried out by bullet or captive-bolt pistol, or by impact by a 'knocker', following which the animal is killed by exsanguination. Exsanguination is effected by 'sticking', whereby a long knife is used to puncture the major blood vessels at the base of the neck, not far from the heart.

Dressing of the carcass varies from location to location, but can be summarised in general terms. The carcass is suspended on an overhead rail enabling removal of the distal parts of the hindlimbs. The head and distal parts of the forelimbs are removed, following which, the hide is removed, usually by an automated hide puller. The thoracic cavity and abdomen are opened and any loose fat cut away. Next, the pelvic girdle is split and the anus and oesophagus are tied off. The gastrointestinal tract is removed, along with the 'plucks' (heart, lungs and trachea). By sawing through the vertebral column, the left and right sides of the carcass are separated, after which the carcass is measured for 'hot weight'. The carcass is washed, and pinned and shrouded

Figure 1.1 Conversion of beef cattle to meat (Eustace, 1981).



over to smooth the sub-cutaneous fat (Gill *et al.*, 1996a).

At this stage, chilling can be achieved using either cold air or water. Using freezing air blasts, the rate of cooling is determined by temperature, relative humidity and air velocity in the meat cooler. Heat is lost in the usual way by conduction, convection and radiation, but can also be by evaporative heat loss as water is lost from the surface. Evaporation will be greater for carcasses with a high volume:surface ratio (*ie*. smaller carcasses) and if there is only a thin covering of sub-cutaneous fat. Initial

chilling is achieved using temperatures as low, and fan speeds as high, as possible without causing excessive carcass weight loss. Once carcass surface temperature and air temperature are comparable, air speed must be reduced to avoid desiccation (Lawrie, 1991). At this point, carcasses can be stored at just above 0°C at a relative humidity of 90% with some slight air movement. Higher humidities will reduce evaporative losses, but will encourage microbial proliferation (Swatland, 1984). An example weekend cooling pattern in an Australian abattoir sees fan speeds set at 110% (~1m/sec) at 8.5°C for the first 8h of chilling, 60% at 9.5°C for the following 12h and 40% at 9.5°C for the remaining time (McPhail, pers. comm.). An overnight chilling pattern will be structured in the same way, but achieves and maintains a low deep-butt temperature over a shorter period of time (for more on temperature monitoring and control, see Sections 1.3.3.1 and 1.3.3.2). If meat is frozen before the onset of *rigor mortis*, 'thaw shortening' will occur, which results in very tough meat. Also, if cooling is too rapid (even if *rigor mortis* has occurred), 'cold shortening' occurs and again, the meat will become tough by the time it reaches the consumer. It is a general rule that carcasses should not be subjected to air less than 5°C with a velocity over 1m/sec within 24h after slaughter (Cutting, 1974). Cold shortening can be prevented by electrical stimulation of carcasses, a process which accelerates the normal decline in muscle pH. Used in countries such as the US, New Zealand, Great Britain and Sweden, electrical stimulation can also lead to improved meat tenderness, colour and appearance (Swatland, 1984). In a typical spray-chilling process, the method of choice in North America, beef sides are cooled overnight and then graded on a sales floor which is maintained at 2 - 4°C. Most sides are moved to a cutting area on the same day that they exit the chiller, but can remain on the sales floor for up to three days if they are chilled immediately before a non-working day (Gill and Jones, 1992b).

After the carcass has been chilled to below a specified surface temperature within a certain time (below 7°C within 24h of stunning in Australian abattoirs (Agriculture and Resource Management Council of Australia and New Zealand (ARMCANZ), 1997)), below a specified deep temperature (below a temperature of

10°C in a Canadian abattoir studied by Gill and Jones (1992b)) or has fallen to a certain deep temperature within a specified time (US Department of Agriculture, 1970), it is cut into pieces in the boning room and packaged into cartons. These are stored for as little time as possible at between 0 and 2°C before being transported to wholesalers and retailers (Gill and Jones, 1992b).

Meat can be 'hot boned' from the carcass while it is still warm, although it is more difficult to handle. This method is desirable when there is limited chilling space and also when drip-loss from carcasses needs to be reduced (Swatland, 1984). In a New Zealand operation investigated by Reichel *et al.* (1991), beef sides were moved to a chilled area (below 10°C) immediately after dressing and splitting, spending no longer than 30 minutes on this floor before moving to the boning room. Sides were then broken down immediately and packed into cartons, which were placed in a freezing store operating at -18°C. The freezer was filled in about 4h and cooling the product to ~-20°C occurred within about 48h.

There are both advantages and disadvantages of hot-boning. Firstly, immediate chilling after slaughtering and dressing requires handling of large and bulky carcasses and a system of overhead rails and slides. Chillers therefore need to have large doors which release cold air when open. Only about 5% of the volume that a carcass takes up in a chiller is converted into saleable meat, and weight losses of 2 - 4% result from evaporation. Boning must then take place at less than 10°C.

Using hot-boning, an animal can be processed in one day, reducing or even eliminating the need to chill between dressing and boning. However, hot boning can result in tough meat because the excised muscles can shorten markedly during the onset of *rigor* because they are free from their skeletal attachments. Also, hot-boning exposes a large surface-area of warm, moist meat to the environment, so subsequent cooling must be efficient in order to reduce the risk of growth of pathogenic bacteria (Williams, 1978).

1.3 Bacterial growth on beef

1.3.1 Contamination of beef

Beef carcasses can become contaminated from various sources during the slaughtering and dressing processes. Bacteria can be derived from the animal itself (for example, from gut contents, hide and faeces); from abattoir workers (hands, clothing and instruments of personnel); and from contact surfaces, such as walls, floors and drains in dressing areas, from walls and floors in chillers, and from cutting boards and tables in boning rooms (Eustace, 1981). Contaminating microorganisms can be classed as either spoilage or pathogenic. Spoilage organisms are those which produce off-odours or flavours and are primarily responsible for quality losses to the food industry (*eg*. pseudomonads, *Brocothrix thermosphacta, Acinetobacter/Moraxella*), while pathogenic organisms are those which cause disease and therefore are of public health significance (*eg*. *Salmonella*, Shiga toxin-producing *E. coli, Campylobacter jejuni*) (Venkitanarayanan *et al.*, 1996).

Typical contaminating microorganisms include micrococci, staphylococci, *Bacillus* spp., coryneforms, Enterobacteriaceae, flavobacteria, pseudomonads, lactic acid bacteria and *Brocothrix thermosphacta* (Dainty and Mackey, 1992). The dominant bacteria on aerobically cold-stored meat are usually *Pseudomonas* spp., which along with other Gram-negative, non-fermentative rods found on beef are the predominating species of surface waters in the environment (Mossel *et al.*, 1975). Lactic acid bacteria normally dominate in vacuum and modified atmosphere packages, which have elevated carbon dioxide levels (Gustavvson and Borch, 1993).

A number of studies have attempted to map the distribution of numbers and types of bacteria over the carcass surface, with varying results. A survey of slaughter operations in Norway showed that the brisket, forerib, flank, groin and round sites (see Figure 1.2 for carcass site locations) consistently had higher total counts than other sites (Dickson and Anderson, 1992). The brisket was found to have the highest total count in six abattoirs in Alberta, Canada (Jericho *et al.*, 1994), while the hindshank

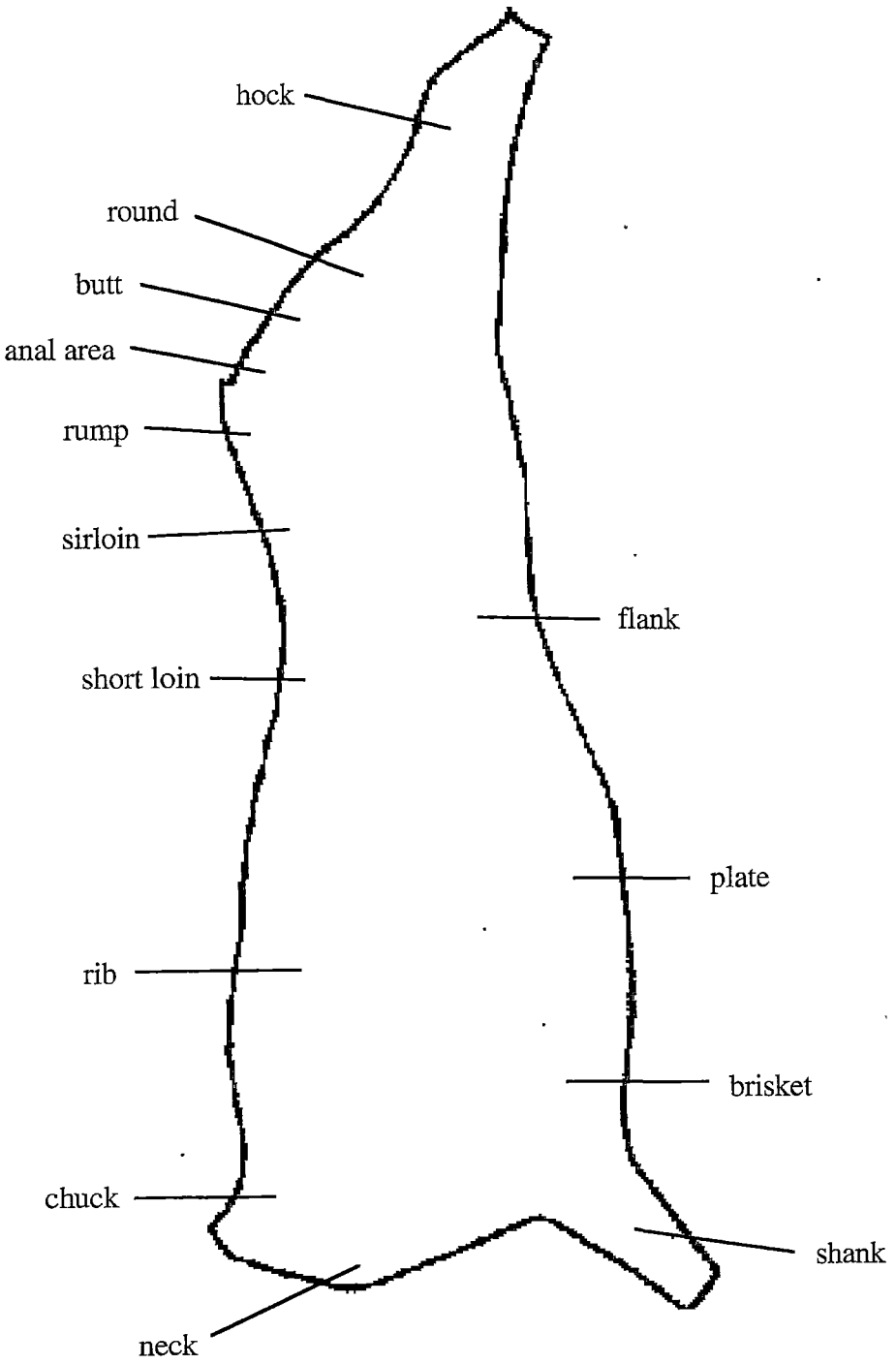
was found to have the highest and the neck the lowest counts in a study at the Kansas State University abattoir (Kenney *et al.*, 1995). Bell (1997) concluded from a study of three different dressing systems in NZ that the highest counts arose at those sites associated with opening cuts and/or those subject to hide contact during hide removal.

With respect to specific bacterial types, Gill *et al.* (1996b) reported that the hock, anal area and rump sites had the highest counts of *Escherichia coli* after skinning. After splitting, *E. coli* contamination was highest at the butt, anal area and rump sites. After trimming and washing, contamination with *E. coli* was relatively moderate compared to the previous sampling, but again was highest at the butt, anal area and rump sites. It was also observed that *E. coli* counts reflected total coliform counts, but not total aerobic counts. In another study (Gill *et al.*, 1996a), the rump site had the highest *E. coli* count (>log 4.0 CFU/cm^2) after skinning compared to the neck and brisket, while after washing, the brisket had the highest count (log 2.7 CFU/cm^2). Total aerobic counts were somewhat different. After skinning, the highest count occurred at the neck (up to log 4.9 CFU/cm^2), while after washing, the highest total count occurred at the brisket (up to log 3.9 CFU/cm^2).

1.3.2 Modes of tissue breakdown

Breakdown of meat occurs at the surface where the accumulation of a liquid film provides a nutrient-rich region. Nutrients diffuse from the tissue into the external film giving rise to an excess supply for microbial growth. If the surface is extensively colonised, attached bacteria will use nutrients before they can reach the liquid film and these nutrients will become limiting. Therefore, two distinct microenvironments will form: one at or very close to the surface where adherent bacteria utilise nutrients diffusing from the underlying tissue; and another in the liquid film colonised by free-floating microorganisms. If this model is correct, nutrient levels in the liquid film will be diminished relatively quickly, explaining the observation that attachment is reduced after a total cell density of 10^5-10^6 CFU/cm^2 is reached (Delaquis and McCurdy, 1990).

Figure 1.2 Regions of the beef carcass (Adapted from Swatland (1984) and Gill *et al.*, (1996a)).



Bacteria growing on meat utilise low molecular weight soluble compounds. Since the rate of degradation of preferred nutrients such as glucose is dependent on their rate of diffusion from underlying tissue, levels of substrate may not be sufficient to meet the demand of resident bacteria and so secondary substrates may be degraded even though high concentrations of the preferred substrate may be still present in the deep tissue. Degradation of nutrients can occur by one of two pathways. Growth by oxidative metabolism has been found to give a final population of ~10^8CFU/cm^2. The final population can reach 10^9CFU/cm^2 and is only limited by rate of diffusion of oxygen into the slime layer. When growth occurs via fermentative metabolism the energy yield is only 10% of that by oxidative means and so only supports 10^7CFU/cm^2. Rate of growth is the same on both muscle and fat tissue and occurs at the expense of the same low molecular weight compounds. However, because the concentration of nutrients is lower in fat tissue, the final population will be lower than on muscle tissue (Gill, 1983).

The physical manifestation of spoilage is different depending on whether metabolism is via oxidative or fermentative pathways. Oxidative or aerobic spoilage is marked by the appearance of slime on the meat surface, which occurs due to the coalescing of bacterial colonies. Discolouration of the meat follows, caused by destruction of meat pigments (*eg.* oxidation of myoglobin to brown metmyoglobin, production of sulphmyoglobin or production of pigment by spoilage microorganisms themselves). Off-odours can also be produced, as can deposits of fat. If spoilage is by fermentative or anaerobic means, characteristics such as foul odours, gas production and souring become evident (Lawrie, 1991).

The onset of spoilage, dominated by the pseudomonads, is marked by attack of glucose. Pseudomonads dominate because of their relatively high growth rate, resulting in a shelf-life of only several days (Gill and Molin, 1991). Of these organisms, *Pseudomonas fragi* is the most successful coloniser due to its ability to use creatine and creatinine (Drosinos and Board, 1994). When cell numbers reach 10^8CFU/cm^2, levels of glucose are either completely depleted or rate of diffusion from

underlying tissue is insufficient to meet demand. The substrate of choice then switches to amino acids, which in turn leads to release of ammonia and a rise in meat pH. (Gill, 1983). The initial metabolism of glucose does not result in production of offensive odours or flavours, but degradation of amino acids leads to the release of malodorous metabolites such as sulfides, esters and acids. The *Acinetobacter/Moraxella* group of microorganisms is also a major part of the aerobic spoilage population and utilises amino acids in preference to glucose but does not produce malodorous by-products and so is of low spoilage potential. However, the growth of this group of bacteria lowers O_2 tension and so enhances the spoilage potential of the pseudomonads and *Shewanella putrefaciens* because these organisms metabolise amino acids even if glucose is present (Lambert *et al.*, 1991).

On DFD meat, pseudomonads are still the dominant spoilage organisms but often degrade amino acids immediately because glucose is absent. As a result, spoilage is detected when cell numbers are in excess of $10^6/cm^2$. In general, the group of microorganisms dominating spoilage will be those with the highest initial numbers but will ultimately be those with the fastest growth rate (Lambert *et al.*, 1991).

1.3.3 Effect of temperature on contaminants

Temperature is probably the most important environmental factor controlling the growth of bacteria on meat. Generally, a finite increase in temperature gives rise to an increase in bacterial growth rate. However, in a mixed bacterial population, the effect will also to be to change the predominating species. On meat, pseudomonads dominate at temperatures up to 20°C, while *Acinetobacter* and Enterobacteriaceae dominate at 30°C (Lambert *et al.*, 1991). This change is brought about by selection of the microorganism with the fastest relative growth rate. The results of Gill and Newton (1977) (Table 1.1) show that pseudomonads have the highest growth rates at chill temperatures on aerobically stored meat and therefore, they will dominate the bacterial population. Their superior growth rate is thought to stem from their ability to metabolise glucose to a form which is not readily utilised by other bacteria. However,

they still predominate when glucose is not limiting and so their dominance is not entirely explained (Dainty and Mackey, 1992). Meat spoilage organisms can be classified as psychrotrophs, mesophiles or thermophiles, which have temperature optima >15, 10 to 40°C and 43 to 66°C, respectively. Another example of temperature affecting the microbiological population on meat occurs in tropical areas. Here, the soil microbiota is dominated by mesophiles, and therefore beef produced in these areas is contaminated mostly by mesophilic bacteria and will keep for longer at chill temperatures than beef produced in temperate regions (Lawrie, 1991).

Table 1.1 Generation times (h) of psychrotrophic bacteria growing aerobically on meat (Gill and Newton, 1977).

	Temperature (°C)			
Isolate	2	5	10	15
*Pseudomonas**	8.2	5.4	3.0	2.0
Pseudomonas†	7.6	5.1	2.8	2.0
Enterobacter	11.1	7.8	3.5	2.4
Acinetobacter	15.6	8.9	5.2	3.1
B. thermosphacta	12.0	7.3	3.4	2.8

*flourescent; †non-fluorescent.

Cessation of growth at the lower temperature limits on meat occurs at -12°C in the absence of liquid water, however growth can occur as low as -20°C in super-cooled liquid. At the upper temperature limits, growth of microorganisms is possible at temperatures far higher than usually experienced on meat, except in the case of certain decontamination procedures (see Section 1.3.6.3) where surface temperature may be raised using hot water or steam treatments. The recorded maximum temperature for growth of microorganisms was in the range 93.5 to 95.5°C in 1970 and as high as 105°C in 1982 (White, 1984). In circumstances where growth is not possible, survival occurs over a much wider range of temperature. For example, microorganisms have

been to found to remain viable for 10h at -252°C and as spores for approximately 2h at 200°C in the dry state (Lawrie, 1991).

Upon entry to the chiller, carcasses have surface temperatures of 25 to 30°C and deep-body temperatures of 38 to 40°C (Eustace, 1981). Because temperature conditions on the carcass surface are close to optimal for psychrotrophs and mesophiles, carcasses must be chilled as quickly as possible, but without causing 'cold shortening'. Once deep-butt temperatures are below 10°C, only 10% of the initial bacterial population can grow. Of this population, only a small fraction are spoilage organisms (Borch *et al.*, 1996). If the temperature of meat is further reduced and frozen, bacterial growth is negligible, but yeasts and moulds will continue to grow to approximately -5°C (Lowry and Gill, 1984).

1.3.3.1 Measurement of temperature

Monitoring of temperature during the slaughtering and dressing processes is rarely carried out in abattoirs and in Australian abattoirs is not carried out at all. Temperature monitoring only begins at the chilling stage and occurs to different extents depending on location. Australian domestic plants measure chiller temperature and only in some cases record temperature histories, while export plants are required to record temperature histories and do so using either continuous multi-pen chart recorders or computerised logging systems. Some plants also record carcass deep-butt and surface temperatures. Most export plants measure deep-butt temperature at the completion of chilling for a selection of carcasses in the chiller. They are required to achieve a deep-butt temperature of 20°C or below within 20h of entry to the chiller, while domestic plants are required to check that the surface temperature is at 7°C or below prior to dispatch (McPhail, pers. comm.). Gill (1987) noted that air temperature monitored during chilling is only useful if its relationship is defined with respect to product temperature. Because gradients of air temperature and flow occur within chillers, air and product temperature must be measured at various points in a loaded

chiller and their relationship defined. Chiller operations can then be modified to give the best possible product temperature.

Collection of temperature histories during meat production can be used to determine the equivalent growth of spoilage or pathogenic bacteria in a procedure termed 'temperature function integration' (see Section 1.5.1). This technique has been suggested for use during conventional cooling processes (Gill *et al.*, 1991a), spray chilling processes (Gill *et al.*, 1991b), hot boning (Reichel *et al.*, 1991), cooling of pig carcasses (Gill and Jones, 1992a) and cooling of beef offals (Gill and Jones, 1992b).

There is no mention in the literature of routine measurement of water activity of carcasses.

1.3.3.2 Devices for measurement of temperature

As mentioned in Section 1.3.3.1, the only point at which temperature is routinely monitored in the fabrication of beef carcasses is at the chilling stage. At this and earlier stages during processing, temperature can be measured and recorded using a number of commercially available physico-chemical or electronic devices. Some of these also interpret temperature history as equivalent bacterial growth. Examples of both have been described previously (McMeekin *et al.*, 1993; Ross and McMeekin, 1995) and so will not be dealt with in detail here.

The development of physico-chemical time-temperature indicators (TTIs) has received particular attention by Labuza and colleagues at the University of Minnesota (Taoukis and Labuza, 1989a, b; Fu *et al.*, 1991; Labuza and Taoukis, 1991; Sherlock *et al.*, 1991; Taoukis *et al.*, 1991). TTIs are based on physical of chemical processes such as migration of a coloured dye through a porous medium or reaction of an enzyme and substrate to give a colour change. They are small, inexpensive devices and as such, can be attached to individual units of food. They are classed as 'full history' monitors if they respond continuously to a time-temperature history, or 'partial history' monitors if they respond only after a certain time-temperature threshold has been exceeded. McMeekin *et al.* (1993) criticised the fact that most TTIs designed to

simulate bacterial growth are based on Arrhenius kinetics when bacterial growth is better described by Bělehrádek kinetics (see Section 1.5 for more detail).

There are many commercially available electronic devices designed to monitor temperature history and in some cases, interpret this as equivalent bacterial growth. All comprise essentially the same components, namely a robust case containing the electronic unit responsible for data storage and a temperature sensor mounted inside a protective probe. Ross and McMeekin (1995) listed 15 examples of commercially available devices, of which three interpreted temperature history as bacterial growth. Other functions varied greatly depending on intended use and gave rise to a range of unit prices from $A25 to $A2000. Measurable temperature range varied from as little as 60°C to as much as 2000°C, number of channels varied from one to 50 and total record capacity varied from 1800 to 300,000. In some cases, data was expressed as a readout on the device, while in others data had to be downloaded onto a computer. Battery life varied from several days to approximately 10 years. Despite their high relative cost compared to physico-chemical TTIs, electronic devices may be more useful to the meat industry as a recorded temperature history can be interpreted in terms of growth of any bacterial type as long as information (*ie.* a mathematical model) exists to do so.

1.3.4 Effect of water activity on contaminants

After temperature, availability of water is perhaps the most important requirement for growth of microorganisms on meat (Lawrie, 1991). A measure of water availability, water activity (a_w), of an aqueous solution is defined as the ratio of its vapour pressure with that of pure water at the same temperature and is expressed as a value from zero to one. Water activity is inversely proportional to the number of solute molecules in a particular compound and is not greatly affected by temperature (Scott, 1953).

In the same way that temperature affects bacterial growth rate, reducing water activity causes a concomitant decrease in growth rate. Once a critical low ater activity

is reached, growth ceases. This is evident in the data of Scott (1953) (Table 1.2) where 14 strains of *Staphylococcus aureus* were grown in a medium adjusted to desired water activity levels using a mixture of NaCl, KCl and Na_2SO_4. The fastest growth rates were observed at water activity values of 0.995 and 0.990, with a steady decline in growth rate as water activity was decreased. Above 0.995, slight inhibiton of growth rate may occcur due to dilution of nutrients. Troller (1987) also noted that addition of sub-inhibitory levels of a humectant extended duration of the lag phase.

The water activity limits for growth vary for different bacterial groups, with most non-halophilic species having optimal growth rates in the region 0.997 to 0.980. Gram-negative rods are the most sensitive to reduced water activity, having observed growth minima in laboratory media of 0.96 to 0.94. Gram positive rods such as *Clostridium* spp. have minima of 0.95 to 0.94, while for *Bacillus* spp. they are 0.93 to 0.90.

Table 1.2 Relationship between growth rate and Gram positive for 14 strains of *Staphylococcus aureus* (Scott, 1953).

a_w	Growth rate (divisions/h)
1.000	1.12
0.995	1.30
0.990	1.30
0.982	1.15
0.971	1.10
0.960	0.97
0.940	0.66
0.920	0.43
0.900	0.15

Gram positive cocci have a wider range of minimal growth water activities of 0.95 to 0.83. Halophilic bacteria, those able to grow in environments of relatively low water

activity, are characterised by an inability to grow in substrates approaching a water activity of 1.00. They are classed as either moderate or extreme halophiles and have observed growth minima of 0.85 and 0.75, respectively (Troller and Christian, 1978). Similar to the response shown to temperature, bacteria can survive at water activities lower than those permitting growth. For example, *E. coli* can grow at a water activity as low as 0.95, but a water activity of 0.53, regardless of temperature, is required to cause cell death. Death at this point is thought to be caused by breakage of DNA strands. Protection of bacterial cells at very low water activity is afforded by polyols such as glycerol, an effect exploited by common culture storage procedures (Hahn-Hägerdal, 1986).

A *caveat* must be issued when describing water activity minima for microorganisms as there exists a recognised solute effect. Chirife and Buera (1996) exemplified this using data for a number of bacteria grown in laboratory media with water activity adjusted using NaCl and glycerol. In most cases, minimum observed water activity in media containing NaCl was higher than that observed in media with glycerol (*eg.* 0.966 and 0.943 for *Clostridium botulinum* E; 0.949 and 0.940 for *E. coli*). However, values for *S. aureus* were lower in NaCl than in glycerol, at 0.860 and 0.890, respectively.

Physiologically, exposure of a bacterial cell to a low water activity substrate initially causes loss of water to the outside environment by osmosis, resulting in cell dormancy or death by plasmolysis. In a process thought to be mediated by accumulation of intracellular K^+, halophiles synthesise compounds termed 'compatible solutes' (Brown and Simpson, 1972) to reduce intracellular water activity. Examples of bacterial compatible solutes are glycylbetaine, proline and γ-aminobutyric acid and were so named because even at very high concentrations they do not interfere with the metabolic and reproductive functions of the cell (Troller, 1987).

The water activity of fresh meat is normally about 0.99 and so does not limit the growth of most spoilage and pathogenic microorganisms (Scott, 1957). Therefore, drying of meat as soon as possible after slaughter assists in the prevention of growth of

contaminants. Scott and Vickery (1939) illustrated this point in a simple experiment where bacterial growth was observed on lean meat in a commercial chiller. The fastest growth rate was seen when a portion of the meat was covered, reducing the rate of drying and giving a final log population density after 24h of 5.5. On a portion uncovered after 10h, growth rate immediately slowed and on a region uncovered for the duration of the experiment, log population density never exceeded the initial level of 3.0.

1.3.4.1 Devices for measurement of water activity

Although there is no routine measurement of water activity during the fabrication of beef carcasses and no reported method for application of existing technology to do so, there exist a number of commercially available devices able to measure water activity. Different methods for water activity determination and devices employing these principles have been described previously (Labuza *et al.*, 1976; Troller, 1983; Stamp *et al.*, 1984). Methods include bithermal equilibrium; vapour pressure manometry; hair, isopiestic and electrical hygrometry; psychrometry; freezing-point depression and dew point. These authors concluded that most methods available were unsatisfactory in more than one way, with recurrent problems being that instruments were very cumbersome or fragile, oversensitive to temperature fluctuations, insensitive in general, unable to measure water activity over a wide range and were overly expensive. In most cases, the procedure involved was relatively lengthy, sometimes requiring several days. More recently, improvements in technology led McMeekin *at al.* (1993) to report that a number of methods have been successfully used, of which the chilled-mirror dew point technique is the most successful. This type of instrument contains a stainless-steel mirror, housed inside the sample chamber, which is continuously heated and cooled. Each time water vapour condenses on the mirror, sample temperature and water activity are measured. A final reading is given when consecutive water activity measurements are within a specified range. This technique is a primary measurement method and as such, has greater

accuracy and precision than methods employing water activity transducers such as hygristors and capacitance relative-humidity sensors. In the dew-point method, temperature of the sample and sensor do not need to be the same, whereas they must be using transducers . An instrument exploiting this principle takes 3-5 minutes to give a water activity reading, which represents a vast improvement over earlier devices (Roa and Tapia de Daza, 1991).

1.3.5 Effect of temperature and water activity combinations

In general, microorganisms tolerate low water activity best at temperatures close to optimal for growth. For example, for *Clostridium botulinum* type B, with an optimum growth temperature of 37-40°C at high a_w, growth occurred at decreasing water activity as temperature was increased from 20 to 40°C (Ohye and Christian, 1967). The same response was shown by Wodzinski and Frazier (1960) for *Pseudomonas fluorescens*, *Enterobacter aerogenes* and *Lactobacillus viridescens*. Leistner and Rodel (1976) termed the inhibition of growth by different environmental parameters the 'hurdle concept', and suggested that successive hurdles could either act synergistically or independently. Davey (1989) concluded that temperature and water activity acted independently of one another. McMeekin *et al.* (1993) noted these trends but cautioned that discrete values for the growth limits of bacteria were unique to the conditions under consideration and so were of limited value. They cited Troller (1987), who stated that laboratory estimates for the minimum a_w for growth of *S. aureus* were from 0.83 to 0.86, but that the 'casual' observer would find it difficult to obtain growth below 0.89 and in many foods below 0.90 to 0.91. Based on Troller's argument, they added that is it better to take into consideration the factors which impinge on microbial growth rather than merely taking note of minimal values for growth. This concept is termed 'predictive microbiology' and is discussed in Section 1.5.

1.3.6 Decontamination strategies and hygiene control

Since the early requirements of nineteenth century meat production hygiene were implemented, a number of newer strategies have been developed. While some have gained wide use within commercial operations, research continues into improvement of general hygiene and carcass decontamination practice.

1.3.6.1 Pre-chilling hygiene practice

Some authors report that initial washing of live animals before slaughter reduces subsequent bacterial contamination (Empey and Scott, 1939), while others disagree unless there is gross soiling of the hide (Roberts, 1980). During and immediately after slaughter, cutting equipment is immersed in or sprayed with water at 82°C in between use with each carcass (Schutz, 1991). This procedure is intended as a pasteurisation step, but immersion or spraying would have to last for about 10s to be effective and is commonly never this thorough (Smith, 1992). Trimming of carcasses is often assumed to remove visible as well as microbiological contamination, but rarely achieves the latter and varies in efficacy depending on the skill of the individual performing the trimming (Reagan *et al.*, 1996). Washing of larger equipment and walls in the dressing area is often done using hot water, but pasteurisation is rarely achieved and bacteria often remain attached to surfaces despite removal of visible soiling. Sammarco *et al.* (1997) suggested that liquid sanitisers which run off equipment and walls directly onto the floor are not as efficient at removing pathogens as sanitisers such as foams which adhere to surfaces.

Rahkio and Korkeala (1997) found an association between airborne bacteria and carcass contamination. Their results indicated that the slaughter line should be designed to prevent air flow from highly contaminated areas to cleaner areas, and also to prevent the movement of workers from areas of high to low contamination.

Inspection occurs after removal of the hide and aims to examine the viscera and carcass for symptoms of disease, and the carcass for visible contamination with hair, dirt, faeces and ingesta (Food and Agricultural Organization of the United

Nations/World Health Organization, 1983). Areas of the carcass soiled with such materials are excised, but it has been demonstrated that soiled areas and those with high bacterial loads do not correlate (Jericho *et al.*, 1993). The same misconception occurs for bruised areas of the carcass as these areas have been shown to have comparable microbiological counts with unbruised areas (Gill, 1995).

Before entering the chiller, carcasses are commonly sprayed with hot or cold water at specified intervals along the dressing line. This serves to wash away visible soiling and in the case of warm water, lowers the temperature of the carcass by evaporative cooling. Achieved most often using a hand-held nozzle, the washing process has been shown to miss certain areas of the carcass and can merely move foreign material from one area on the carcass to another (Dickson and Anderson, 1992). Its effectiveness is also dependent on the temperature of the spray water, spray pressure, volume of water used and the rate of travel of carcasses through the spray (Anderson *et al.*, 1975).

1.3.6.2 Post-chilling hygiene practice

Hygiene control in the chiller is limited to the effects of low temperature air blasting, which cause a decrease in carcass temperature and some surface drying (see Section 1.2.2 for details of the chilling process). With large carcasses such as beef, both temperature reduction and surface drying are hindered if sides are too close or touching in the chiller and/or if spraying with water has taken place immediately before or just after loading of the chiller. In the case of spraying, which occurs during the first few hours of chilling in some parts of North America, (Gill, 1995) there exists a balance between reduction of carcass soiling and the need to keep the surface dry. In many cases, chilling is actually designed to minimise water loss from carcasses because a reduction in weight leads to economic loss (Jones and Robertson, 1988). When there is minimal drying of carcasses during chilling, the only hurdle to microbial growth is low temperature, and as mentioned, achieving low temperature can often be inefficient if chillers are over-loaded. The logistics of loading a chiller can also mean

that the last carcasses to arrive may receive several hours less refrigeration than those loaded first (Gill and Jones, 1992a) and these may also experience elevated air temperatures due to the number of warm sides already present. For these reasons, different carcasses may experience varied and sometimes inadequate rates of chilling (Gill, 1995).

1.3.6.3 Recent developments in hygiene control

From the discussion in Sections 1.3.6.1 and 1.3.6.2, it can be seen that current hygiene control in abattoirs can be improved at many stages in the beef production process. Recent developments into slaughter-line hygiene and decontamination of carcasses have already been extensively reviewed (*eg.* Dickson and Anderson, 1992; Lambert *et al.*, 1991; Smulders, 1995) and so the following are only given as examples.

Investigations into decontamination have focused on a number of different steps in the beef production process. With regard to the slaughtering and initial dressing procedures, Mackey and Derrick (1979) demonstrated that the slaughter instruments could contaminate deep tissues of the carcass with marker organisms and that abattoir workers themselves were a source of contamination. However, when instruments and workers were eliminated in an automated system, overall bacterial load on carcasses was the same but differently distributed over the surface (Whelehan *et al.*, 1986). Trimming of fat from carcasses has been found to reduce bacterial numbers in some studies (Prasai *et al.*, 1995a), but in others there was no significant reduction (Prasai *et al.*, 1995b). A reduction in total count by 1.9-log CFU/cm^2 was observed by Reagan *et al.* (1996) after a combination of trimming and washing.

Studies involving carcass washing with water have experimented with varying water pressure and temperature. For example, Gorman *et al.* (1995a) found that higher warm water pressures were more effective than lower pressures in reducing faecal contamination and bacterial counts on carcass adipose tissue, while Gorman *et al.* (1995b) observed higher total count reductions (3.0-log) using hot water than a

combination of warm water and hand-trimming (1.4-2.3-log). Dorsa *et al.* (1996) also confirmed the successful use of high-pressure warm water washes using a commercial carcass washer. Experimentation has also occurred with different methods of water dispensation. Attempts have been made to mechanise the spraying of water to avoid inefficient cleaning using a single, hand-held nozzle and resulted in the construction of wash cabinets of varying design employing banks of spray nozzles (Dickson and Anderson, 1992).

The effects of water in the form of steam were employed in a steam-vacuuming procedure which was found to be at least as effective as knife trimming in decontaminating carcasses with areas of visible contamination (Kochevar *et al.*, 1997). Steam used as a pasteurisation treatment, where the carcass surface instantaneously reaches 90.5°C, reduced total counts by 1.4-log and totally eliminated *E. coli* from a starting population density of 0.6-1.53-log (Nutsch *et al.*, 1997). Phebus *et al.* (1997) found that pasteurisation consistently provided greater pathogen reductions on beef carcasses than trimming or hot water/steam vacuum spot cleaning.

The use of organic acids in carcass washes has received widespread attention in the literature. Acid efficacy is thought to depend on the pH-lowering effect, extent of dissociation and the nature of the acid molecule. Antimicrobial activity varies among organic acids and in general is increased if pH and extent of dissociation are reduced (Smulders, 1995). Differences arise in antimicrobial activity due to differences in pK_a. For example, more that 50% of propionic acid molecules will exist in the undissociated form below a pH of 4.86 ($pK_a = 4.86$), whereas a pH of 3.75 is required in order to have more than 50% of formic acid ($pK_a = 3.75$) molecules in the antimicrobial form. Choice of organic acids based on their pK_a has been exploited in the fish silage industry but has not been reported for use in the decontamination of meat carcasses (Raa and Gildberg, 1982).

Hardin *et al.* (1995) demonstrated that washing carcasses with cold water followed by spraying with warm 2% acetic or lactic acid resulted in greater reductions of pathogenic bacteria than washing and trimming alone. Comparing lactic acid,

water, and chlorine treatments, Kenney *et al.* (1995) showed that 3% lactic acid sprays after rail inspection and again after an 8h spray-chilling cycle best reduced carcass contamination. Some authors have warned that acid treatment pre-adapts bacteria to subsequent acid challenges (Foster and Hall, 1990; Leyer and Johnson, 1992), but Dickson and Anderson (1995) found that salmonellae attached to lean beef tissue and pre-adapted to lactic acid were just as sensitive to subsequent lactic acid rinses as non-adapted cells.

Use of chlorine and other compounds as decontaminants has also been documented. Spraying with water was found to be just as effective as chlorine dioxide on fecally contaminated carcass tissue (Cutter and Dorsa, 1995). Other compounds gave better results, with small reductions in total counts reported after application of ozone and hydrogen peroxide (Reagan *et al.*, 1996), reduction of *Brocothrix thermosphacta* after treatment with nisin immobilised in a gel (Cutter and Siragusa, 1996) and inhibition of pathogenic bacteria after addition of sodium triphosphate (Dickson *et al.*, 1994).

1.3.6.4 Modern hygiene control - HACCP

Traditional quality assurance in beef production from slaughter to chilling involves inspection of carcasses and rejection or treatment of sub-standard product. This system can become wasteful if inspecton is overly strict or inefficient if line speeds are too high. Also, inspection can only maintain and not improve meat quality (Gill, 1995). A new system originated from the work of the Pillsbury Company, who in 1959 were required to manufacture food for use in zero gravity on space missions for The National Aeronautics and Space Agency (NASA). Apart from needing certain physical attributes, there needed to be as close as possible to 100% assurance that the food was free from pathogens and chemical toxins (Bauman, 1992). The new system, termed the Hazard Analysis Critical Control Point (HACCP) system, was described in terms of six steps by the International Commission on Microbiological Specifications for Foods (ICMSF, 1988). These can be summarised as:

1. Identification of hazards and assessment of severity of risk to the various stages of food manufacture and use.
2. Determination of critical control points (CCPs) where control can either be exercised to prevent a hazard (CCP1) or to minimise the risk of a hazard (CCP2).
3. Specification of criteria that serve to control an operation at a specified CCP.
4. Establishment and use of procedures to monitor each CCP to see that it is under control.
5. Taking corrective action if a CCP is not under control.
6. Verification of the efficacy of the HACCP programme.

Unlike traditional practices, HACCP provides a systematic approach to hazard identification, assessment and control. It focuses on those factors that directly affect the microbiological and toxicological safety of foods and can be used at all points in the food production chain.

CCPs in the meat production process have been putatively identified (ICMSF, 1988). They are animal production on the farm, skinning, evisceration, chilling and transportation to cutting plants. Of these, animal production, skinning and evisceration are major sources of contamination and are all CCP2s. Chilling, a minor source of contamination, is the only CCP1, while transportation is a CCP2 and a minor contamination source. Once identified, criteria are specified for each CCP and monitoring takes place to ensure that the specified standards are met during production. Control is achieved during skinning, for example, by pulling the hide downwards and making sure that the outside of the hide does not contact the exposed carcass surface. Control of other CCPs has been discussed elsewhere (ICMSF, 1988), but could also be effected using a decontamination step, many of which were discussed in Section 1.3.6.3. Despite the ICMSF's identification of CCPs, Gill (1995) warns that there is still much confusion in the development of HACCP systems for raw meat processing. This author suggests that microbiological data for assessing the risk of

each step in slaughter and dressing operations is generally lacking. Microbiological sampling should only take place after CCPs and not after every step, and should involve screening for total counts, coliform counts and *E. coli* as a marker of pathogenic bacterial growth (Gill, 1995).

Limitations of the HACCP system are that there is a need to educate non-professional food handlers, a need for acceptance of the system by food inspectors and the public and not only food processors, differences in opinion, even among experts, as to which processing steps are CCPs, and the possibility that HACCP may give the public false assurance about the quality of a product (Jay, 1996). Because of these limitations and due to the fact that risks can not always be quantified in meat processing operations, Berends *et al.* (1996) suggested using an HACCP-like approach which is more closely related to risk assessment.

1.4 Significance of *Escherichia coli* to beef production

1.4.1 *E. coli* as an indicator organism

The notion of using an indicator organism was postulated over 100 years ago after the discovery that the bacterium now known as *Escherichia coli* was present almost universally in human excreta. Since then, the indicator of choice shifted to coliforms and then to faecal coliforms, but the recent development of sensitive testing methods have meant that once more, *E. coli* is the most widely used indicator organism for non-pathogenic enterics in foods (Hartman *et al.*, 1986). As a result, the ICMSF (1978) dubbed *E. coli* "the classical indicator of the possible presence of enteric pathogens in water, shellfish, dairy products and other foods." Protocols devised for the isolation of *E. coli* include the most-probable-number (MPN) method, a fluorogenic method exploiting the ability of *E. coli* to hydrolyse glucuronide conjugates such as 4-methylumbelliferyl-ß-D-glucuronide (MUG), a membrane-filter method, various rapid confirmatory tests and plating methods using selective agars (Hartman *et al.*, 1986). The presence of *E. coli* in foods usually indicates direct or

indirect pollution of faecal origin, although it is not always indicative of the presence of salmonellae and other pathogens. It is advantageous to monitor the numbers of a mesophilic indicator during food processing when environmental conditions cannot be monitored. Monitoring also reveals steps in a process which give rise to significant increases in indicator numbers. This represents a tool able to be exploited in a HACCP plan (see Section 1.3.6.4).

Use of *E. coli* as an indicator organism with respect to meat processing was recommended by Harris and Stiles (1992) who noted that, unlike other Enterobacteriaceae, *E.coli* survives well on meats. It therefore indicates the sanitation exercised during the processing of meats better than previously used indicators such as coliform bacteria or total Enterobacteriaceae. Gill *et al.* (1996b), taking swab samples from the surface of beef carcasses during the dressing process reported that were was a good correlation between *E. coli* and coliform counts, but a weak correlation between *E. coli* and aerobic, 25°C counts. They suggested that assessment of the hygienic adequacy of stages in the dressing process (*eg.* for HACCP purposes) should be based on *E. coli* counts and possibly coliform counts, but not on aerobic, 25°C counts.

1.4.2 Shiga toxin-producing *E. coli*

It was demonstrated in the previous section that the generic *E. coli* species is a useful indicator of enteric pathogens on meat. A group of enteric pathogens of particular concern to the meat industry in recent years, also a sub-class of *E. coli*, are the Shiga toxin-producing *E. coli* (STEC). This group, belonging to the enterohaemorrhagic *E. coli* (EHEC), was formerly known as the verotoxigenic (VTEC) or Shiga-like toxin-producing (SLTEC) *E. coli.* EHEC are distinguished from other groups by a combination of their disease symptoms and virulence factors. The disease-defining symptom of EHEC is haemorrhagic colitis (HC), or bloody diarrhoea, while the main virulence factors are toxins. All EHEC produce Shiga toxin 1 (Stx1) and/or Shiga toxin 2 (Stx2), formerly known as verotoxin 1 (VT1) and verotoxin 2 (VT2). The ability for toxin production is thought to have arisen from a bacteriophage

originating from *Shigella*. Shiga toxin is a 70kDal protein comprising a single A subunit (32kDal) and five B subunits (7.7kDal). The A subunit contains an N-glycosidase that inactivates the 28S ribosome in eucaryotic cells, thereby blocking protein synthesis. Tissue specific binding is effected by the B subunits which have an affinity for globotriaosylceramide (Gb_3) receptors on the surface of eucaryotic cells. Endothelial cells are particularly high in Gb_3 receptors, accounting for concentrated toxin activity in the colon and renal glomeruli, resulting in HC and haemolytic uraemic syndrome (HUS), respectively. However, pathogenesis requires more than just Shiga toxin production as evidenced by the existence of Stx-positive non-pathogenic *E. coli.* Pathogenesis is also thought to be mediated by genes encoding for outer membrane proteins associated with attachment (Buchanan and Doyle, 1997).

STEC are designated by their somatic, O, and flagellar, H, antigens. Those isolated from humans belong to over 150 O:H serotypes, most of which belong to the 200 or more serotypes isolated from animals, foods and other sources. The serotype most frequently associated with human HC and thought to be responsible for 85-95% of HUS cases is O157:H7. This serotype was first recognised in 1982 when it caused an outbreak due to contaminated ground beef sandwiches (Riley *et al.*, 1983; Wells *et al.*, 1983). Consumption of food contaminated with *E. coli* O157:H7 normally leads to the onset of HC within 1-2 days. This is characterised by mild, non-bloody diarrhoea and abdominal pain over the first 1-2 days, followed by a period of severe abdominal pain and overtly bloody diarrhoea during the next 4-10 days. Several severe life-threatening complications of HC can occur, of which HUS is the most common. Occurring most often in children under the age of 10, this complication arises approximately a week after the onset of gastrointestinal symptoms and is characterised by microangiopathic haemolytic anaemia (intravascular destruction of red blood cells), thrombocytopaenia (lowered platelet counts), oligo-anuria (lack of urine formation), edema (swelling) and acute renal failure. Over half of HUS patients require dialysis and the mortality rate is 3-5% (Buchanan and Doyle, 1997).

The growth and survival of STEC with respect to environmental parameters such as temperature, water activity and pH are thought generally to be no different to those of non-pathogenic *E. coli.* However, differences have been observed for *E. coli* O157:H7 above 44°C, a temperature region used traditionally to isolate *E. coli* selectively. Doyle and Schoeni (1984) reported that a single strain did not grow well, if at all, above 44°C, while Raghubeer and Matches (1990) observed that a single strain would not grow above 41°C within 48h. Palumbo *et al.* (1995) questioned these results in finding that over 20 strains grew to at least 45°C, but noted that growth was culture medium-dependent in some cases.

Due to food-poisoning outbreaks involving acidic foods, several studies have aimed to find whether *E. coli* O157:H7 survives for long periods of time in products such as mayonnaise (Zhao and Doyle, 1994), fermented sausage (Clavero and Beuchat, 1996), apple cider (Zhao *et al.*, 1993) and Cheddar cheese (Reitsma and Henning, 1996). Survival has been shown to occur for weeks and even months, but in none of the above investigations was a non-pathogenic *E. coli* strain used concurrently and so acidic resistance is not necessarily a trait unique to STEC. A point worthy of note, because it is not immediately intuitive, is that survival was extended in these foods when the storage temperature was lowered. For example, *E. coli* O157:H7 survived in apple cider for 2-3 days at 25°C but for 10-31 days at 8°C (Zhao *et al.*, 1993).

Serotype O157:H7 has been found to be different biochemically from other serotypes in that it does not produce ß-glucuronidase (Wells *et al.*, 1983) and is generally unable to ferment sorbitol, the latter characteristic having been exploited in the development of a selective growth medium, sorbitol MacConker agar (March and Ratnam, 1986). This diagnostoc tool, like others developed more recently should be used with caution as not all O157:H7 strains behave identically and as mentioned, there are many other non-O157:H7 STEC which are potentially pathogenic.

A list of outbreaks caused by O157:H7, not intended to be exhaustive, is given in Table 1.3. STEC serotypes other than O157:H7 have been responsible for ten reported outbreaks since 1984, with nearly half of these incidents involving serotype

Table 1.3 Examples of major outbreaks of *E. coli* O157:H7 infections (Doyle, 1991; Dairy Industry Quality Centre, 1996).

Year	Location	Population	Number of cases (deaths)	Suspected vehicle
1982	Oregon	Community	26	Ground beef
1982	Michigan	Community	21	Ground beef
1982	Oregon	Nursing home	31	Ground beef Person-person
1984	Nebraska	Nursing home	34	Ground beef
1984	North Carolina	Day-care centre	36	Person-person
1985	Ontario	Nursing home	73	Ham, turkey, cheese sandwiches Person-person
1985	England	Community	24	Handling potatoes
1986	Ontario	Kindergarten	46	Raw milk
1986	Alberta	Nursing home	16	Ground beef
1986	Washington	Community	37	Ground beef
1987	England	Community	26	Turkey roll sandwiches
1987	Utah	Half-way houses	51	Ground beef
1988	Minnesota	High school	30	Ground beef
1990	Missouri	Community	~240	Water
1992/3	USA*	Community	583 (4)	Ground beef
1994	Scotland	Community	45	Milk
1996	Japan	Primary schools	~10, 000 (11)	Water cress
1996	USA†	Community	69 (1)	Fresh apple juice
1997	Colorado	Community	17	Ground beef

*Washington, California, Idaho and Nevada.

†Connecticut and New York.

Table 1.4 Examples of major outbreaks of *E. coli* Stx-producing non-O157:H7 infections (Johnson *et al.*, 1996).

Year	Serotype	Location	Population	Number of cases (deaths)	Suspected vehicle
1984	O145:H-	Japan	School	100	Unknown
1986	O111:H-	Japan	Orphanage	22	Unknown
1988	O26:H11	Czech rep.	Community	5	Tap water
1988	O157:H-	Germany	Community	6	Unknown
1991	O111:H-	Japan	School	234	Unknown
1991	O?:H19	Japan	School	89	Unknown
1992	O111:H-	Italy	Community	9	Unknown
1994	O104:H21	USA	Community	11	Unknown
1995	O111:H-	Australia	Community	100+ (1)	Mettwurst

O111:H- (H- denotes a lack of motility) (Johnson *et al.*, 1996) (Table 1.4). While O157:H7 is the leading cause of HC and HUS in the United States, Canada, Great Britain, and regions of Europe, O157:H- and O111:H- outbreaks are more common in Australia.

The food most frequently implicated in STEC-derived food poisoning outbreaks has been ground beef, with the largest outbreak occurring in four states of the US in 1992/3 involving 538 cases and 4 deaths (Centers for Disease Control, 1993). Other beef-derived products implicated in food-poisoning outbreaks have included dry-cured salami (Centers for Disease Control, 1995a) and garlic mettwurst (Centers for Disease Control, 1995b). These latter outbreaks illustrate STECs' ability to survive in fermented, acidic meats. A small outbreak in the UK (Willshaw *et al.*, 1994) highlighted another important trait of STEC; that of its very low infective dose. The contaminated food responsible for the outbreak, raw beef-burger, had an *E. coli* O157 count of <2/25 g. Other outbreaks have been linked to foods as diverse as

potatoes (Morgan *et al.*, 1988), raw milk (Anonymous, 1986), ham, turkey and cheese sandwiches (Carter *et al.*, 1987), drinking water (Swerdlow *et al.*, 1992) and acidic foods such as apple cider (Besser *et al.*, 1993) and mayonnaise (Weagant *et al.*, 1994).

Because the majority of STEC outbreaks have involved *E. coli* O157:H7, much of the published work on STEC has focused on this serotype. Additionally, most outbreaks of *E. coli* O157:H7 infection have occurred as a result of contaminated beef and so this product has received the most attention in studies of foods as possible bacterial reservoirs. Several authors have found a low but nonetheless consistent prevalence of *E. coli* O157:H7 populations in beef cattle. This was particularly evident in a study by Hancock *et al.* (1997) who observed that the frequency of *E. coli* O157 in feedlot cattle faeces in the US was low at 1.8% of samples, but 63% of feedlots participating in the study had at least one positive faecal sample. Doyle and Schoeni (1987), sampling from ground beef obtained from grocery stores in the Madison-area in the US and the Calgary, Alberta, Canada-area found *E. coli* O157:H7 in six (3.7%) of 164 samples. Non-O157:H7 STEC was found in three (2.9%) of 105 beef samples from Rio de Janeiro City in Brazil (Cerqueira *et al.*, 1997). In two large studies conducted in Ontario, evidence of STEC was found in 17% of 1790 cattle in 1988 (Wilson *et al.*, 1992) and 45% of 1478 cattle in 1992 (Clarke *et al.*, 1994). *E. coli* O157:H7 was isolated from 0% of animals tested in 1988 and 0.6% of animals tested in 1992.

Fewer studies have focused on sources of STEC other than beef. Workers in Germany found STEC most frequently in sheep (66.6%), goats (56.1%) and cattle (21.1%), with lower prevalence rates in chickens (0.1%), pigs (7.5%), cats (13.8%) and dogs (4.8%) (Beutin *et al.*, 1993). Abdul-Raouf *et al.* (1996) sampling foods in Egypt found *E. coli* O157:H7 in 2 (4%) of 50 chicken samples, 1 (4%) of 25 lamb samples and 3 (6%) of 50 milk samples. Doyle and Schoeni (1987) found a similarly low frequency of *E. coli* O157:H7 in pork (4 (1.5%) of 264 samples), poultry (4 (1.5%) of 263 samples) and lamb (4 (2.0%) of 205 samples). Those authors also isolated this organism from a bulk tank of unpasteurised milk. Therefore, it appears that STEC,

including serotypes of particular public health significance such as *E. coli* O157:H7, has an animal reservoir and is present at low levels globally.

1.5 Predictive microbiology

Predictive microbiology or 'quantitative microbial ecology' as it has become known (McMeekin *et al.*, 1997) has been reviewed extensively in the literature (Baird-Parker and Kilsby, 1987; Gould, 1989; McMeekin and Olley, 1986; McMeekin and Ross, 1996; Skinner, Larkin and Rhodehamel, 1994) and was recently the subject of a monograph (McMeekin *et al.,* 1993). It is based on the premise that bacterial responses to environmental parameters such as temperature, water activity and pH are reproducible and can be summarised in the form of mathematical equations or models.

Predictive microbiology was first alluded to by Scott (1937) who suggested that the growth of spoilage microorganisms on chilled beef might be predicted by prior knowledge of the growth rates of spoilage bacteria at known temperatures. However, the discipline of predictive microbiology did not begin to evolve until the culmination of two separate lines of research (Ross, 1993): that of Olley and Ratkowsky (1973) on the spoilage rate of fish after the publication of a model relevant to fish spoilage (Spencer and Baines, 1964); and that on the reduction of *Clostridium botulinum* spores (*eg.* Baird-Parker and Freame, 1967). The term 'predictive microbiology' was coined by Roberts and Jarvis (1983) who suggested that the growth responses of microorganisms should be modelled with respect to environmental parameters rather than relying on "*ad hoc* microbiological examination". During that decade, various groups published models for the *growth* of bacteria with respect to one or more environmental parameters (*eg.* Ratkowsky *et al.,* 1982, 1983; Broughall *et al.*, 1983). Later in that decade, models were used in the development of computer software such as Food Micromodel and the Pathogen Modeling Program (Buchanan, 1991). More recently it has been recognised that the ability to predict the rate of *growth* of a bacterial population in a food ('kinetic modelling') is not sufficient. In some cases, the

presence of a toxin or single bacterial cell may be unacceptable and so the need arose to model the *probability* of a certain event occurring.

Whiting and Buchanan (1993) divided predictive models into three types: primary, secondary and tertiary. 'Primary' models are those which measure bacterial response to a single set of conditions over time (*eg.* Gompertz function (Gibson *et al.*, 1987)). This response is summarised in the form of specific values for parameters in the primary model. 'Secondary' models describe the response of one or more primary model parameter values to changes in one or more cultural conditions. An example of secondary models are the Bělehrádek- or square-root-type models (see Section 1.5.1) which calculate changes in growth rate with respect to changes in factors such as temperature, water activity and pH. User-friendly or applications software using one or more secondary models have been termed 'tertiary' models. These can extend the use of secondary models by allowing calculation of accumulated bacterial growth of one or more strains over changing environmental histories (temperature function integration).

1.5.1 Kinetic modelling

Ross (1993) identified four main types of kinetic models, of which the first type is relevant to this work:

Bělehrádek- or square-root-type models
Arrhenius-type models
Modified Arrhenius, or 'Davey' models
Polynomial, or 'Response Surface' models.

Ohta and Hirahara (1977) found that a plot of the square-root of nucleotide breakdown in fish muscle was linear. From this observation, Ratkowsky *et al.* (1982) formulated a model to describe the effect of temperature on growth rate of bacteria between the minimum and just below the optimum temperature:

$$\sqrt{k} = b(T - T_{min}) \quad (1)$$

where k is growth rate, T is temperature, T_{min} is the sub-optimal temperature at which growth rate is predicted to be zero and b is a coefficient to be estimated.

Ross (1987) noted that Eqn. 1 was a special case of the temperature function of Bělehrádek (1930) which is used widely in other biological sciences. However, Eqn. 1 does not model growth above optimum growth temperatures (T_{opt}) and so Ratkowsky *et al.* (1983) constructed a model able to predict the rate of bacterial growth throughout the entire biokinetic temperature range. This non-linear regression model is given by

$$\sqrt{k} = b(T - T_{min})(1 - \exp(c(T - T_{max}))) \quad (2)$$

where k, T, T_{min} and b have the same meaning as previously, T_{max} is the super-optimal temperature at which growth rate is predicted to be zero and c is a coefficient to be estimated.

McMeekin *et al.* (1987) further modified Eqn. 2 to include a term for water activity:

$$\sqrt{k} = b(T - T_{min})\sqrt{a_w - a_{w_{min}}} \quad (3)$$

where k, T, T_{min} and b have the same meaning as previously, a_w is water activity and $a_{w_{min}}$ is the sub-optimal water activity at which growth rate is predicted to be zero.

Eqn. 3 was expanded to describe growth over the entire biokinetic temperature range by Miles (1994):

$$\sqrt{k} = \frac{b(T - T_{min})(1 - \exp(c(T - T_{max})))}{\sqrt{(a_w - a_{w_{min}})(1 - \exp(d(a_w - a_{w_{max}})))}} \quad (4)$$

where k, T, T_{min}, b, c, a_w and a_{wmin} have the same meaning as previously and d is a coefficient to be estimated.

Additional modifications to Eqn. 2 have been made to include a term for pH (Adams *et al.*, 1991) and both water activity and pH terms (McMeekin *et al.*, 1992; Presser *et al.*, 1997).

An extension of secondary models of particular relevance to the prediction of spoilage of beef is temperature function integration (TFI). TFI allows the consideration of environmental histories such as chilling regimes and was alluded to in work as far back as Scott (1936, 1937) and later by Nixon (1971). It was developed for the determination of *E. coli* proliferation on cooling beef by The Meat Industry Research Institute of New Zealand (MIRINZ) (Gill *et al.*, 1988).

Predictive models (*eg.* Eqn. 1-4) calculate bacterial growth rates in terms of generations per unit time (*eg.* gen h^{-1}). Integration operates by converting growth rate into bacterial numbers at different time intervals and then calculating the accumulated population over total time. Assuming that a population adjusts immediately to changing environmental conditions, bacterial numbers are given by,

$$\text{growth rate}(\text{gen}\,h^{-1}) \times \text{time} = \text{number of generations} \quad (5)$$

where generations are equivalent to population doublings.

Integration therefore requires calculation of the area under a graph of rate *versus* time. This can be done manually (*eg.* Ronsivalli and Charm, 1975) or more easily using a software-based system (*eg.* Ross, 1993). Some of the devices discussed in Section

1.3.3.2 enable not only collection of temperature history data but also integration of data with respect to bacterial growth.

1.5.2 Probability modelling

As already mentioned, probability modelling arose due to a need to predict the likelihood of an unacceptable event occurring, such as the growth of pathogens or toxin production. Ross and McMeekin (1994) discussed how this is achieved in the simplest case. Replicate samples of known inocula are grown under defined environmental conditions over a set period of time. The number of samples positive for growth or toxin production is assessed and a model is derived relating the probability of growth/toxin production under the specific environmental conditions.

The use of probability models was reported as long ago as the early 1970s when Genigeorgis *et al.* (1971) modelled 'decimal reduction' of *Staphylococcus aureus*. Their model was used in subsequent studies using other microorganisms and combinations of environmental factors (Ross and McMeekin, 1994). Initiation of cell growth was measured within 20 days of inoculation. The probability of a single cell initiating growth was given by,

$$p = R_G / G_I \qquad (6)$$

where R_G is the number of cells initiating growth and R_I is the number of cells inoculated into the system.

The logarithm of this ratio, $\log(R_I/R_G)$, represents the number of decimal reductions of a staphylococcal population resulting from its exposure to a particular environment. The effect of environmental conditions on p was modelled by the polynomial,

$$\log(R_I / R_G) = a + b_1(\%NaCl) + b_2(pH) + b_3(\%NaCl)^2 + b_4(pH)^2 + b_5(\%NaCl)(pH) \quad (7)$$

Lindroth and Genigeorgis (1986) applied a different form of model to the initiation of growth and toxigenesis of *C. botulinum* spores:

$$p(\%) = \frac{MPN \times 100}{inoculum} \quad (8)$$

where MPN is the number of spores which have initiated growth and toxigenesis and inoculum is the number of spores initially present.

Probability can also be expressed in the following way and as such has a value from zero to one:

$$p = \frac{e^y}{1+e^y} \quad (9)$$

where y is a function of the variables modelled by a polynomial.

Lindroth and Genigeorgis (1986) used a model based on Eqn. 9 and included terms for lag time and initial inoculum level:

$$\log_{10} p(\%) = 5\left(\frac{e^y}{1+e^y}\right) - 3 \quad (10)$$

where y represents the effects of environmental variables and is expressed as:

$$y = b_1 + b_2 T + b_3(S_t - LP) + b_4 T(S_t - LP) \quad (11)$$

where b_1-b_4 are coefficients to be estimated, T is temperature, S_t is elapsed time and LP, time to toxigenesis, is modelled by:

$$LP = a + b_5T + b_6(1/T) + b_7(I) \qquad (12)$$

where a, b_5-b_7 are coefficients to be estimated and I is the inoculum concentration.

Ratkowsky and Ross (1995) modified a kinetic model with parameters for temperature, water activity, pH and nitrite concentration such that it could predict growth *versus* no growth. Modification was achieved by taking the logarithm of both sides of the equation and replacing the left-hand side with a logit term (logit (p), an abbreviation of ln[p/(1-p)]). Probability could be set depending on the level of stringency required. An example of a model incorporating terms for four environmental parameters is:

$$\begin{aligned} \text{logit}(p) = {} & b_0 + b_1 \ln(T - T_{min}) \\ & + b_2 \ln(pH - pH_{min}) + b_3 \ln(a_w - a_{w_{min}}) \\ & + b_4 \ln(NO_{2_{max}} - NO_2) \end{aligned} \qquad (13)$$

where b_0-b_4 are coefficients to be estimated, pH_{min} and NO_{2min} are the sub-optimal pH and nitrite concentration, respectively, at which growth rate is predicted to be zero and T, T_{min}, a_w and a_{Wmin} have the same meaning as previously.

This was an example of a 'generalised linear regression model' where cardinal parameters (in this case, T_{min}, a_{Wmin}, pH_{min} and NO_{2min}) were assumed to be known from previous kinetic data. Ratkowsky (pers. comm.) modified this form of equation so that all parameters were estimated. The resulting model type, a 'generalised non-linear regression model' (Ratkowsky, 1993), has received little attention in the statistical literature.

Ross and McMeekin (1994) noted that the probability of detectable growth, when plotted as a function of time, is a sigmoid curve which has an upper asymptotic value equivalent to the maximum probability of growth given infinite time. This implies, they stated, that bacterial growth responses are increasingly variable under conditions stressful to the organism and that growth rates become increasingly variable as a function of generation time. Thus, probability models provide information as to the kinetic behaviour of microorganisms and so there is no clear distinction between these and kinetic models. Integration of kinetic and probability models rather than the use of each model type separately provides the greatest tool for applying predictive microbiology to food safety.

1.6 Objectives of this thesis

Since the emergence of STEC as a major cause of food-poisoning outbreaks in the last decade, much has been published on the growth characteristics of several serotypes, with most work focusing on *E. coli* O157:H7. Research into predictive modelling has, in many cases, been driven by the nature of food-poisoning outbreaks and not pro-actively to reduce the risk of outbreaks before they occur. An objective of this work is to assess existing models developed for non-pathogenic *E. coli* for predicting the growth of STEC strains by comparison of model predictions with observed growth of STEC strains in liquid growth medium and with published data for growth of *E. coli* in foods.

As discussed in Section 1.5.2, kinetic modelling of STEC growth is not adequate when only a few cells are required to cause disease. Accordingly, another aim is to define the boundary between growth and no growth for an STEC strain with different combinations of temperature and water activity, and using the growth/no growth data to develop a probability model to predict the likelihood of growth of STEC within the environmental constraints tested.

As mentioned in Section 1.3.4.1, water activity at the surface of red meat carcasses cannot be measured *in situ* directly. Were such a method available it could be used to predict the extent of proliferation of carcass surface bacteria during chilling. This information could be used to optimise chilling regimes.

Chapter 2. Growth Response of STECs to Temperature and Water Activity Change

2.1 Summary

Models were developed for the temperature dependence of growth rate of non-pathogenic *E. coli* strains and temperature/water activity dependence of growth of a Shiga toxin-producing *E. coli* (STEC) strain. The suitability of these models for predicting the growth rate of nine STEC strains was assessed. Model parameters were compared to analogous fitted parameters of STEC models using the approximate t-test. Bias and accuracy factors were also calculated. Growth rates of STEC strains were found to be adequately described by models for non-pathogenic *E. coli.* Predictions of a non-pathogenic *E. coli* model and an STEC model were also found to describe sufficiently well published growth rate data for non-pathogenic *E. coli* on mutton carcase surfaces and *E. coli* O157:H7 in ground roasted beef, milk, and on cantaloupes and watermelons. In addition, an *E. coli* O157:H7 strain was found to grow in the region 44 to 45.5°C.

2.2 Introduction

Food poisoning outbreaks due to Shiga toxin-producing *Escherichia coli* (STEC) have increased dramatically in incidence over the past decade. STEC comprise many pathogenic serotypes, of which O157:H7 is the most studied. This serotype was first recognised in 1982 when it caused an outbreak due to contaminated ground beef sandwiches (Riley *et al.*, 1983; Wells *et al.*, 1983). It has been implicated in other outbreaks involving beef-derived foods, such as dry-cured salami (Centers for Disease Control, 1995a) and hamburger patties, which caused over 500 persons to become ill and four people to die in a single outbreak (Centers for Disease Control, 1993). In July 1997, an outbreak in Denver, Colorado involving at least 17 cases led to the recall of 25 million pounds of ground beef potentially contaminated with *E. coli* O157:H7 (Centers for Disease Control, 1997). Other outbreaks have been associated with a variety of foods including fruits, vegetables, milk and water, and even acidic foods such as cider and mayonnaise. STECs other than O157:H7 have been responsible for ten reported outbreaks since 1984 (Johnson *et al.*, 1996), with nearly half of these incidents involving *E. coli* O111:H-. This serotype has caused 3 deaths; one each in Japan (Kudoh *et al.*, 1994), Italy (Caprioli *et al.*, 1994) and Australia (Centers for Disease Control, 1995b).

Temperature/water activity models for the growth rate of non-pathogenic *E. coli* strains were developed previously in this laboratory. Temperature models were also generated for non-pathogenic *E. coli* from literature data. A comparison between models for non-pathogens and data for the growth of pathogenic strains was undertaken. Non-pathogenic *E. coli* model predictions and those of an STEC model were then compared to literature data for the growth of non-pathogenic *E. coli* on meat and *E. coli* O157:H7 in a variety of food products and nutrient medium. Simultaneously an investigation was undertaken of conflicting reports in the literature that STEC strains can (Palumbo *et al.*, 1995) or cannot (Doyle and Schoeni, 1984) grow well in the range 44 to 45.5°C.

2.3 Materials and Methods

2.3.1 Sources of isolates

Escherichia coli O157:H7 and O111:H- strains were obtained from the Royal Melbourne Children's Hospital (RMCH), while seven STEC strains were obtained from the Department of Pathology, University of Tasmania, Royal Hobart Hospital (RHH). Serotyping and Shiga toxin assays were performed by workers at the RMCH and RHH. Identification of those isolates to species level was verified using API strips (API 20E, Biomérieux Vitek Inc., USA). M23, a non-pathogenic strain, was obtained from the culture collection of the Department of Agricultural Science, University of Tasmania. All strains were maintained on Plate Count Agar (PCA) slopes at 4°C and sub-cultured every three months (Appendix 2.2).

Sources of isolation and toxigenicity information of 11 STEC strains are given in Appendix 2.1 (Table A2.1). Six strains were isolated from water, one from potable water and the remainder from environmental samples. Two strains were isolated from meat samples and three from human patients, two of whom had HUS. Most strains synthesised at least one Shiga toxin (NT, O81:H-, ONT:H8, O88:H- (R172)), while O157:H-, O157:H7 and O111:H- strains synthesised both Stx1 and Stx2. O157:H7 and O111:H- were isolated in Victoria while all other strains were isolated in Tasmania.

2.3.2 Generation of temperature growth models

Temperature-growth rate models were developed for nine strains of STEC. Each strain was incubated in nutrient broth (NB) (Appendix 2.3) at 37°C for approximately 18h. Approximately 0.5ml of this culture was inoculated aseptically into NB in L-shaped, optical quality glass tubes (L-tubes) to give an initial absorbance of ~0.05 and incubated in water baths (Ratek Instruments, Australia) or in a temperature gradient incubator (Model TN3, Toyo Kagaku Sangyo Ltd., Japan) with shaking (50±10 rpm). Generation times (GT) at each temperature were calculated from %transmittance data (540 nm) using a Gompertz function as described in McMeekin *et al.* (1993, Appendix

2A.9). The temperature of each tube was measured in quadruplicate using a Fluke 51 K/J thermometer (John Fluke MFI Co. Inc., USA). GTs were corrected to equivalent viable count values using a previously determined ratio of 1.5 (Dalgaard *et al.*, 1994). Derived growth curves were fitted to Eqn. 2 (Ratkowsky *et al.*, 1983) using the SAS PROC.NLIN procedure (SAS Institute, 1989).

2.3.3 Generation of temperature/water activity growth models

A temperature/water activity-growth rate model for *E. coli* NT (strain R31) was generated using the method outlined in Section 2.3.2, except that growth rate observations were carried out in NB containing 0-9% (w/w) NaCl. Data of Chirife and Resnik (1984) was used as a guide in order to convert [NaCl] to water activity values. Water activity of each broth was verified using a water activity meter (Aqualab model CX2, Decagon Devices, USA). Growth curves were fitted to a square-root model containing temperature and water activity terms (Eqn. 3) (McMeekin *et al.*, 1987).

2.3.4 Comparison of STEC temperature models with the M23 and Barber model

A temperature-growth rate model was developed previously for *E. coli* M23 (Ross, pers. comm.). M23 was obtained from the culture collection, Department of Agricultural Science, University of Tasmania. The value b was determined by equating the model to the fastest recorded growth of *E. coli* at optimum conditions of temperature, pH and water activity (Barber, 1908; Spector, 1956). Thus the model is purposefully conservative and intended to predict the fastest growth rate probable. A pH term was not included because other results (Presser *et al.*, 1997) suggest that at the pH of the foods for which published *E. coli* growth rate data are available, the influence of pH is small.

The model is given by:

$$\sqrt{k} = 0.0080(T - 4.26)(1 - \exp(0.363(T - 47.3))) \quad (14)$$

where k and T have the same meaning as previously.

The data of Barber (1908) for the growth of non-pathogenic *E. coli* with respect to temperature was fitted to the four-parameter square-root model (Eqn. 2).

To compare temperature models for the growth of STECs with the models for non-pathogenic *E. coli* ('generic' models; Eqns. 14 and 16), fitted parameters (T_{min}, T_{max}, b, and c) for each STEC model were compared statistically to analogous parameters of the generic models using the approximate t-test (Appendix 2.4), at a confidence interval of 95% ($p = 0.05$). Statistical comparison of optimum temperature (T_{opt}) values was not possible because T_{opt} is not estimated in the modelling process but, rather, is subsequently derived from other parameters (Appendix 2.5). STEC models were also compared to the generic models using bias and accuracy factors (Ross, 1996). The bias factor is defined as the geometric mean of the ratio of predicted to observed generation times, while the accuracy factor describes the geometric mean of the absolute values of this ratio. Only data acquired below 40°C was considered in the comparison using bias and accuracy factors in order to reduce the influence of large differences between predictions and observations in the super-optimal range. A comparison between predictions of Eqn. 14 and STEC models was also made after making Eqn. 14 less conservative by reducing the b value by 5%.

2.3.5 Comparison of R31 and modified M23 model predictions with literature data

The predictions of two models containing both temperature and water activity terms were compared to literature data for growth rate of *E. coli* in food systems and nutrient medium. The models used were a modified version of the M23 model described

previously, and the R31 model described in Section 2.3.3. The M23 model was modified by addition of a term for water activity:

$$\sqrt{k} = 0.304(T - 4.26)(1 - \exp(0.363(T - 47.3))) \sqrt{(a_w - 0.955)} \quad (15)$$

where k, T and a_w have the same meaning as previously.

Predictions of the generic M23 (Eqn. 15) and R31 (Eqn. 17) temperature/water activity models were compared to published data for the growth of non-pathogenic *E. coli* on meat and *E. coli* O157:H7 in food and laboratory growth media. Published GTs, including those derived by Sutherland *et al.* (1995) from two different primary models (a modified Gompertz model and the D-model (Baranyi *et al.*, 1993)) and those predicted by the Pathogen Modelling Program (Buchanan, 1991), were compared to the same data.

2.4 Results

2.4.1 Temperature growth models

The data of Barber (1908) fitted to the four-parameter square-root model is presented in Figure 2.1. The resulting model is given by:

$$\sqrt{k} = 0.0068(T - 3.34(1 - \exp(0.303(T - 49.2))) \quad (16)$$

where k and T have the same meaning as previously.

Growth rate data for all STECs fitted to the four-parameter square-root model are presented in Figures 2.2a-i. Parameter values for square root models fitted to the growth rate data are listed in Table 2.1. In most cases, fitted parameters of the STEC

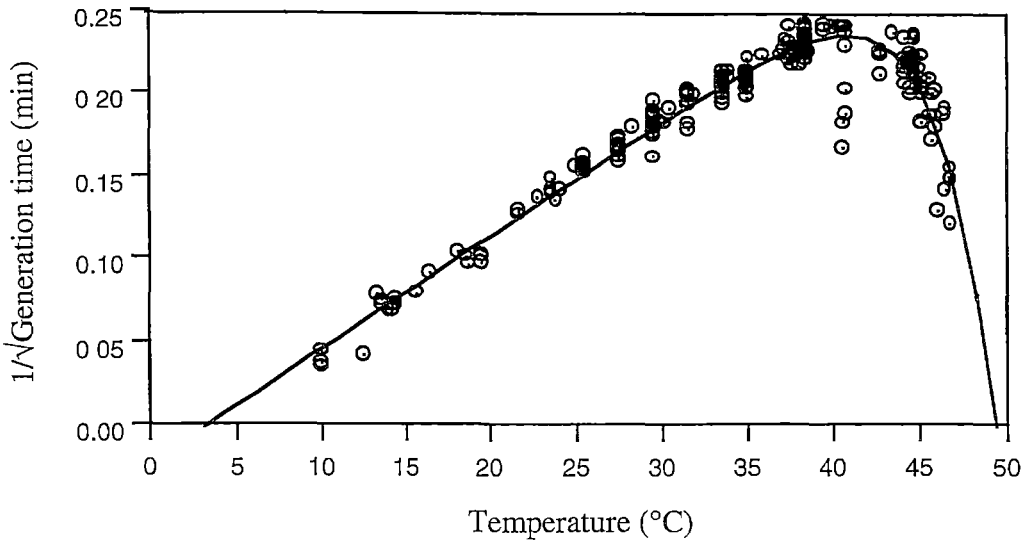


Figure 2.1 Four-parameter square-root plot for growth of *E. coli* (data of Barber (1908)).

models were not significantly different to those of the generic models for non-pathogenic *E. coli* (Table 2.2). The fitted parameters T_{min}, b and c for all STEC strains were not significantly different to the respective parameters of the M23 model (Eqn. 14), and T_{min}, T_{max} and c for all STEC strains were not significantly different to the respective parameters of the Barber model (Eqn. 16). T_{opt} values for STECs were, at the most, 0.5°C below and 2.5°C above the value for Eqn. 14 and 1.4°C below and 1.7°C above the value for Eqn. 16. In all but two cases (O157:H- and O157:H7), values for T_{max} for STEC models were significantly different to that for Eqn. 14. In all but two cases (ONT:H8 and O157:H7), values for b for STEC models were significantly different to that for Eqn. 16. One T_{min} value for an STEC model (O88:H-(R172)) was significantly different to that for Eqn. 16. Values for T_{min} and c for Eqn. 14 were not significantly

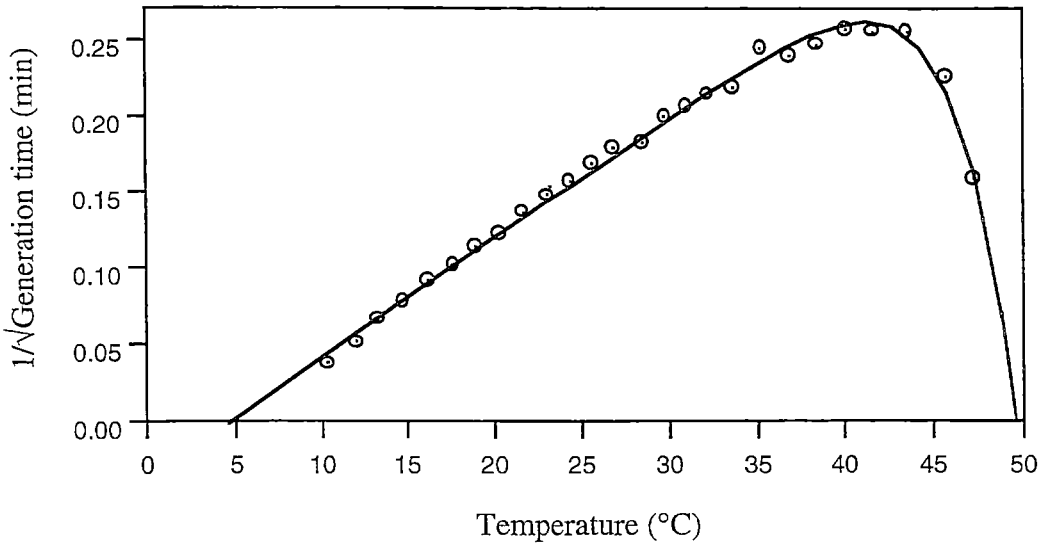


Figure 2.2a Four-parameter square-root plot for growth of *E. coli* O126:H21 (R10).

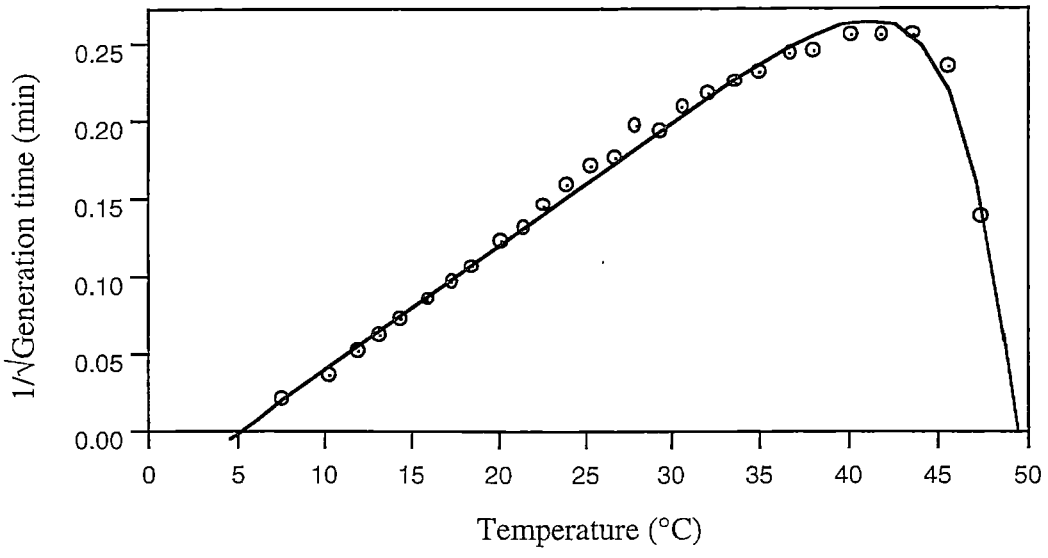


Figure 2.2b Four-parameter square-root plot for growth of *E. coli* NT (R31).

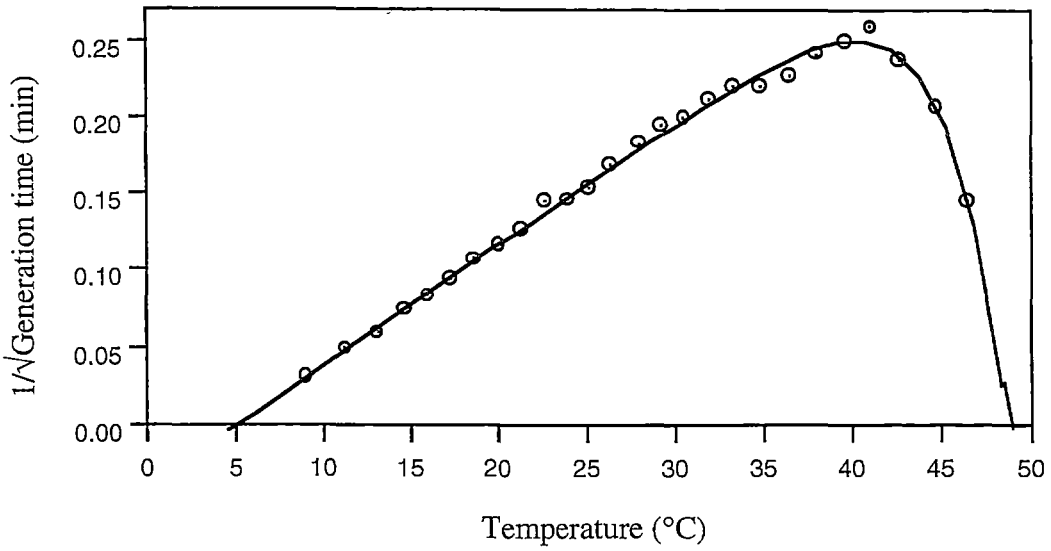


Figure 2.2c Four-parameter square-root plot for growth of *E. coli* O81:H- (R91).

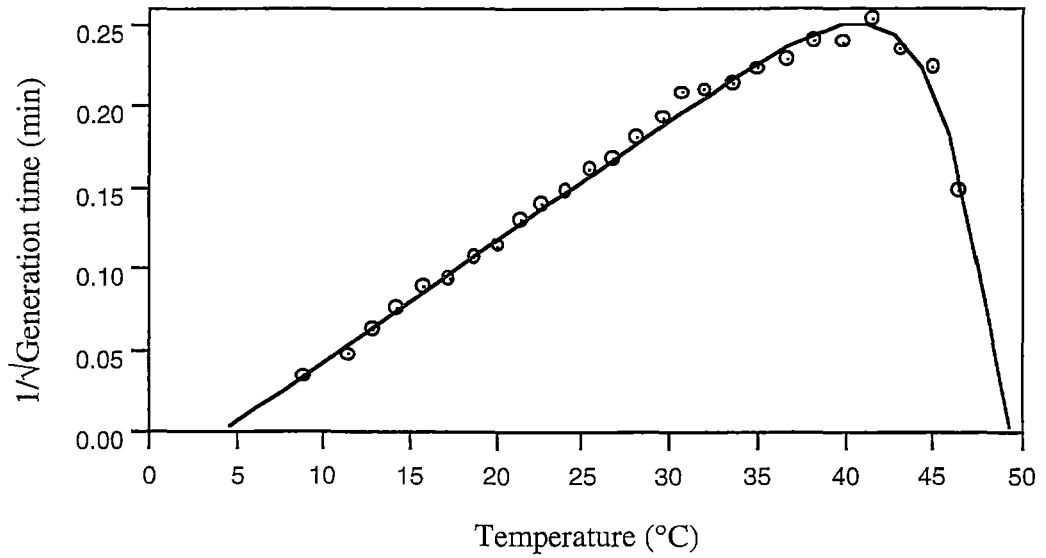


Figure 2.2d Four-parameter square-root plot for growth of *E. coli* ONT:H8 (R106).

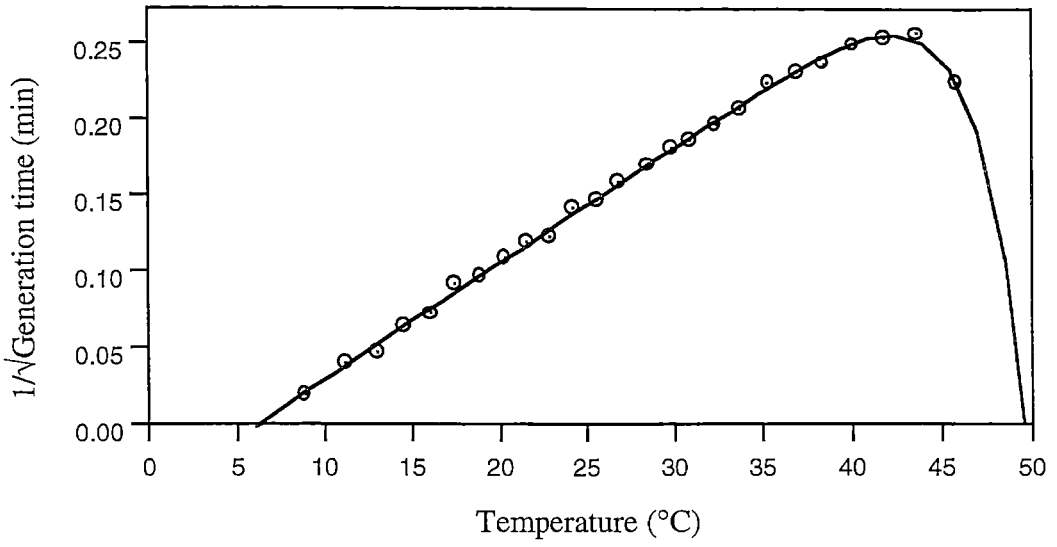


Figure 2.2e Four-parameter square-root plot for growth of *E. coli* O88:H- (R171).

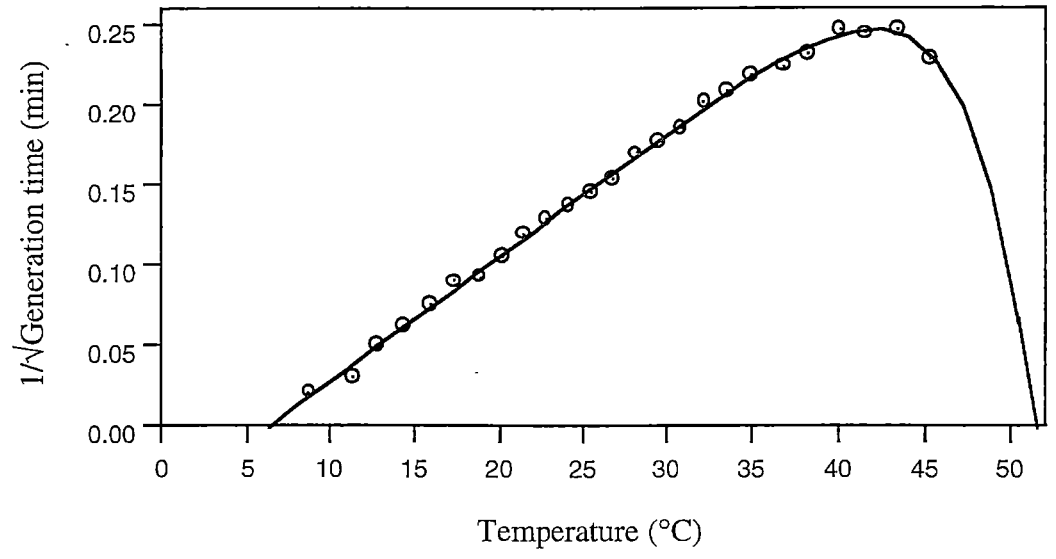


Figure 2.2f Four-parameter square-root plot for growth of *E. coli* O88:H- (R172).

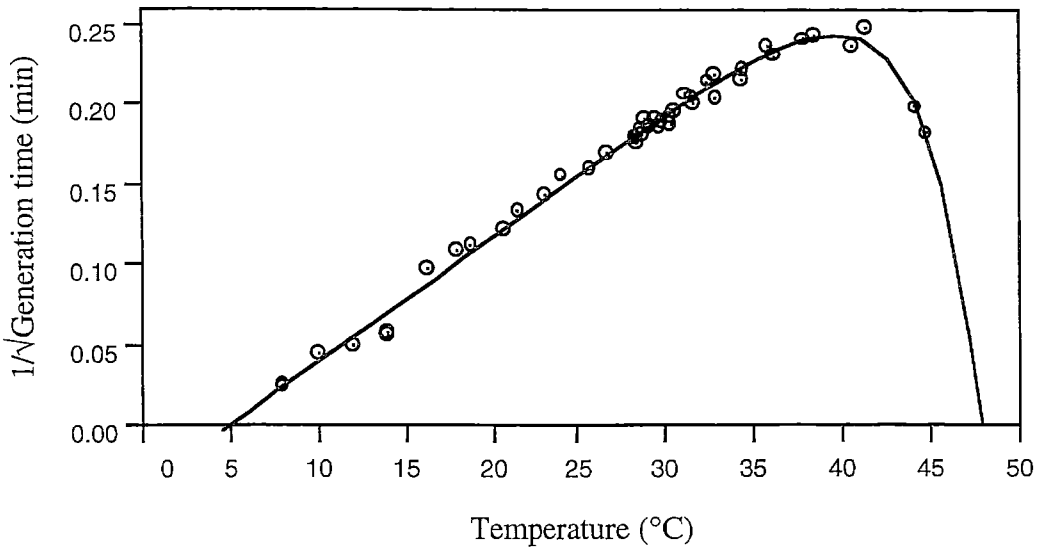


Figure 2.2g Four-parameter square-root plot for growth of *E. coli* O157:H-.

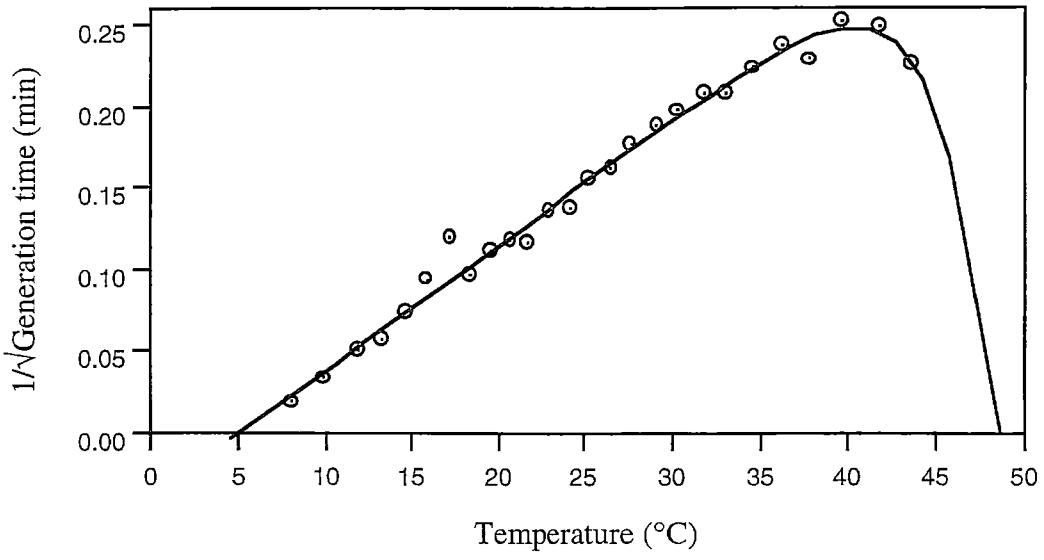


Figure 2.2h Four-parameter square-root plot for growth of *E. coli* O157:H7 (EH9).

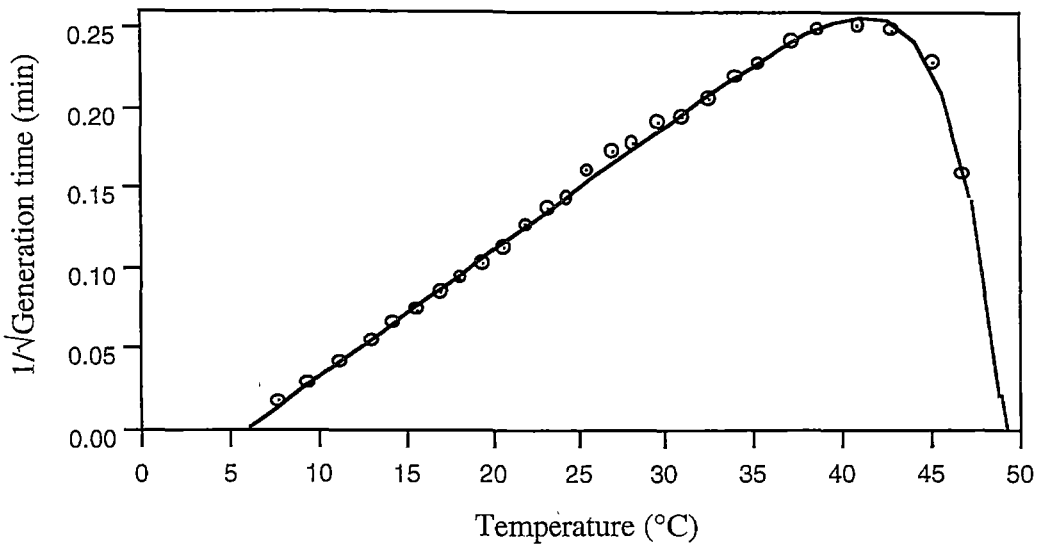


Figure 2.2i Four-parameter square-root plot for growth of *E. coli* O111:H- (EH39).

Table 2.1 Fitted model parameter values for non-pathogenic *E. coli* and STECs.

	Model parameters					Data range*
Strain/Serotype	T_{min}	T_{opt}	T_{max}	b	c	(°C)
M23	4.263±1.041	40.05	47.32±0.490	0.0080±0.0004	0.3625±0.0819	7.8 - 46.7
Barber	3.339±1.184	40.91	49.21±0.448	0.0068±0.0003	0.2911±0.0360	5.9 - 50.0
O126:H21	4.758±0.874	41.15	49.52±0.509	0.0078±0.0004	0.2940±0.0560	5.0 - 53.6
NT	5.113±0.904	41.12	49.23±0.453	0.0080±0.0004	0.3069±0.0600	5.0 - 53.1
O81:H-	5.072±0.732	40.23	48.61±0.388	0.0078±0.0003	0.2878±0.0440	3.5 - 52.7
ONT:H8	4.232±1.005	40.75	48.58±0.533	0.0074±0.0004	0.3268±0.0704	3.6 - 52.9
O88:H- (R171)	6.187±0.453	42.33	49.60±0.899	0.0076±0.0002	0.3648±0.0847	3.0 - 52.6
O88:H- (R172)	6.413±0.545	42.56	51.26±1.737	0.0077±0.0003	0.2749±0.0738	3.5 - 52.8
O157:H-	4.994±0.632	39.55	47.84±0.733	0.0078±0.0003	0.2901±0.0615	8.0 - 48.7
O157:H7	5.008±1.251	40.34	48.15±3.371	0.0076±0.0006	0.3220±0.2337	1.7 - 46.1
O111:H-	5.889±0.467	41.35	48.85±0.331	0.0079±0.0002	0.3442±0.0470	1.8 - 46.8

*Range tested, not necessarily growth observed.

Table 2.2 Results of an approximate t-test (95% confidence interval) to determine whether parameters of models for non-pathogenic *E. coli* were significantly different from those of STEC models.

	Significant difference ($p<0.05$)?							
	M23				Barber			
Serotype	T_{min}	T_{max}	b	c	T_{min}	T_{max}	b	c
O126:H21	no	yes	no	no	no	no	yes	no
NT	no	yes	no	no	no	no	yes	no
O81:H-	no	yes	no	no	no	no	yes	no
ONT:H8	no	yes	no	no	no	no	no	no
O88:H- (R171)	no	yes	no	no	no	no	yes	no
O88:H- (R172)	no	yes	no	no	yes	no	yes	no
O157:H-	no	no	no	no	no	no	yes	no
O157:H7	no	no	no	no	no	no	no	no
O111:H-	no	yes	no	no	no	no	yes	no
Comparison between M23 and Barber data					no	yes	yes	no

different to those of Eqn. 16, while values for T_{max} and b were significantly different.

A comparison of STEC data and Eqn. 14 and Eqn. 16 predictions in terms of bias and accuracy factors is given in Table 2.3. When compared to Eqn. 14, the bias factor for all STEC models was below 1. The lowest level of agreement occurred for an O88:H- (R172) model, which, on average had growth rates that were 66% of those predicted by Eqn. 14. The highest level of agreement occurred for an O126:H21 model, which, on average had growth rates that were 88% of those predicted by Eqn. 14. The average bias for all STEC models indicated that growth rates were 77% of those

Table 2.3 Assessment of agreement of STEC models with M23 model, using data below 40°C. Values in brackets are for the M23 model with b reduced by 5%.

	M23		Barber	
Serotype	Bias	Accuracy	Bias	Accuracy
O126:H21	0.88 (0.98)	1.14 (1.08)	1.10	1.19
NT	0.87 (0.97)	1.16 (1.10)	1.09	1.21
O81:H-	0.83 (0.93)	1.20 (1.09)	1.04	1.14
ONT:H8	0.85 (0.95)	1.18 (1.06)	1.06	1.12
O88:H- (R171)	0.67 (0.75)	1.50 (1.34)	0.83	1.24
O88:H- (R172)	0.66 (0.74)	1.52 (1.36)	0.82	1.25
O157:H-	0.84 (0.94)	1.20 (1.10)	1.04	1.19
O157:H7	0.81 (0.88)	1.26 (1.19)	0.97	1.21
O111:H-	0.77 (0.85)	1.30 (1.18)	0.94	1.19

predicted by the Eqn. 14. In absolute terms, the accuracy of Eqn. 14 was, at best, ±14% (O126:H21) and at worst, ±52% (O88:H- (R172)), compared to the data for pathogenic strains. Average accuracy was ±27%.

When Eqn. 14 was made less conservative by reducing the value of b by 5% (from 0.0080 to 0.0076), bias and accuracy values were greatly improved (Table 2.3). Eqn. 14 predictions still 'failed-safe', but the highest level of agreement (O126:H21) between observed and predicted growth rates increased from 88% to 98% and the lowest level of agreement (O88:H- (R172)) increased from 66% to 74%. Average bias was 89%. Accuracy was, at best, ±6% (ONT:H8) and at worst, ±36% (O88:H- (R172)), compared to the data for pathogenic strains. Average accuracy was ±17%.

When STEC models were compared to Eqn. 16, bias values were scattered below and above 1. Below 1, the poorest agreement was for the O88:H- (R172) model which had growth rates that were 82% of those predicted by Eqn. 16, while the highest level of agreement was for the O157:H7 model at 97%. Above 1, the highest level of agreement was for the O81:H- and O157:H- models (104%) while the poorest

agreement was for the O126:H21 model (110%). Accuracy of Eqn. 16 was, at best, ±12% (ONT:H8) and at worst, ±25% (O88:H- (R172)), compared to the STEC models. Average accuracy was ±19%.

The M23 model and data for three STECs (O88:H- (R172), the model for which showed the poorest agreement with the M23 model, and O157:H7 and O111:H-, two strains of particular public health significance) are compared graphically in Figure 2.3. Almost all STEC data in the sub-optimal temperature region fell below the predicted curve, with *E. coli* O88:H- (b) being the slowest growing strain. In the super-optimal temperature region there were several cases of under-prediction by the model, with some data points falling above the curve.

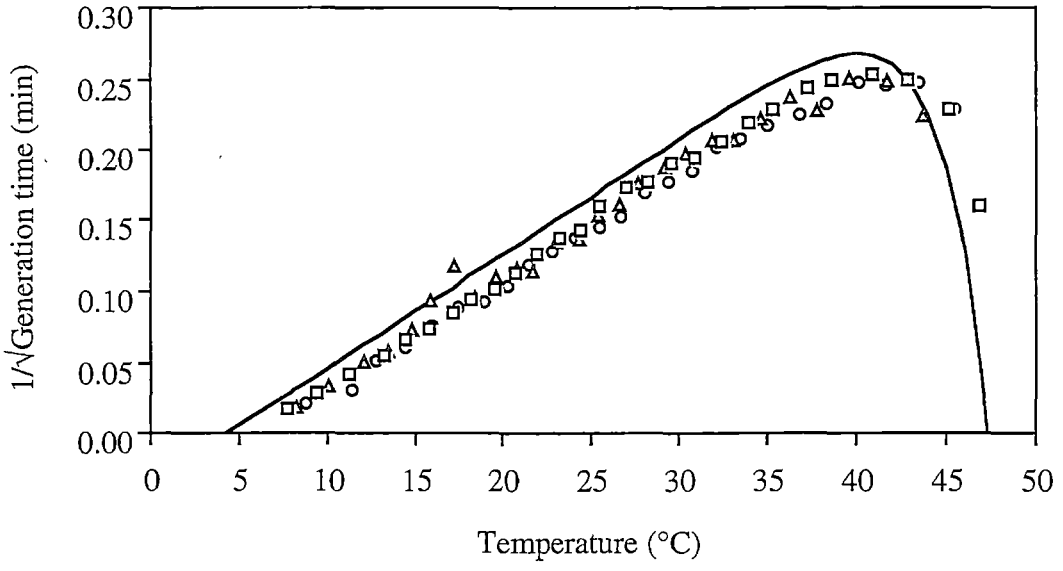


Figure 2.3 Four-parameter square-root plots of three STECs compared with a predicted curve generated by a model for non-pathogenic *E. coli* M23. O, *E. coli* O88:H- (R172); Δ, *E. coli* O157:H7; □, *E. coli* O111:H-.

A log/log plot of predictions and corresponding observations under 40°C for the same three STECs is shown in Figure 2.4. Almost all data lies below the line of equivalence, indicating that model predictions were 'fail-safe'. Observed growth rates

were adequately described by model predictions, but the level of accuracy of predictions is not apparent from Figure 2.4 due to the log/log transformation of data. The largest variation between observed and predicted GT occurred at the lower temperature limits of growth.

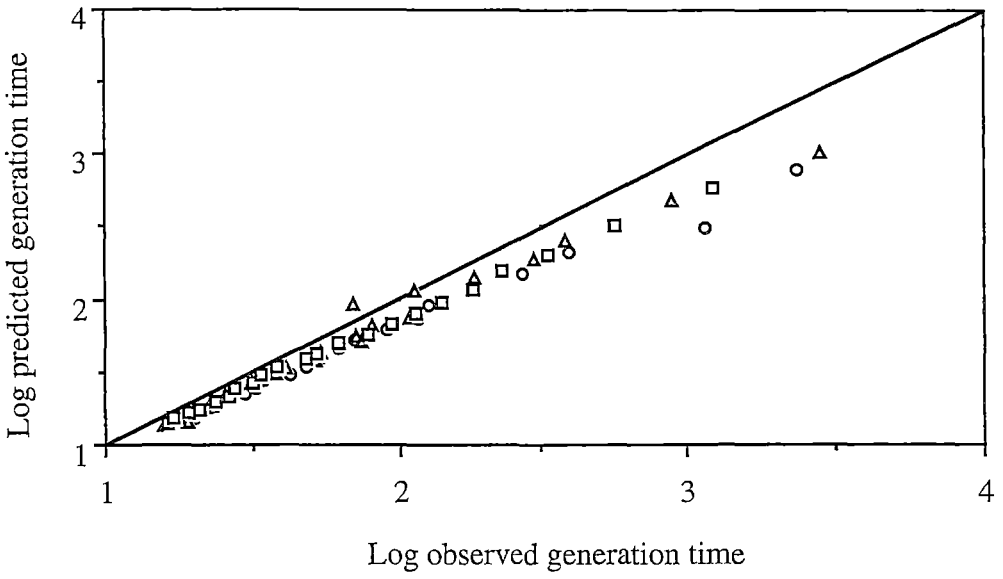


Figure 2.4 Comparison of observed values for GT of three STEC strains with those predicted by a model for *E. coli* M23, using data below 40°C. O, *E. coli* O88:H-(R172); Δ, *E. coli* O157:H7; □, *E. coli* O111:H-.

2.4.2 Growth of STECs at the upper temperature limits

Growth rate data near the upper temperature limits for growth of STECs are given in Table 2.4. Generation time was calculable by the Gompertz equation only when growth reached an optical density of greater than 0.69 (%transmittance < 20%). The Gompertz equation could not fit a sigmoid curve to data accurately when there was no data in this region. Where it did not reach this level, data is given when measurable growth still occurred. For all STECs except O157:H-, measurable growth (absorbance >0.3) occurred up to at least 45°C. In many cases there was also measurable growth at

between 46 and 47°C. For all strains with the exception of O157:H-, cell yield exceeded 10^8 CFU/ml in the region 44-45.5°C after 24h while for most strains cell yield after 24h exceeded 10^8 CFU/ml at the upper temperature limits (46-47°C). Exceptions were a non-typable strain which reached $10^{7.9}$ CFU/ml at 47.4°C and an O157:H7 strain which reached $10^{5.2}$ CFU/ml at 46.1°C. Cell yield was calculated from optical density data using the results of Dalgaard *et al.* (1994).

Table 2.4 Highest temperature at which GTs were able to be calculated for STECs and equivalent cell yield after 24h. Data is also given where measurable growth occurred but when GT could not be calculated using a Gompertz function.

Serotype	Temperature (°C)	GT (min) or [highest absorbance attained]	8-log CFU/ml after 24h? or [log CFU/ml after 24h]
O126:H21	47.2	40.22	yes
NT	47.4	53.17	[7.85]*
O81:H-	46.5	45.84	yes
ONT:H8	47.1	47.21	yes
O88:H- (R171)	45.9	20 03	yes
O88:H- (R172)	45.4	19.10	yes
O157:H-	44.8	30.17	yes
O157:H7	43.6	10.41	yes
O157 H7	46.1	[0.69]	[5.20]*
O111:H-	46.8	28.05	yes

*Calculated from data of Dalgaard *et al.* (1994).

2.4.3 Temperature/water activity growth models

The growth rate response of an *E. coli* NT strain (R31) with reference to temperature and water activity is presented in Figure 2.5. Response to combinations of temperature and water activity agreed with previous observations (*eg.* Wodzinski and Frazier (1960), Ohye and Christian (1967), Doe and Heruwati (1988)); growth rates at

low water activities were highest at temperatures close to optimal. When the dataset was fitted to a model incorporating temperature and water activity terms (Miles, 1994; Eqn. 4) a response surface was generated and is shown in Figure 2.6. The model is given by:

$$\sqrt{k} = \frac{0.0455(T - 4.20)(1 - \exp(0.353(T - 49.0)))}{\sqrt{(a_w - 0.963)(1 - \exp(56.4(a_w - 1.03)))}} \qquad (17)$$

where k, T and a_w have the same meaning as previously.

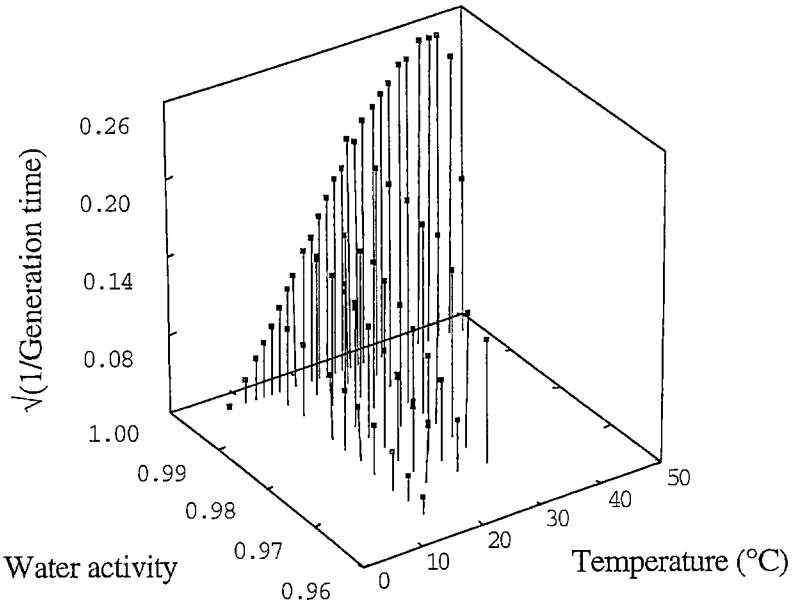


Figure 2.5 Observed growth rate of *E. coli* NT (R31) with respect to temperature and water activity.

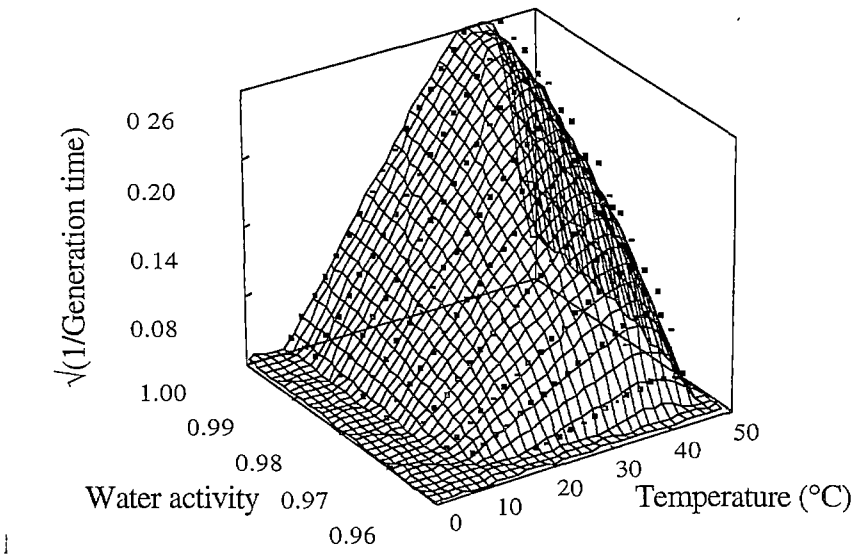


Figure 2.6 Response surface for growth rate of *E. coli* NT (R31) with respect to temperature and water activity.

2.4.4 Comparison of model predictions with literature data

A comparison between generic M23 (Eqn. 15) and R31 (Eqn. 17) model predictions and literature data for rate of growth of non-pathogenic *E. coli* on meat is given in Table 2.5. There was good agreement between both Eqn. 15 and Eqn. 17 predictions and data for growth rates of *E. coli* on the surface of mutton carcases (Smith, 1985). Predictions by the Pathogen Modelling Program for the growth of *E. coli* O157:H7 also showed very good agreement, and were better than those of a modified Gompertz model and the D-model. Predictions of Eqns. 15 and 17 for growth rates of *E. coli* on sliced beef (lean) (Gill and Newton, 1980) and sliced beef (fat and lean) (Grau, 1983) were poor, as were predictions made by the three published models. The predictions of all models 'failed-safe' except for three predictions of Eqn. 17 when compared to data of Smith (1985). Eqn. 17 performed better than Eqn. 15 for all three

Table 2.5 Comparison of published and predicted generation times for non-pathogenic *E. coli* in meat.

Temp (°C)	pH	NaCl (%w/v)	Substrate	Inoculum (log cfu/ml)	GT_{publ} (h)	GT_{pred} (h)$_{M23}$	GT_{pred} (h)$_{R31}$	Bias	Accuracy	Reference
20	5.5	0.5	sliced beef (lean)	4	1.8	1.12	1.22	0.43[1]	2.31[1]	Gill and Newton (1980)
20	5.5	0.5	sliced beef (lean)	4	2.1	1.12	1.22	0.47[2]	2.12[2]	
30	5.5	0.5	sliced beef (lean)	4	1.2	0.42	0.46	0.27[3]	3.65[3]	
30	5.5	0.5	sliced beef (lean)	4	1.4	0.42	0.46	0.45[4]	2.20[4]	
								0.62[5]	1.62[5]	
25	5.6	0.5	sliced beef (fat)	3	0.72	0.65	0.71	0.53[1]	1.90[1]	Grau (1983)
25	6.9	0.5	sliced beef (lean)	3	1.6	0.65	0.71	0.57[2]	1.75[2]	
25	6.5	0.5	sliced beef (lean)	3	1.53	0.65	0.71	0.46[3]	2.17[3]	
25	6.4	0.5	sliced beef (lean)	3	1.40	0.65	0.71	0 50[4]	1.99[4]	
25	6.2	0.5	sliced beef (lean)	3	1.45	0.65	0.71	0.65[5]	1.61[5]	
25	5 9	0.5	sliced beef (lean)	3	1.20	0.65	0.71			
25	5.7	0.5	sliced beef (lean)	3	1.13	0.65	0.71			
25	5.6	0 5	sliced beef (lean)	3	1.10	0.65	0.71			
25	5.5	0.5	sliced beef (lean)	3	1.24	0.65	0 71			
8.2	5.7*	0.5	mutton carcase surface	4	17.2	17.87	19.05	0.90[1]	1.18[1]	Smith (1985)
10	5.7*	0.5	mutton carcase surface	4	6.9	8 42	9.07	0.97[2]	1.13[2]	
15	5.7*	0.5	mutton carcase surface	4	2.6	2.41	2.62	0.79[3]	1.29[3]	
20	5.7*	0.5	mutton carcase surface	4	1.4	1.12	1.22	0.78[4]	1.34[4]	
25	5.7*	0.5	mutton carcase surface	4	0.78	0.65	0.71	0.88[5]	1.20[5]	
30	5.7*	0.5	mutton carcase surface	4	0.52	0.42	0.46			
35	5.7*	0 5	mutton carcase surface	4	0 37	0.30	0.33			
40	5.7*	0.5	mutton carcase surface	4	0.3	0.25	0.26			

GT_{publ} = published generation time; $GT_{predM23}$ = predictions from M23 model; $GT_{predR31}$ = predictions from R31 model; *data of Smith (1985) originally reported at pH 5.7- 6.3, but adjusted to pH 5.7 by Sutherland *et al.* (1995); bias and accuracy values indicate comparison of published data with [1]M23 model, [2]R31 model, [3]modified Gompertz model (Sutherland *et al.*, 1995), [4]D-model (Baranyi *et al.*, 1993), [5]Pathogen Modelling Program (Buchanan, 1991).

Table 2.6 Comparison of published and predicted generation times for *E. coli* O157:H7 in growth media and food.

Temp (°C)	pH	NaCl (%w/v)	Substrate	Inoculum (log cfu/ml)	GT_{publ} (h)	GT_{pred} (h)$_{M23}$	GT_{pred} (h)$_{R31}$	Bias	Accuracy	Reference
21	6.1	0.5	ground roasted beef	5.0	1.57	0.99	1 08	0.70[1]	1.43[1]	Abdul-Raouf *et al.* (1993)
30	6.1	0.5	ground roasted beef	5.0	0.56	0.42	0.46	0.74[2]	1.34[2]	
21	6 0	0.5	ground roasted beef	4 8	1.35	0.99	1.08	0.67[5]	1.50[5]	
30	6.0	0.5	ground roasted beef	4.8	0.68	0.42	0.46			
25	7.0	0.5	cantaloupe	3	1.51	0.65	0.71	0.61[1]	1.63[1]	del Rosario and Beuchat (1995)
25	5.6	0.5	watermelon	3	0.77	0.65	0.71	0.65[2]	1.53[2]	
								0.64[5]	1.57[5]	
25	7.3*	0.5	Trypticase soy broth	3	1.46	0.65	0.71	0.51[1]	1.98[1]	Doyle and Schoeni (1984)
30	7.3*	0.5	Trypticase soy broth	3	0.58	0.42	0.46	0.53[2]	1.89[2]	
37	7.3*	0.5	Trypticase soy broth	3	0.49	0.27	0.29	0.53[5]	1.88[5]	
40	7.3*	0.5	Trypticase soy broth	3	0.57	0.25	0.26			
42	7 3*	0.5	Trypticase soy broth	3	0.64	0.25	0.25			
44.5	7.3*	0.5	Trypticase soy broth	3	1.08‡	0.42	0.30			
45	7.3*	0.5	Trypticase soy broth	3	1.26‡	0.52	0.32			
45.5	7.3*	0.5	Trypticase soy broth	3	1.21‡	0.70	0.35			
12	5.7	0.5	minced beef	3 - 4	6.0	4.30	5.02	0.79[1]	1.30[1]	Walls and Scott (1996)
12	6.3	0.5	minced beef	3 - 4	3.9	4.30	5.02	0.91[2]	1.20[2]	
20	5.7	0.5	minced beef	3 - 4	1.5	1.12	1.22	0.85[5]	1.18[5]	
20	6.3	0.5	minced beef	3 - 4	1.3	1.12	1.22			
35	5.7	0.5	minced beef	3 - 4	0.4	0.28	0.33			
35	6.4	0.5	minced beef	3 - 4	0 4	0.28	0.33			
15	6.9	0.5	unpasteurised milk	3	3.26	2.41	2.62	0.59[1]	1.68[1]	Wang *et al.* (1997)
8	7.1	0.5	homogenised milk	3	24.96‡	19.84	21.11	0.65[2]	1.54[2]	
15	7.1	0.5	homogenised milk	3	3.84	2.41	2.62	0.54[5]	1.86[5]	
22	7.1	0.5	homogenised milk	3	1.25	0.88	0.96			
8	7.1	0.5	low fat milk	3	10.56‡	19.84	21.11			
15	7.1	0.5	low fat milk	3	3.13	2.41	2.62			
22	7.1	0.5	low fat milk	3	2.25	0.88	0.96			
8	7 1	0.5	skim milk	3	20.64‡	19.84	21.11			
15	7.1	0.5	skim milk	3	3.8	2.41	2.62			
22	7.1	0.5	skim milk	3	2.09	0.88	0.96			

Abbreviations as in Table 2.6; *pH not specified, therefore assumed; ‡treatment outside bounds of Pathogen Modelling Program, therefore not included in model comparison.

datasets. The performance of the modified Gompertz- and D-models could only be assessed with respect to the datasets in Table 2.5 as predictions were taken from Sutherland *et al.* (1995) in which only these datasets were considered.

In most cases, predictions of Eqns. 15 and 17 were adequate when compared to published growth rate data for *E. coli* O157:H7 in ground roasted beef, cantaloupe and watermelon, milk and Trypticase soy broth (Table 2.6). Predictions of the Pathogen Modelling Program were similar, but in several cases (8, 44.5, 45 and 45.5°C) the temperatures encountered on foods and in broth were outside the bounds of the model and so its performance was not able to be compared with that of Eqns. 15 and 17. Overall, model predictions 'failed-safe'. Again, Eqn. 17 performed better than Eqn. 15 for all datasets.

2.5 Discussion

Comparison of STEC growth rate data with Eqn. 14 in terms of model parameters indicated that there was good agreement between the two. Of most relevance to the growth of pathogens in food is the range from refrigeration to room temperature, encompassing both good storage practice as well as possible product temperature abuse. In this range, predictions of the generic model agreed very closely with observed growth rates of STECs. Predictions at temperatures approaching T_{max} were less accurate, but the performance of the models in this temperature range is not generally relevant to foods stored at room temperature or below.

Comparison of STEC growth rate data with Eqn. 16 in terms of model parameters indicated similar agreement with that for Eqn. 14, in that there was a discrepancy between one parameter of the generic model and that of STEC models. While the discrepancy occurred for a comparison of T_{max} values of Eqn. 14 and STEC models, it arose in a comparison of b values of Eqn. 16 and STEC models. However, predictions of Eqn. 16 at temperatures approaching T_{max} should be more accurate than those of Eqn.

14 as there was not a significant difference between T_{max} values of Eqn. 16 and any STEC model T_{max} value.

In most cases, growth of the STECs in liquid medium at a fixed water activity was adequately described by the generic temperature models for non-pathogenic *E. coli.* The accuracy factor showed that there was good agreement between values of predicted and observed growth rates, with the only unusually high accuracy values occurring for a comparison between predictions of Eqn. 14 and growth rates of both *E. coli* O88:H- strains. As mentioned below, this is thought to arise due to *E. coli* O88:H-'s unusually slow growth rate. Calculation of the bias factors also indicated good agreement between predictions of Eqns. 14 and 16 and observed growth rates of STECs. In the comparison involving Eqn. 14 bias factors were below 1 meaning that on average, Eqn. 14 over-predicted growth rates of STECs. Thus, when compared to data generated in this thesis, the M23 model performed as expected and consistently generated 'fail-safe' predictions. In the comparison between Eqn. 16 and STEC data, bias values were scattered below and above 1, indicating that predictions of Eqn. 16 were not always 'fail-safe'. Unlike Eqn. 14, this model was not made to predict the fastest growth rate probable and so would not be expected to make 'fail-safe' predictions in all cases, despite consistently making more accurate predictions than Eqn. 14.

The higher accuracy of Eqn. 16 stems from its bias, which is scattered below and above 1 and not always below 1 like that of Eqn. 14. When the b value of Eqn. 14 was reduced by 5%, the accuracy of Eqn. 14 surpassed that of Eqn. 16 in nearly all cases. The bias was also greatly improved and all predictions still 'failed-safe'. This example illustrates the importance of considering both accuracy and bias factors. A consideration of accuracy alone would lead to the selection of Eqn. 16 as the model of choice for predicting the growth rate of the STEC strains used in this study. However, model choice must be based on a combination of accurate *and* 'fail-safe' predictions. It was on this premise that Eqn. 14 was used in subsequent comparisons.

Notable was the high level of similarity in the growth characteristics of most STEC strains. However, growth rates of both O88:H- strains were ≈30% slower than

generic model predictions. Because both O88:H- datasets were similar, it would appear that O88:H- is an unusually slow-growing serotype. Discrepancies between observed growth rates and model predictions could also be attributed partly to slightly elevated T_{min} values for both O88:H- strains, compared to other strains.

The similarity between STEC strains and the conservative nature of Eqn. 14 was also apparent from Figs. 2.3 and 2.4. The largest variation between observed and predicted GT, at the lower temperature limits of growth, was thought to result from the larger inherent variability of bacterial growth responses close to growth-limiting temperatures (Ratkowsky *et al.*, 1991).

When any data derived from turbidimetric experiments is converted to equivalent viable count data (see Section 2.3.2), it must be remembered that the conversion factor used (a factor of 1.5 in this case) is the average of a number of conversion factors for specific bacteria, with some having relatively large standard deviations. There exists no known conversion factor for *E. coli* in the literature, therefore the factor for a morphologically similar bacterium, *Pseudomonas*, was chosen from the literature (Neumeyer, 1995) This and other conversion factors (*eg.* Dalgaard *et al.*, 1994) were averaged from a number of factors which is a flawed process in itself as it may not be mathematically valid to take the average of a series of ratios (Ratkowsky, pers. comm). In addition, each of the ratios in the series averaged by Dalgaard *et al.* (1995) was the result of varying numbers of growth curves and so each carried a varying degree of weight. Therefore, when turbidimetric data is compared to a model derived from viable count methods, the conversion factor used has an inherent error which must be considered in subsequent comparisons.

When compared to published data for the growth of non-pathogenic *E. coli* on meat, two generic models (Eqns. 15 and 17) performed similarly to three published models for *E. coli* O157:H7. Poor agreement with the data of Gill and Newton (1980) and Grau (1983) could not be explained, but the performance of all of the published models was similar. Similar to this study, a model derived by Gibson *et al.* (1988) for the growth rate response of salmonellae agreed well with observed growh of non-

pathogenic *E. coli* on mutton carcass surface. Comparison of predictions with literature growth rate data for *E. coli* O157:H7 in ground roasted beef, melons, milk of varying fat content and Trypticase soy broth yielded similar results. Growth rate predictions of Eqns. 15 and 17 were 1.5-2.0-fold faster than observed in most cases, but the Pathogen Modelling Program performed similarly. Some of this overprediction may be accounted for by lack of accurate water activity values for the foods considered. Also, it is known that the Gompertz function overpredicts the exponential growth rate, so that rates not derived from fitted Gompertz functions will be lower than analogous Gompertz function-based estimates (Whiting and Cygnarowicz-Provost, 1992; Baranyi *et al.*, 1993). The polynomial models of Sutherland *et al.* (1995) and the Pathogen Modelling Program are based on Gompertz-derived growth rate estimates. Again, Eqn. 17 performed slightly better than Eqn. 15 but this was due to the deliberately conservative nature of Eqn. 15. Despite Eqn. 17 giving more accurate predictions of growth of *E. coli* in real food systems it sometimes 'failed-dangerous'. The 'fail-safe' nature of Eqn. 15 is more desirable than that of Eqn. 17 despite the former being less accurate.

Nonetheless, that three models derived from independent data sets in independent laboratories are essentially consistent, but deviate markedly from independent published reports of *E. coli* growth rates in foods, begs comment. The models do predict well the growth rates reported in some of the data sets (*eg.* Walls and Scott, 1996; Smith, 1985). This suggests that either there is a deficiency in all of the models, or that some published data are not truly representative of the growth potential of bacteria in foods.

There are contradictory reports in the literature regarding the growth of *E. coli* O157:H7 at super-optimal temperatures. Doyle and Schoeni (1984) reported that for a single strain of O157:H7, growth was poor in the range 44 to 45.5°C, a temperature region used traditionally to isolate *E. coli* selectively, while Raghubeer and Matches (1990) observed that for a single strain, growth did not occur above 41°C within 48h. All STECs used in this study, except O157:H-, grew at temperatures up to 45°C, with many showing substantial growth (absorbance >0.3) at 46 and 47°C. In this temperature region, only two strains tested did not attain a population density after 24h of greater than

10^8 CFU/ml. This is consistent with the results of Palumbo *et al.* (1995) who showed that over 20 strains of O157:H7 grew to at least 45°C, and reinforces that temperatures in the range 44 to 45.5°C are suitable for isolating most STEC strains.

The present study suggests that there is little difference in the growth responses of different strains of *E.coli*, including pathogenic strains, and that generic models for the growth of non-pathogenic laboratory strains of *E. coli* are sufficient to describe the growth of many STECs. While some strains were shown to grow more slowly, there was a high level of similarity in the growth of fast growing strains, which facilitates the development of a 'fail-safe' model. The generic models (Eqns. 15 and 17) performed as well as three published models developed for predicting the growth rate of pathogenic *E. coli* in a variety of foods and in nutrient medium. STECs were not exceptional in that their growth was adequately described by the generic models and, because there was sufficient viability between 44 and 45.5°C to enable detection in diagnostic procedures using incubation in this temperature range.

Appendix 2.1 Sources of Isolates

Table A2.1 Source and toxin-production of STEC strains obtained from the Royal Hobart Hospital and Royal Melbourne Children's Hospital.

		Isolation		
Serotype	Code	Source	Location	Toxins
O126:H21	R10	Water	Lauderdale Canal	-
NT*	R31	Water	Gypsy Bay	Stx1
O81:H-	R91	Pork	-	Stx2
ONT*:H8	R106	Beef	-	Stx2
O88:H-	R171	Potable Water	-	-
O88:H-	R172	Water	McRobies Gully	Stx1
O134:H-	R216	Water	Tyenna River	-
O86:H-	R218	Water	Tyenna River	-
O157:H-	-	Diarrhoea Patient	-	Stx1, Stx2
O157:H7	EH9	HUS Patient	-	Stx1, Stx2
O111:H-	EH39	HUS Patient	-	Stx1, Stx2

*NT = non-typable.

Appendix 2.2 Maintenance of Bacterial Cultures

Cultures were maintained aerobically on standard plate count agar (PCA) slopes at 4°C and sub-cultured every three months from stock cultures. Stock cultures were prepared by growing strains overnight on PCA plates and suspending resulting colonies in 1ml of 15% (v/v) glycerol in NB. Each suspension was aseptically pipetted into sterile vials containing 10-15 3mm beads and aspirated to remove air-bubbles. Vials were stored at -70°C.

Appendix 2.3 Growth Media

Nutrient Broth (Oxoid, CM 67) was prepared according to the manufacturer's instructions. Sterilisation was achieved by autoclaving for 15min at 121°C. When NaCl was added, sterilisation was achieved by autoclaving for 30min at 105°C to avoid formation of a precipitate.

Plate Count Agar (Oxoid, CM 463) was prepared according to the manufacturer's instructions and sterilised.

Appendix 2.4 Example Approximate t-Test Calculation

To test whether there is a significant difference between T_{min} of the M23 temperature model and T_{min} for STEC O126:H21 (R10) at a confidence interval of 95% (p=0.05):

M23 model

T_{min}= 4.263±1.041

Degrees of freedom = data points - parameters

= 53

STEC R10

T_{min}= 4.758±0.874

Degrees of freedom = 21

Now,

$$t = \frac{4.758 - 4.263}{\sqrt{(0.874)^2 + (1.041)^2}}$$

$$\therefore t = \frac{0.495}{1.359}$$

$$\therefore t = 0.364$$

t has an approximate t-distribution with 53 + 21 = 74 degrees of freedom.

The critical value for t at p = 0.05 and 74 degrees of freedom is ~2.0 (from a table of the distribution of t, *eg.* Bishop (1966)). Since |0.364| < 2.0, the null hypothesis is accepted. That is, there is no significant difference between the T_{min} for the M23 model and T_{min} for STEC R31.

Appendix 2.5 T_{opt} Calculation

T_{opt} was calculated from a knowledge of other model parameters by solving the following equation:

$$\exp(c \times (T_{max} - T_{opt}) - 1 - c \times (T_{opt} - T_{min}) = 0$$

This was performed using the "Solver" routine of Excel software (Microsoft Excel Version 4.0 © 1985 - 1992. Microsoft Corporation, 1 Microsoft Way, Redmond, WA, USA).

Appendix 2.6 Experimental Data

The following is 'raw' tubidimetric data.

Table A2.2 Temperature growth rate data for STEC strains O126H21, NT and ONT:H8 (Figs. 2.2a-c).

O126:H21 (R10)		NT (R31)		ONT:H8 (R91)	
Temperature (°C)	Generation time (min)	Temperature (°C)	Generation time (min)	Temperature (°C)	Generation time (min)
10.4	1083.20	7.6	3630.73	9.0	1638.64
12.1	579.72	10.3	1147.58	11.3	631.26
13.3	344.37	12.0	558.35	13.1	430.64
14.7	248.99	13.2	393.11	14.7	270.42
16.2	181.36	14.5	289.08	15.9	217.85
17.6	145.28	16.0	207.33	17.3	169.32
18.9	116.49	17.4	161.82	18.6	131.40
20.3	101.46	18.5	133.93	20.0	111.41
21.6	80.46	20.2	100.01	21.3	93.42
23.0	69.12	21.5	87.97	22.7	71.36
24.3	61.20	22.7	71.08	24.0	70.09
25.6	52.63	24.1	60.12	25.2	63.46
26.8	46.81	25.4	52.07	26.4	52.46
28.6	45.32	26.7	48.57	28.1	44.46
29.8	37.78	27.9	38.83	29.4	39.42
31.0	35.42	29.3	40.23	30.6	37.53
32.2	32.77	30.6	34.63	32.0	33.36
33.7	31.49	32.1	31.95	33.4	30.94
35.3	25.17	33.6	29.80	34.9	30.93
36.9	26.15	35.0	28.19	36.5	29.06
38.5	24.68	36.7	25.52	38.0	25.66
40.1	22.93	38.0	25.10	39.7	24.19
41.7	23.05	40.1	23.07	41.2	22.34
43.5	23.05	41.9	23.22	42.8	26.51
45.7	29.42	43.6	23.04	44.8	34.95
47.2	60.32	45.6	27.32	46.5	70.81
		47.4	79.76		

Table A2.3 Temperature growth rate data for STEC strains O81:H-, O88:H- (R171) and O88:H- (R172) (Figs. 2.2d-f).

O81:H- (R106)		O88:H- (R171)		O88:H- (R172)	
Temperature (°C)	Generation time (min)	Temperature (°C)	Generation time (min)	Temperature (°C)	Generation time (min)
8.9	1315.06	8.9	4191.57	8.7	3496.27
11.4	675.18	11.2	986.62	11.3	1727.58
12.8	382.28	13.0	680.34	12.8	590.48
14.3	264.07	14.5	377.22	14.3	399.30
15.8	187.96	16.1	293.81	15.9	270.00
17.2	172.05	17 4	178.58	17.4	187.82
18.7	131.31	18.9	163.66	18.9	175.59
20.1	115.18	20.3	128.29	20.2	137.37
21.4	89.32	21.5	105.51	21.5	106.09
22.6	76.22	22.9	100.12	22.8	91.71
24.1	68.51	24.3	75.52	24.2	80.00
25.5	57.92	25.6	69.88	25.6	71.78
26.7	52.93	26.8	59.62	26.8	63.76
28.1	45.90	28.5	52.38	28.2	52.29
29.6	40.21	29.8	45.80	29.5	48.10
30.8	34.53	31.0	43.24	30.8	43.78
32.0	34.02	32.4	38.89	32.2	36.75
33.6	32.75	33.8	35.10	33.6	34.59
35.1	30.10	35.4	29.87	35.0	31.62
36.7	28.56	36 9	28.32	36.9	29.88
38.2	25.95	38.4	26.85	38.3	27.85
39.9	26.22	40.1	24.24	40.1	24.58
41.6	23.45	41.8	23.51	41.6	25.11
43.2	27.22	43.6	22.98	43.5	24.56
45.0	29.98	45.9	30.05	45.4	28.64
46.5	68.76				

Table A2.4 Temperature growth rate data for STEC strains O157:H- and O157:H7 (Figs. 2.2g-h).

O157:H-		O157:H- (continued)		O157:H7 (EH9)	
Temperature (°C)	Generation time (min)	Temperature (°C)	Generation time (min)	Temperature (°C)	Generation time (min)
8.0	1762.01	29.5	27.32	8.1	4213.46
8.0	1711.36	29.7	28.89	9.9	1331.27
10.0	519.10	29.9	28.06	11.9	579.50
10.0	495.80	30.3	26.85	13.3	451.64
12.0	413.44	30.5	26.12	14.6	278.18
12.0	413.67	31.2	23 44	15.8	170.01
14.0	300.19	31.5	23.85	17.1	104.97
14.0	315.28	31.6	24.94	18.3	162.93
16.2	105.47	32.4	21.73	19.5	122.39
18.0	85.31	32.8	20.92	20.7	108.59
18.8	79.52	32.9	24.01	21.6	111.41
20.7	67.60	34.3	21.57	22.9	81.70
21.5	56.17	34.4	20.28	24.3	79.51
23.2	48.49	35.8	17.89	25.4	62.01
24.1	41.39	36.2	18.72	26.6	57.05
25.8	38.98	37.9	17.17	27.7	48.04
26.8	34.68	38.5	16.92	29.2	42.17
28.4	31 40	40.5	17.83	30.4	38.56
28.4	30.80	41.4	16.15	31.8	34.71
28.5	28.38	44.1	25.38	33.1	34.65
28.5	32.42	44.8	30.17	34.6	30.04
28.7	29.16			36.3	26.60
28.7	30.59			37.8	28.76
28.9	27.58			39.6	23.73
28.9	27.34			41.8	24.28
29.1	27.58			43.6	29.57
29.2	28.63				

Table A2.5 Temperature growth rate data for STEC strain O111:H- (Fig. 2.2i).

O111:H-	
Temperature (°C)	Generation time (min)
7.7	4939.47
9.4	1844.49
11.2	852.50
13.1	499.26
14.3	344.46
15.7	271.66
17.1	210.06
18 2	169.91
19.5	142.28
20.7	116.85
22.0	93.01
23.3	79.13
24.4	72.78
25.6	57.81
27.0	50.22
28.2	47.35
29.7	41.09
31.0	39.76
32.5	35.17
34.1	31.07
35.3	28.86
37.3	25.50
38.7	24.10
41.0	23.58
42.9	24.13
45.2	28.54
46.8	58.89

Table A2.6 Temperature/water activity growth rate data for STEC strain NT (Fig. 2.5).

NT								
T (°C)	a_w	GT* (mın)	T (°C)	a_w	GT* (mın)	T (°C)	a_w	GT* (min)
7.6	1.00	2420.49	20.0	0.99	72.97	30.0	0.98	36.74
10 3	1.00	765.05	20.0	0.99	81.17	30.0	0.98	36.58
12.0	1.00	372.24	20.0	0.98	90.50	30.0	0.98	46.26
13.2	1.00	262.07	20.0	0.98	112.04	30.0	0.98	65.26
14.5	1.00	192.72	20 0	0.98	142.47	30.0	0.97	75.32
15.0	0.99	162.91	20.0	0.97	206.82	30.6	1.00	23.09
15.0	0.99	176.95	20.0	0.97	248.34	32.1	1.00	21.30
15.0	0.99	200.20	20.2	1.00	66.68	33.6	1.00	19.87
15.0	0.98	225.79	21 5	1.00	58 64	35.0	1.00	18.79
15.0	0.98	251.74	22.7	1.00	47.38	36.7	1.00	17 01
15 0	0.98	303.39	24.1	1.00	40.08	38.0	1.00	16.73
15.0	0.97	420.70	25.0	0.99	54.85	40.1	1.00	15.38
15.0	0.97	641.87	25.0	0.99	56 98	41.9	1.00	15.48
15.0	0.97	1058.06	25.0	0.99	56.86	43.6	1.00	15.36
16.0	1.00	138.22	25.0	0.99	66.06	45.6	1.00	18.22
17.4	1.00	107.88	25.0	0.98	75.68	47.4	1.00	53.17
18.5	1.00	89.29	25.0	0.98	90.87			
20.0	0.99	63.07	25.0	0.98	116.91			
20.0	0.99	68.85	25.0	0.97	143.30			
20.0	0.99	65.82	25.0	0.97	277.89			
20.0	0.99	74.77	25.4	1.00	34.71			
20.0	0.98	90.02	26.7	1.00	32.38			
20 0	0.98	132.83	27.9	1.00	25.88			
20.0	0.97	182.83	29.3	1.00	26.82			
20.0	0.97	220.25	30.0	0.99	30.68			
20.0	0.99	63.68	30 0	0.99	32.53			
20.0	0.99	68.27	30.0	0.99	32.52			

*GT = generation time.

Chapter 3. Growth/No Growth of an STEC Strain

3.1 Summary

Two methods to determine the limits of growth of pathogenic *E. coli* strains (O126:H21, R10 and a non-typable strain, R31) as a function of combinations of temperature and water activity were assessed. A gradient plate method was investigated for generation of 'growth/no growth' data , but protocols available for detection of bacterial growth were found to lack sensitivity. A broth-based method was found to be more suitable and allowed the generation of quantitative results. From these data the growth/no growth boundary for combinations of temperature and water activity was defined for an STEC strain and modelled using a 'generalised non-linear regression model'. The model fitted with an approximate concordance rate of 96.5%.

3.2 Introduction

Much of the effort expended on generating predictive microbiology databases has focused on kinetic data, in which growth rates of microorganisms are determined in the normal biokinetic temperature range and under optimal or close to optimal water activity, pH and nutrient status. This strategy is adequate when the desired information is the extent of growth of food spoilage organisms. However, it is often important to assess the risk of outgrowth of pathogenic organisms, in which case, numbers as low as 1 cell per unit of food can be unacceptable. In such a case, that which must be modelled is the probability of a pathogenic organism growing at all under the extrinsic and intrinsic conditions experienced by the food product (see also Section 1.5.2).

The use of probability models appeared in the literature in the early 1970s when Genigeorgis *et al.* (1971), motivated by a need to predict safe combinations of conditions to prevent pathogen growth and toxin formation, modelled decimal reduction of *Staphylococcus aureus.* As discussed by Ross and McMeekin (1994), that model was used in subsequent studies using other organisms and combinations of environmental factors. A further development was the ability to predict whether or not an organism will grow under a given set of environmental conditions. Ratkowsky and Ross (1995) modified a kinetic model incorporating temperature, pH, water activity and nitrite concentration such that it could predict growth *vs* no growth. The modification involved taking the logarithm of both sides of the model equation and replacing the left-hand side with a logit term (logit (p), an abbreviation for ln[p(/1 - p)], where p is the probability of growth occurring). From this model the sets of combinations of conditions leading to a selected probability of growth could be determined. That was an example of a 'generalised linear regression model', where parameters such as T_{min} had been determined previously. Ratkowsky (pers. comm.) modified this equation so that all parameters are estimated and termed the model a 'generalised non-linear regression model'. This model was subsequently used by Presser *et al.* (In Press) to model the

growth/no growth boundary of a non-pathogenic *E. coli* strain with respect to temperature, pH, lactic acid concentration and water activity.

This study tested the suitability of a 'generalised non-linear regression model' for fitting growth/no growth data for a pathogenic *Escherichia coli* strain under combinations of temperature and water activity. Two methods were assessed for the acquisition of data: a gradient plate method and a broth method.

3.3 Materials and Methods

3.3.1 Gradient plate method

Agar plates were prepared by the technique of Wimpenny and Waters (1984). A water activity gradient was established by overlaying a wedge of agar containing 80g/20ml agar NaCl (Waters and Lloyd, 1985; Appendix 3.1) onto a wedge of PCA (Appendix 2.2) in 12×12cm square Petri-plates (Greiner Labortechnik, Germany). Agar was prepared as a stock solution from which 20ml-lots were pipetted into McCartney bottles and sterilised. Sterilised bottles were held at 80°C in water baths (Ratek Instruments, Australia), after which PCA containing NaCl was poured into Petri-plates which were raised approximately 3mm at one end. Once this layer had set, plates were laid flat and 20ml PCA was overlayed onto the first layer. Plates were prepared in a laminar flow cabinet. Once solidified, plates were sealed inside plastic bags and allowed to equilibrate for 24h at room temperature.

Equilibrated gradient plates were spread with a lawn of *E. coli* O126:H21 (R10) by inoculating 0.1ml of a culture previously grown in nutrient broth (Appendix 2.2) at 37°C for approximately 18h. The culture was spread evenly over plates using a sterile glass rod. A strip 1cm wide down the edge of each plate, running parallel with the NaCl gradient, was left uninoculated. Plates were incubated at 37°C for up to a week.

Determination of the amount of bacterial growth was attempted using a biuret method for protein estimation (Pelley *et al.*, 1978). In the biuret method, agar samples

were taken from plates using a 0.5mm-diameter cork-borer and added to the reaction mixture. The incubation temperature of 60°C recommended by Pelley et al. (1978) was increased to 80°C in order to melt the agar sample. Alongside each sample taken from the inoculated region, a sample was taken directly adjacent in the uninoculated region in order to assay for protein in the growth medium. %transmittance of the reaction mixture was read on a spectrophotometer (Varian Techtron, USA) at 545nm. A standard graph was constructed by adding varying concentrations of bovine serum albumin (BSA) (Sigma Chemical Co., USA) to the biuret reaction mixture and recording change in %transmittance at 545nm.

3.3.2 Broth-based method

Nutrient broth (NB, pH 7.4) (Appendix 2.2) was adjusted to desired water activity levels using NaCl and the tables of Chirife and Resnik (1984) as a guide. Broths were sterilised (121°C at 15psi for 15min) and 2ml of each broth was aseptically pipetted into four replicate wells in well plates (24 Multi-Well Plates, Linbro, USA). In each well plate, one set of four wells was used for each NaCl concentration and another set of four wells was filled with 2ml NB containing no NaCl. Initially, NaCl was added to NB at concentrations of 4.0-8.75% (w/w) at 0.5% increments. Once the approximate location of the growth/no growth boundary was determined, the range of NaCl concentrations used was decreased. In other experiments, 10ml of broth was aseptically pipetted into L-shaped, optical quality glass tubes (L-tubes). NaCl was added to NB at concentrations of 5.5-8.0% (w/w) at 0.5% increments to give a set of six tubes. One set of tubes was inserted into the TGI, with the tube containing the lowest NaCl concentration (5.5% (w/w)) inserted into the first slot at the low-temperature end of the TGI. The remaining tubes in the set were inserted in order of increasing NaCl concentration. The next set was inserted in the same order immediately after the first set, and so on. A duplicated series of tubes was inserted into the opposite side of the TGI. The water activity of aliquots of each broth was measured using a water activity meter (Aqualab model CX2, Decagon Devices, USA) (Appendix 3.1).

A non-typable STEC strain (R31) was incubated in NB at 37°C for approximately 18h. A 0.5ml aliquot was pipetted into 10mL NB in L-tubes, which were pre-incubated at 37°C in a water bath (Ratek Instruments, Australia). When this culture was growing exponentially (absorbance = 0.8), 20µl was pipetted into each well of previously prepared well-plates. This level of inoculation resulted in turbidity that was just detectable by the unaided eye. This culture was also used to inoculate two out of four wells containing NB only to serve as positive controls. Plates were sealed inside plastic bags and incubated in water baths or in incubators (Qualtex, Watson Victor Ltd., Australia) for up to 50 days. For broths in L-tubes, 100µl of inoculum was used and incubation was carried out on a temperature gradient incubator (TGI) (Model TN3, Toyo Kagaku Sangyo Ltd., Japan). The temperature of each tube was measured in quadruplicate using a Fluke 51 K/J thermometer (John Fluke MFI Co. Inc., USA). In most cases it was not possible to replicate tubes on the TGI with respect to temperature as opposing tubes were not held at exactly the same temperature.

Plates and tubes were observed daily and scored positively for growth in the case of visible increase in turbidity or a deposit in the base of a well. If after 50 days there was neither turbidity nor a deposit, a loopful of culture was streaked onto PCA (Appendix 2.2) according to the ecometric method of Mossel *et al.* (1983). This method avoided the need to serially dilute a culture and streak dilutions onto many plates for each treatment. A single plate was divided into four equal sectors and a loopful of culture was streaked four times in lines of decreasing length in each of the four sectors. Streaking was performed consistently each time by placing plates on a template. Growth of the test oganism was recorded to have occurred if there was only one colony type and if proliferation on ecometric plates exceeded that of a control, *ie.* one inoculated with a loopful of an initial culture. All positive cultures were presumptively identified as *Escherichia coli* by streaking onto Eosin Methylene Blue Agar (Appendix 3.1) and incubating overnight at 37°C.

3.3.3 Modelling growth/no growth data using 'generalised non-linear regression'

The response of *E. coli* at a given temperature/water activity combination was scored from 0 to 1 depending on the fraction of replicate wells or tubes exhibiting growth and no growth. For example, no growth in any well or tube for a single treatment was scored as 0, while growth in all wells or tubes was scored as 1. Growth in a quarter of tubes or wells was scored as 0.25 and so on. Data was fitted to a 'generalised non-linear regression' model using the SAS PROC.NLIN procedure (SAS Institute, 1990). The form of the model is similar to Eqn. 13 (Section 1.5.2) and is given by:

$$\begin{aligned} \text{logit}(p) = {} & b_0 + b_1 \ln(a_w - a_{w_{min}}) \\ & + b_2 \ln(T - T_{min}) + b_3 \ln(a_w - a_{w_{min}}) \ln(T - T_{min}) \\ & + b_4 (\ln(T - T_{min}))^2 \end{aligned} \qquad (17)$$

where b_0-b_4 are coefficients to be estimated and logit(p), p, a_w, a_{Wmin}, T and T_{min} have the same meaning as previously.

Goodness-of-fit of the model to observed data was assessed by a number of criteria calculated during the fitting procedure: concordance and discordance rate, an r^2-value, the Hosmer-Lemeshow goodness-of-fit statistic (Hosmer and Lemeshow, 1989) and the c-value. Ideal values are:

Concordance rate	100%
Discordance rate	0%
r^2	1
Hosmer-Lemeshow goodness-of-fit statistic	as close as possible to degrees of freedom

c-value 1

Descriptions of these criteria are given in SAS version 6.12 (SAS, 1997) and Nagelkerke (1991).

The growth/no growth boundary was determined by fixing Eqn. 13 fitted to the data at a probability level of 0.1 or 0.5. At known temperatures, corresponding water activity values were calculated using the "Solver" routine of Excel software (Microsoft Excel Version 4.0 © 1985 - 1992. Microsoft Corporation, 1 Microsoft Way, Redmond, WA, USA). The values were plotted graphically to give the growth/no growth boundary.

3.4 Results

3.4.1 Gradient plate method

A standard graph constructed using BSA in the range 0-0.008g in the biuret reaction mixture is shown in Figure 3.1. Initial experiments using a maximum 0.01g BSA per tube to calibrate the spectrophotometer failed to detect any protein even from samples containing substantial bacterial growth. The sensitivity of the assay was increased by finding the smallest amount of BSA which gave a full-scale deflection on the spectrophotometer. This was found to be 0.1mg. Using this BSA level as the standard, results were generated for a series of agar plugs from a single gradient plate (Table 3.1). There was only a small deflection for all samples and little difference between the measured protein content of inoculated and uninoculated agar plugs. When the protein content was increased by assaying four plugs at a time (Table 3.1) there was a larger deflection, but little difference, if any, between inoculated and uninoculated agar.

Calculation of the expected cell mass on four agar plugs is as follows:
assuming that cell density on plugs is 10^{10} CFU/cm^2 and protein mass of a single cell is 1.56×10^{-13}g (Neidhardt, 1996),

area of a single plug $= \pi \times \text{radius}^2$

$= \pi \times 0.25^2$

$= 0.20\text{cm}^2$

$\therefore$ area of four plugs $= 0.80\text{cm}^2$

$\therefore$ four agar plugs contain 0.8×10^{10} CFU/cm^2

$= 8 \times 10^9$ CFU/cm^2

$\therefore$ maximum cell mass on four agar plugs

$= (8 \times 10^{9)} \times 1.56 \times 10^{-13}\text{g}$

$= 1.25 \times 10^{-3}\text{g}$

$= 1.25\text{mg}.$

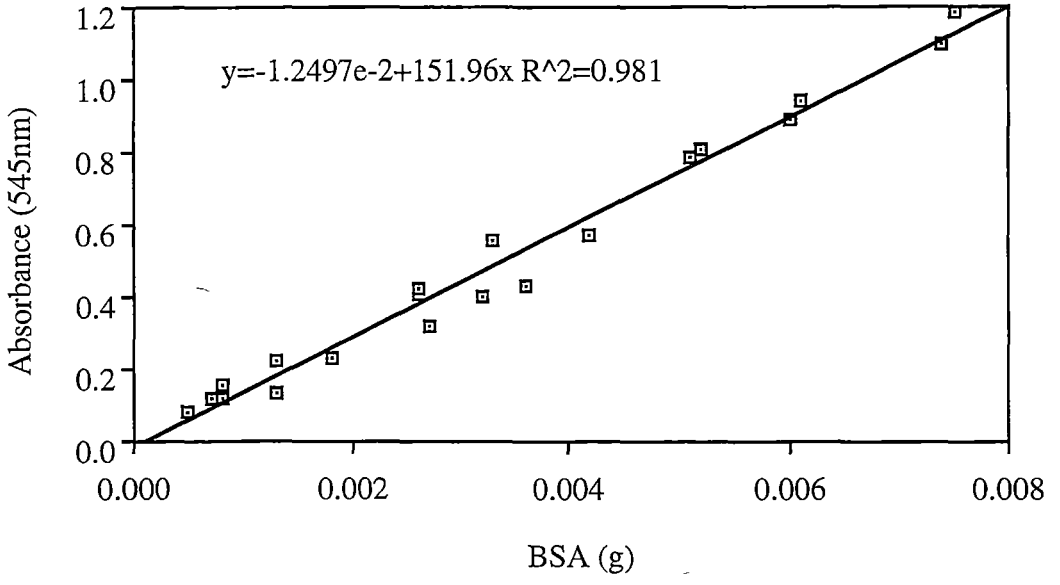


Figure 3.1 Standard graph of relationship between BSA (g) in biuret reaction mixture and absorbance (545nm).

Table 3.1 Results of a protein assay (545nm) for determination of extent of bacterial growth on gradient plates. Protein mass was calculated from the standard graph (Figure 3.1).

Sample	Distance from low [NaCl] end (cm)	Absorbance - inoculated agar	Protein mass (mg)	Absorbance - uninoculated agar	Protein mass (mg)
1 agar plug	1	0.0287	0.271	0.0182	0.202
	2	0.0241	0.241	0.0155	0.184
	3	0.0315	0.290	0.0164	0.190
	4	0.0287	0.271	0.0223	0.229
4 agar plugs	1	0.0731	0.563	0.0600	0.477
	2	0 0696	0.540	0.0752	0.577

3.4.2 Broth-based method

Water activity of broths was calculated from the data of Chirife and Resnik (1984) and agreed very well with measured values (Appendix 3.2).

Results of growth/no growth experiments using a broth-based method are presented in Appendix 3.2, with a plot of results given in Figure 3.2. For simplicity, a growth symbol in Figure 3.2 was only assigned when growth occurred in 50% or more of wells or tubes at a single condition. The minimum permissible water activity for growth occurred at between 25 and 30°C, with the minimum observed water activity for growth, 0.948, occurring at 25°C (4/4 cases) and 28°C (1/4 cases). The minimum water activity for growth increased rapidly below 20°C and increased slightly above 30°C. At 37°C, there was no growth (0/8) at a water activity of 0.949 and a positive growth score of only 12.5% (1/8) at a water activity of 0.951.

In most cases, the boundary between growth and no growth was well defined. At 10, 20, 25, 28 and 34°C, a decrease in water activity by a single interval (0.001 - 0.003 water activity units) at the boundary gave rise to a transition from growth in all

samples to no growth in all samples. At 9, 28 and 37°C, the transition from growth to no growth was not as sudden.

The fitted 'generalised non-linear regression model' which defines the boundary between growth and no growth (Eqn. 18) is:

$$\begin{aligned}\text{logit}(p) = {} & 94.1 + 50.6 \times \ln(a_w - 0.916) \\ & + 17.9 \times \ln(T - 5.10) - 9.63 \times \ln(a_w - 0.916) \\ & \times \ln(T - 5.10) - 8.22 \times (\ln(T - 5.10))^2\end{aligned} \qquad (18)$$

where p, a_w and T have the same meaning as previously.

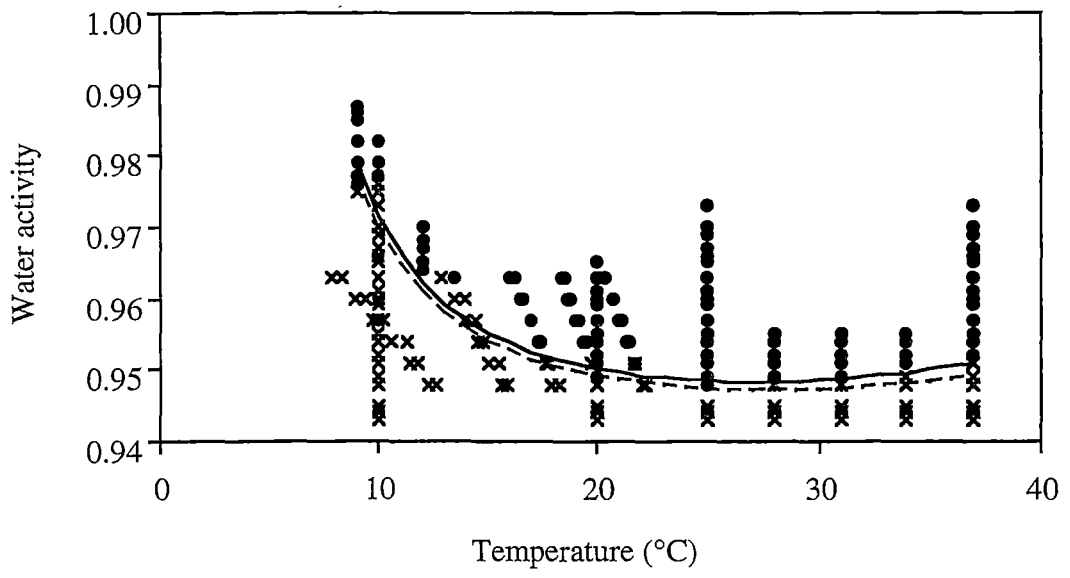


Figure 3.2 Growth/no growth boundary of *E. coli* NT (R31) with respect to temperature and water activity and fitted to a 'generalised non-linear regression' model (Eqn. 18). ×, no growth; •, growth; solid line, model prediction (p = 0.5); dashed line, model prediction (p = 0.1).

The probabilities of growth predicted by the fitted model were set by varying the value of p. Figure 3.2 shows two sets of model predictions; one made at p = 0.5 and

another at p = 0.1. The predicted boundary at p = 0.1 moved only slightly on the no growth side compared to the boundary predicted at p = 0.5.

The fit converged with a concordance rate of 96.5%. Most observations above 20°C close to the growth/no growth boundary agreed with model predictions. Data below 20°C accounted for the 3.5% discordance, with several points falling on the wrong side of the predicted boundary. The goodness-of-fit of the model to observed data was described by an r^2-value (0.800), the Hosmer-Lemeshow goodness-of-fit statistic (52.1 with 8 degrees of freedom (DF)) and a c-value (0.965).

T_{min} was fixed at 5.1°C (T_{min} for STEC R31 calculated from kinetic data; see Table 2.2) in the fitting process while all other parameters converged to the values shown in Eqn. 18., including a_{wmin}, which converged at 0.916. Allowing T_{min} to be estimated in the fitting process resulted in an unrealistically low value for T_{min} compared to the value found from kinetic studies (see Chapter 2). Fixing the value of T_{min} 1.5°C lower at 3.6°C (an arbitrary reduction in T_{min} to gauge the effect on the goodness-of-fit of the model to observed data) resulted in a higher a_{wmin} (0.930) and an improved fit. The resulting model is given by

$$\begin{aligned} \text{logit}(p) = {} & 45.4 + 41.5 \times \ln(a_w - 0.930) \\ & + 41.1 \times \ln(T - 3.60) - 8.85 \times \ln(a_w - 0.930) \\ & \times \ln(T - 3.60) - 12.1 \times (\ln(T - 3.60))^2 \end{aligned} \qquad (19)$$

where p, a_w and T have the same meaning as previously.

r^2, the goodness-of-fit statistic and the c-value were calculated to be 0.816, 10.7 (8 DF) and 0.969, respectively. Concordance rate was 96.8%. Fixing T_{min} at a lower value still (-2°C) resulted in a higher converged value for a_{wmin} (0.935) and a better fit to observed data as indicated by improved fitting criteria with the exception of one (goodness-of-fit statistic). r^2, the goodness-of-fit statistic and the c-value were calculated to be 0.836, 22.3 (8 DF) and 0.975, respectively.

3.5 Discussion

Determination of the growth/no growth boundary using a gradient plate method only progressed to the stage of assaying for bacterial growth. Using the most sensitive level of detection possible on the spectrophotometer, there was a small deflection using a single inoculated agar plug and a larger deflection using four inoculated agar plugs. However, the deflection for uninoculated agar plugs was very similar to that of inoculated plugs in all cases, indicating that the protein content of the agar was comparable to that of the bacterial growth on inoculated samples. Providing the assay could have been corrected to allow for protein concentration of the agar, the protein present was insufficient to cause a measurable spectrophotometric deflection, even using the most sensitive level of detection possible. Calculated cell mass on four agar plugs was 1.25mg assuming a cell density of 10^{10} CFU/cm^2. Therefore, the protein assay should have been easily sensitive enough considering a standard plot was constructed using 0.1mg BSA and less. The fact that only a small deflection was observed indicates that cell density was considerably less than 10^{10} CFU/cm^2, which is not surprising as assaying was done from cultures growing under salt stress. In this case, a cell density of 10^{10} CFU/cm^2 would be unlikely. Assuming a cell density of 10^8 CFU/cm^2 (equivalent to 0.0125mg protein) which is still relatively high for a population under salt stress, the protein present would be at the limits of detection which probably explains the very small amount of protein detected using spectrophotometry. Visual detection of growth was considered to be insufficient at the growth/no growth boundary, where only a small increase in cell numbers above inoculum levels would be expected. McClure and Roberts (1986) found that a stain specific to the group of organisms being targeted was required for visual determination of the growth/no growth boundary. A further weakness of this method is that the NaCl gradient begins to deteriorate even after 72h (McClure *et al.*, 1989), whereas incubation times of many weeks may be required to resolve the lag time of cells growing under salt stress. Other workers (*eg.* McClure and Roberts, 1986; McClure *et al.*, 1989) mapped bacterial growth on gradient plates using

scanning laser densitometry. They achieved very accurate determinations of the growth/no growth boundary and even information about the extent of bacterial growth within the growth region. As visual detection on agar was considered to be insufficient for exact determination of the growth/no growth boundary, a different approach was sought.

The growth/no growth boundary was mapped successfully using a broth-based method. By adding NaCl to NB at 0.25% (w/w) increments (Table A3.1), water activity increments obtained were of the order of 0.001-0.003 units, which is at the limit of the resolution of the water activity meter. Therefore, the smallest water activity increments verifiable in this laboratory were attained.

Contrary to the results of workers such as Ohye and Christian (1967) and Wodzinski and Frazier (1960) (see Section 1.3.5), low water activity was not tolerated best at temperatures close to optimal for growth rate. Growth at the lowest water activity (0.948) occurred at temperatures in the range 25-30°C, while the minimum observed water activity for growth at the optimal growth temperature (37°C) was 0.951. Only one of eight replicated samples grew at a water activity of 0.951, while eight of eight replicated samples grew at a water activity of 0.952. This raises a question about the physiological state of the bacterial cell at close to optimal temperatures. Previously the response of bacteria to environmental constraints was thought to be optimal at optimal growth temperatures (Ohye and Christian, 1967; Wodzinski and Frazier, 1960). In survival studies at least, bacteria have been found to be afforded better protection against one constraint when temperatures were far from optimal for growth. For example, *E. coli* O157:H7 survived in mayonnaise (pH 3.6-3.9) for longer at 5°C (34-55 days) than at 20°C (8-21 days) (Zhao and Doyle, 1994). The same appears to be true for the growth/no growth response of this STEC strain with respect to temperature and water activity. Cells incubated at optimal temperatures were more vulnerable to salt-stress than cells incubated 7-12°C lower at 25-30°C. This observation was also noted by McMeekin *et al.* (1997) who cited the results of Clavero and Beuchat (1996). These authors, like Zhao and Doyle (1994) found that *E. coli* O157:H7 survived better at 5°C than 20°C or

30°C regardless of the pH or water activity. Therefore, it appears that survival *and* growth of *E. coli* under low water activity and other constraints occurs more favourably at temperatures distant from T_{opt}. That is, incubation of cells at temperatures close to T_{opt} renders them more vulnerable to other stresses. A possible mechanism for this response has been proposed by Ross (In Press) in describing cell yield with reference to temperature. It was noticed from kinetic studies of *E. coli* that the cell yield (a measure of the conversion of available energy into biomass) was maximal between approximately 15 and 30°C and that above and below this range, yield quickly declined. While growth rate was optimal at about 37°C, cell yield was not, possibly due to denaturation of a rate-limiting enzyme below 15°C and above 30°C. The concept of a rate-limiting enzyme was proposed over 50 years ago (Crozier, 1924; Sizer, 1943). Ross (In Press) proposed that at optimal growth temperature and under no other rate-limiting environmental constraint, the cell is stressed but this is not apparent from the observed growth rate. When an additional environmental constraint is added (*eg.* salt stress), the stress previously undetectable at optimal temperatures becomes apparent as a slowing of growth rate or inability to grow at all. Ross (In Press) showed that if growth rate data is shown as an Arrhenius plot (ln growth rate versus 1/absolute temperature) (Arrhenius, 1889) most of the growth rate plot is a straight line but there are deviations at high and low temperatures. These deviations have been attributed to denaturation of one or more rate-limiting macromolecules, possibly enzymes.

It is important to note that experiments in the present study were performed in broth of pH 7.4, whereas from the tables of Sutherland *et al.* (1995), beef carcass surface pH can range from 5.5 to 6.5 with a lactic acid concentration of approximately 9.0mg/g (Gill, 1982) (equivalent to approximately 125mM lactic acid assuming that beef tissue is 70% water) . Presser *et al.* (In Press) found that the growth/no growth boundary for a non-pathogenic *E. coli* strain with reference to pH varied depending on the lactic acid concentration. At high lactic acid concentration (500mM) and a probability level of 0.5, the boundary occurred at a pH of ≈5.8, but at low lactic acid concentration (0mM), the boundary occurred at an approximate pH of 3.5-4.0. At the calculated lactic

acid concentration of beef (125mM), the boundary occurred at a pH of ≈5.0. Consequently, studies in broth at pH 7.4 are 'fail-safe' because at pH and lactic acid concentration of beef, the probability of growth would be further reduced compared to that predicted by Eqn. 18.

Previously, data similar to growth/no growth data (*ie.* binary or response/no response data) had only been fitted using a 'generalised linear regression model'. Lemeshow (1994) used this form of model for estimating the probability of hospital mortality of adult intensive care unit (ICU) patients. As discussed in Section 1.5.2, Ratkowsky (pers. comm.) modified a linear model previously developed by Ratkowsky and Ross (1995) to give a 'generalised non-linear regression model' in which all parameters can be estimated in the modelling process. Parameter values can be fixed if desired (Ratkowsky, pers. comm.). When this form of model was fitted to the growth/no growth data described here for an STEC strain, T_{min} converged to a value below zero and so it was fixed to a more realistic value (Chapter 2, Table 2.1) in subsequent runs. It was initially fixed at 5.10°C, a value found from kinetic studies for the non-typable STEC strain (R31) (Chapter 2, Table 2.1). However, using a number of different goodness-of-fit criteria, the fit was improved by lowering the value of T_{min} by 1.5°C. Concordance rate improved from 96.5% to 96.8% by lowering T_{min} to 3.6°C. Unlike the other goodness-of-fit criteria, concordance rate did not appear to be a sensitive indicator of model performance, as when T_{min} was lowered it remained very close to 100%. Presser *et al.* (In Press) also reported a very high concordance rate (97.3%) between observed and predicted growth/no growth data despite 6.5% of observed data falling on the 'wrong' side of the predicted boundary. According to two out of three criteria (r^2 and the c-value), the fit was further improved by fixing T_{min} to -2.0°C, which explains why when T_{min} was allowed to be estimated in the fitting process, it did so at an unrealistically low value. Repeatedly decreasing the value of T_{min} also repeatedly increased the converged value of a_{wmin}. At a fixed T_{min} of -2.0°C, a_{wmin} converged closest to the value for *E. coli* of 0.963 found in Chapter 2. It is therefore evident that this type of model is not completely suitable for modelling the

bacterial growth/no growth response. Although a good description of the growth/no growth boundary is achievable, the fact that T_{min} is best fixed at at least 5°C below the value calculated from kinetic studies begs comment. Lemeshow (1994) stated that developers of models should not be satisfied unless the area under the 'receiver operating characteristic (ROC) curve' (given by the c-value in this study) exceeds 0.70. That c-values in this study were of the order of 0.965-0.975 gives a good indication that there was a high level of agreement between observed and predicted data despite inconsistencies in the value of the converged T_{min}.

The discrepancy between improvement of fit of Eqn. 17 to observed data and concomitant convergence to more and more unrealistic values for T_{min} could be explained by the fact that T_{min} does not have a physical interpretation, but is an extrapolated value dependent on the kinetic model and type of data used. For example, McMeekin *et al.* (1993) found that the value of T_{min} varied for a given bacterium depending on the type of kinetic model used to fit observed data. Also, Neumeyer (1995) observed differences in the calculation of T_{min} for pseudomonad growth rate data fitted by the two-parameter square-root model (Ratkowsky *et al.*, 1982) depending on the amount of data used. Different values for T_{min} were calculated when data between 0 and 15°C was used compared to data between 0 and 30°C. Therefore, the use of T_{min} in a model such as a generalised non-linear regression model must be questioned when calculation of T_{min} is kinetic model- and data range-dependent. This was evident from the present study because as the fit by the model to observed data was improved, T_{min} converged to a seemingly unrealistic value.

In response to the convergence by Eqn. 18 to unrealistically low values of T_{min}, Ratkowsky (pers. comm.) modified this equation by removing the cross-product ($\ln(a_w - a_{w_{min}})\ln(T-T_{min})$) and squared ($(\ln(T-T_{min}))^2$) terms and adding a term for T_{max}. The form of the new model is given by:

$$\begin{aligned} \text{logit}(p) = {} & b_0 + b_1 \times \ln(a_w - a_{w_{min}}) \\ & + b_2 \times \ln(T - T_{min}) \\ & + b_3 \times \ln(1 - \exp(b_4(T - T_{max}))) \end{aligned} \qquad (20)$$

The form of Eqn. 20 is in agreement with the original methodology for development of models for predicting the growth/no growth boundary (Ratkowsky and Ross, 1995), which suggested that growth/no growth models could be derived by taking the logarithm of both sides of a kinetic model and replacing the left-hand side by logit (p). Eqn. 20 was fitted to observed data for the growth/no growth of STEC R31. The value for T_{max} had to be fixed at 49.23°C, the value found from kinetic modelling (Chapter 2, Table 2.1), as there was no growth/no growth data above 37°C. Therefore, convergence at a realistic value for T_{max} using Eqn. 20 was unlikely. Values for T_{min} and a_{wmin} converged at 3.41°C and 0.943, respectively, which compare favourably with values found from kinetic modelling in Chapter 2 (T_{min}, 5.1°C; a_{wmin}, 0.963). Rate of concordance was 97.2%. The predicted boundary by Eqn. 20 was almost identical to that predicted by Eqn. 18. Eqn. 20 would be the model of choice for predicting the growth/growth boundary because it converged to a more realistic value of T_{min} compared to Eqn. 18, it contains fewer terms than Eqn. 18 and agrees with the original methodology for deriving growth/no growth models proposed by Ratkowsky and Ross (1995).

An important feature of the model used in this study is that the level of probability can be set depending on the level of stringency required. Two sets of model predictions were compared, one at $p = 0.5$ and another at $p = 0.1$. Predictions made at $p = 0.5$ (50:50 chance that *E. coli* will grow at the boundary) represent a relatively conservative series of estimates, with the predicted response on the boundary being no better than a coin toss. Model predictions were made more stringent by making $p = 0.1$ (10% chance of a false prediction), with only a slight drop in the predicted growth/no growth boundary. That the predicted boundary changed only slightly upon altering the level of probability by a relatively large amount indicates that there is an abrupt transition between growth and no growth at the boundary.

The highly consistent kinetic growth response of pathogenic and non-pathogenic *E. coli* serotypes described in Chapter 2 suggests that the growth/no growth boundary described here may be suitable for predicting the response to combinations of

temperature and water activity of other pathogenic and non-pathogenic *E. coli* serotypes. The growth/no growth response of *E. coli* to combinations of temperature and water activity is particularly relevant to meat processing, where *E. coli* is used as a marker organism for other faecal contaminants (see Section 1.4.1) as well as being as an organism of concern itself. The growth/no growth boundary described here would be particularly useful in the region around 10°C, which is the temperature range typically encountered in meat chillers. It must be remembered that according to the results of Presser *et al.* (In Press), predictions in the present study are 'fail-safe' as growth/no growth data was generated in broth with a higher pH than that found at the surface of red-meat carcasses. If both the temperature and water activity of meat carcasses could be assessed during chilling, the likelihood of outgrowth of *E. coli* could be inferred from the growth/no growth data described here. Temperature monitoring is easily achieved and has been discussed in Section 1.3.3, while monitoring of water activity is the subject of Chapter 4.

Appendix 3.1 Growth Media

Agar containing NaCl comprised 15g agar (Leiner Davis Gelatin, Australia), 2g Nutrient Broth (Oxoid CM67), 80g NaCl and 1g sodium lauryl sulphate in 1 litre of distilled water. Sterilisation was achieved by autoclaving for 15min at 121°C.

Eosin Methylene Blue Agar (Levine) (Oxoid, CM69) was prepared according to the manufacturer's instructions and sterilised.

Appendix 3.2 Experimental Data

Table A3.1 Water activity of nutrient broth with added NaCl verified using a water activity meter.

%NaCl (w/w) in Nutrient Broth	Measured water activity*	Estimated water activity‡	Difference (meas. - estimated)
0.50	0.995	0.994	0.001
0.75	0.993	0.992	0.001
1.00	0.99	0.991	-0.001
1.25	0.989	0.989	0
1.50	0.988	0.988	0
1.75	0.986	0.987	-0.001
2.00	0.986	0.986	0
2.25	0.983	0.984	-0.001
2.50	0.983	0.983	0
2.75	0.982	0.981	0.001
3.00	0.979	0.98	-0.001
3.25	0.977	0.978	-0.001
3.50	0.976	0.977	-0.001
3.75	0.975	0.975	0
4.00	0.973	0.974	-0.001
4.25	0.97	0.972	-0.002
4.50	0.969	0.97	-0.001
4.75	0.967	0.968	-0.001
5.00	0.966	0.967	-0.001
5.25	0.965	0.965	0
5.50	0.963	0.964	-0.001
5.75	0.961	0.962	-0.001
6.00	0.96	0.961	-0.001
6.25	0.959	0.959	0
6.50	0.957	0.957	0
6.75	0.955	0.955	0
7.00	0.954	0.954	0
7.25	0.952	0.952	0
7.50	0.951	0.951	0
7.75	0.949	0.949	0
8.00	0.948	0.947	0.001
8.25	0.945	0.945	0
8.50	0.944	0.943	0.001
8.75	0.943	0.941	0.002

*Using Aqualab model CX2 water activity meter; ‡using data of Chirife and Resnik (1984).

Table A3.2 Change in absorbance of biuret reaction mixture with varying BSA concentration (Fig. 3.1).

Bovine serum albumin (g)	Absorbance (545nm)
0.0005	0.08
0.0007	0.12
0.0008	0.12
0.0008	0.15
0.0013	0.23
0.0026	0.41
0.0026	0.42
0.0033	0.55
0.0051	0.78
0.0061	0.94
0.0075	1.19
0.0005	0.08
0.0018	0.23
0.0036	0.43
0.0052	0.81
0.0013	0.13
0.0027	0.32
0.0032	0.40
0.0042	0.57
0.0060	0.89
0.0074	1.10

Table A3.3 Growth/no growth response of *E. coli* NT (R31) with varying temperature and water activity (Fig. 3.2).

Temp (°C)	a_w	Growth	n*	Temp (°C)	a_w	Growth	n*	Temp (°C)	a_w	Growth	n*
7.70	0.963	0	1	15.60	0 948	0	1	25.00	0.960	4	4
8.20	0.963	0	1	15.90	0.948	0	1	25.00	0.959	4	4
8.80	0.960	0	1	16.00	0.963	1	1	25.00	0.957	4	4
9.00	0.987	4	4	16.20	0.963	1	1	25.00	0.955	4	4
9.00	0.986	4	4	16.50	0.960	1	1	25.00	0.954	4	4
9.00	0.985	8	8	16.60	0.960	1	1	25.00	0.952	4	4
9.00	0.982	8	8	17.00	0.957	2	2	25.00	0.951	4	4
9.00	0.979	4	4	17.30	0.954	1	1	25.00	0.949	4	4
9.00	0.977	4	4	17.50	0.954	1	1	25.00	0.948	4	4
9.00	0.976	3	4	17.70	0.951	0	1	25.00	0.945	0	4
9.00	0.975	1	4	17.80	0.951	0	1	25.00	0.944	0	4
9.30	0.960	0	1	18.00	0.948	0	1	25.00	0.943	0	4
9.70	0 957	0	1	18 30	0.948	0	1	28.00	0.955	4	4
10.00	0.982	4	4	18.40	0.963	1	1	28.00	0.954	4	4
10.00	0.979	4	4	18.50	0.963	1	1	28.00	0.952	4	4
10.00	0.977	4	4	18.70	0.960	1	1	28.00	0.951	4	4
10.00	0.976	0	4	18.80	0.960	1	1	28.00	0.949	4	4
10.00	0.975	0	4	19.00	0.957	1	1	28.00	0.948	1	4
10.00	0.973	0	4	19.20	0.957	1	1	28.00	0.945	0	4
10.00	0.970	0	4	19.40	0.954	1	1	28.00	0.944	0	4
10.00	0.969	0	4	19.50	0.954	1	1	28.00	0.943	0	4
10.00	0.967	0	4	19.80	0.951	0	2	31.00	0.955	4	4
10.00	0.966	0	4	20.00	0.973	4	4	31.00	0.954	4	4
10.00	0.965	0	4	20.00	0.970	4	4	31.00	0.952	4	4
10.00	0.963	0	4	20.00	0.969	4	4	31.00	0.951	4	4
10.00	0 961	0	4	20.00	0.967	4	4	31.00	0.949	3	4
10.00	0.960	0	4	20 00	0.966	4	4	31.00	0.948	0	4
10.00	0.959	0	4	20.00	0.965	4	4	31.00	0.945	0	4
10.00	0.957	0	4	20.00	0.963	4	4	31.00	0.944	0	4
10.00	0.955	0	4	20.00	0.961	4	4	31.00	0.943	0	4
10.00	0.954	0	4	20 00	0.960	4	4	34.00	0.955	4	4
10.00	0.952	0	4	20.00	0.959	4	4	34.00	0.954	4	4
10.00	0.951	0	4	20.00	0.957	4	4	34.00	0.952	4	4
10.00	0.949	0	4	20.00	0.955	4	4	34.00	0.951	4	4
10.00	0.948	0	4	20.00	0.954	4	4	34.00	0.949	0	4
10.00	0.945	0	4	20.00	0.952	4	4	34.00	0.948	0	4
10.00	0.944	0	4	20.00	0.951	4	4	34.00	0.945	0	4
10.00	0.943	0	4	20.00	0.949	4	4	34.00	0.944	0	4
10.20	0.957	0	1	20.00	0.948	0	6	34.00	0.943	0	4
10.60	0 954	0	1	20.00	0.945	0	4	37.00	0.973	4	4
11.30	0.954	0	1	20.00	0.944	0	4	37.00	0.970	4	4
11.40	0.951	0	1	20.00	0.943	0	4	37 00	0.969	4	4
11.80	0.951	0	1	20.10	0.963	1	1	37.00	0.967	4	4
12.00	0.969	4	4	20.40	0.963	1	1	37.00	0.966	4	4
12.00	0.967	4	4	20.70	0.960	2	2	37.00	0.965	4	4
12.00	0.966	4	4	21.00	0.957	1	1	37.00	0.963	4	4
12.00	0 965	4	4	21.10	0.957	1	1	37.00	0.961	3	4
12.00	0 963	4	4	21.30	0.954	1	1	37.00	0.960	2	4
12.20	0.948	0	1	21.40	0.954	1	1	37.00	0.959	4	4
12.60	0.948	0	1	21.70	0.951	1	2	37.00	0.957	4	4
12.90	0.963	0	1	22.10	0.948	0	1	37.00	0.955	8	8
13.50	0.960	0	1	22.20	0.948	0	1	37.00	0.954	8	8
13.50	0 963	1	1	25.00	0.973	4	4	37.00	0.952	8	8
13.90	0.960	0	1	25.00	0.970	4	4	37.00	0.951	1	8
13.90	0.957	0	1	25.00	0.969	4	4	37.00	0.949	0	8
14.40	0.957	0	1	25.00	0.967	4	4	37.00	0.948	0	8

Table A3.3 continued...

Temp (°C)	a_w	Growth	n*	Temp (°C)	a_w	Growth	n*	Temp (°C)	a_w	Growth	n*
14.50	0.954	0	1	25.00	0.966	4	4	37.00	0.945	0	8
14.80	0.954	0	1	25.00	0.965	4	4	37.00	0.944	0	8
15.00	0.951	0	1	25.00	0.963	4	4	37.00	0.943	0	8
15.50	0.951	0	1	25 00	0.961	4	4				

*Number of samples used.

Chapter 4. Measurement of Beef Carcass Surface Water Activity

4.1 Summary

Two methods were assessed for the measurement of water activity at the surface of beef carcasses. The first method attempted to find a relationship between water activity and the conductivity across agar surfaces of varying NaCl concentration. Conductivity was measured between electrodes different distances apart on agar surfaces containing 0-25.5% NaCl. Excessive scatter of data points did not permit the formulation of a relationship between conductivity and water activity.

The second method involved excising thin samples of tissue from beef carcasses using a skin-grafting scalpel and measuring water activity using a water activity meter. Sample thickness was varied, and it was found that the thinnest sample size permissible using a skin-grafting scalpel (approximately 1mm) best represented surface water activity. This thickness also caused minimal damage to the carcass.

Water activity was mapped over the carcass surface and at different stages during chilling at two Australian abattoirs. From temperature and water activity profiles of carcasses during chilling at one abattoir, predicted bacterial populations were calculated and compared to those observed. Most predictions of the changes in population density of pseudomonads and coliforms 'failed-safe' compared to observed population densities.

Starch was assessed for its water activity-lowering ability. Hydrolysed starch was found to significantly lower the water activity of beef samples.

4.2 Introduction

The beef slaughtering and dressing process was outlined in Chapter 1 (Section 1.2.2). Although processing operations can vary from country to country and from plant to plant, the basic method is conserved. In the first stage of processing in any operation, beef cattle are reduced to carcass sides (see Figure 1.2 for a diagram of a beef carcass side showing carcass regions) by removal of the hide, head, distal parts of the limbs and all organs and by sawing through the vertebral column (Gill *et al.*, 1996a). Thereafter, sides are chilled immediately using either freezing air blasts or water sprays, or are boned first ('hot boning'), cut into smaller pieces and packaged into cartons before chilling takes place. Spray-chilling of sides is the method of choice in North America (*eg.* Gill and Jones, 1992b), while most Australian beef processors use air-chilling (McPhail, pers. comm.). 'Hot boning' is used when there is limited chiller space and when drip-loss from carcasses needs to be reduced (Swatland, 1984).

During the carcass dressing process, carcasses are suspended via a hook through one hind-limb to an overhead rail. The rail system extends from the slaughter area to the chilling area and then to the boning room. In a typical air-chilling process, carcass sides are moved into the chiller (a well-insulated room with air-tight doors) on rails. Inside the chiller, rails are arranged in parallel rows and sides are arranged to fill the rows. Ideally, rows are spaced sufficiently to allow air-flow between them, as are carcass sides on each row. Cold air-flow is directed either from above or from the side.

The concept of water activity was introduced in Chapter 1 (Section 1.3.4) and was defined as a measure of the availability of water in an aqueous solution (Scott, 1953). After temperature, water availability is perhaps the most important factor governing bacterial growth rate on carcasses (Lawrie, 1991) (see Section 1.3.4). Like a reduction in temperature, a decrease in water activity causes a decrease in bacterial growth rate until a critical low water activity is reached, at which point bacterial growth ceases. The potential for water activity reduction during beef production is most obvious during chilling operations using freezing air blasts (*ie.* in most Australian

abattoirs). Water activity is not a determining factor in spray-chilling or ' hot boning' as there is no chance for significant evaporative water-loss in these processes. Because water activity is not monitored during beef production, the effect of drying stages such as air-chilling on the proliferation or inhibition of spoilage organisms and pathogens is not known. This is despite the fact that there is an ability to measure water activity by a number of means (see Section 1.3.4.1) and there are reports of the measurement of water activity of meat samples (Lovett, 1978). Optimisation of chilling regimes is only achieved currently by reference to temperature effects on bacterial growth and not water activity effects as well. Most spoilage and pathogenic bacteria cease to grow in the water activity region of 0.90-0.95 (Troller and Christian, 1978). If carcasses are dried to this extent during chilling then the chilling pattern could be relaxed without having any detrimental effects on microbiological quality, thereby saving energy.

In this chapter two different approaches to measure beef carcass surface water activity are assessed. The first is the use of conductivity as a measure of water activity. It was hypothesised that the conductivity between two electrodes on the carcass surface could be related to water activity. The second approach is a more direct method using a skin-grafting scalpel to take surface samples for measurement in a water activity meter. The second approach was found to be preferable and was used to map the water activity over the surface of carcasses. Changes in water activity were monitored for the duration of chilling and on a number of beef sides at different locations in the chiller. Bacteria were enumerated on carcasses and compared to predictions based on temperature and water activity history and using a combination of probability and kinetic modelling.

Starch compounds were assessed for their possible use as edible outer-coatings on beef by determining their water activity-lowering effect on cut beef tissue, adipose tissue and minced beef. Reduction in water activity of beef samples by hydrolysed starch was assessed in terms of the growth rate response of *E. coli.*

4.3 Materials and Methods

4.3.1 Conductivity method

Plate Count Agar (PCA) plates (Appendix 2.3) were prepared with added NaCl at concentrations of 0.5, 5.5, 10.5, 15.5, 20.5 and 25.5% w/w. Water activity of these agar media was estimated from the tables of Chirife and Resnik (1984) and verified using a water activity meter (Aqualab model CX2, Decagon Devices, USA). Conductivity of these media between two stainless steel electrodes (11×22mm) placed on the surface of the agar was measured using a multimeter (Arlec DM 1050) at an electrode separation distance of 20 or 50mm.

4.3.2 Tissue excision method

4.3.2.1 Preliminary investigations

PCA (Appendix 2.3) plates were prepared with the addition of NaCl at concentrations of 0, 0.5, 5.5, 10.5, 15.5, 20.5 and 25.5% (w/w). At each NaCl concentration, plates containing 5, 10 and 15ml agar were prepared which gave agar thicknesses of 1.0, 1.5 and 2.0mm, respectively. Water activities of the media were estimated from the tables of Chirife and Resnik (1984) and verified using a water activity meter (Aqualab model CX2, Decagon Devices, USA). Once the agar had been poured and cooled, plates without lids were kept at ambient temperature to allow dehydration and water activity measured at 0, 4 and 24h.

A skin-grafting scalpel (SGS) (Kaiser's, UK) which uses standard razor blades (Stainless Gillette Blue Blades, USA) was used to excise tissue from a cut of beef with the carcass surface tissue intact. The SGS was set to cut samples 1.0mm and 3.0mm thick. Samples were transferred to water activity meter sample cups (3.9mm in diameter; 1.1mm in height) and placed outer surface-up using forceps. Samples were cut to fit sample cups as closely as possible so that only the outer surface of the excised sample was exposed to the sample chamber. Three different samples containing the

surface tissue were taken at each thickness setting and water activity measured in a water activity meter.

4.3.2.2 Water activity mapping of beef carcasses

Carcass surface samples were taken using an SGS. Samples approximately 25mm×25mm were excised from the carcass surface by cutting downwards with the SGS using a sawing motion. Samples were transferred to samples cups and water activity measured using the method described in Section 4.3.2.1. Water activity measurements were made immediately. If there was a small delay between sampling and water activity measurement, samples were sealed inside sample cups using a lid to form an air-tight seal. The water activity meter was placed directly outside the chiller in order to limit, as much as possible, the time between sampling and measurement.

Plant A (Tasmania)

Carcass sides were arranged on rails in four parallel rows inside the chiller. Initially, water activity measurement was performed on a single beef carcass side (outside rail) at nine different sites located symmetrically over the outside surface of the carcass. Samples were taken from sites 1-9 (shown in Figure 4.1) immediately after chiller loading and 6h later. Carcasses were chilled in an overnight chilling regime.

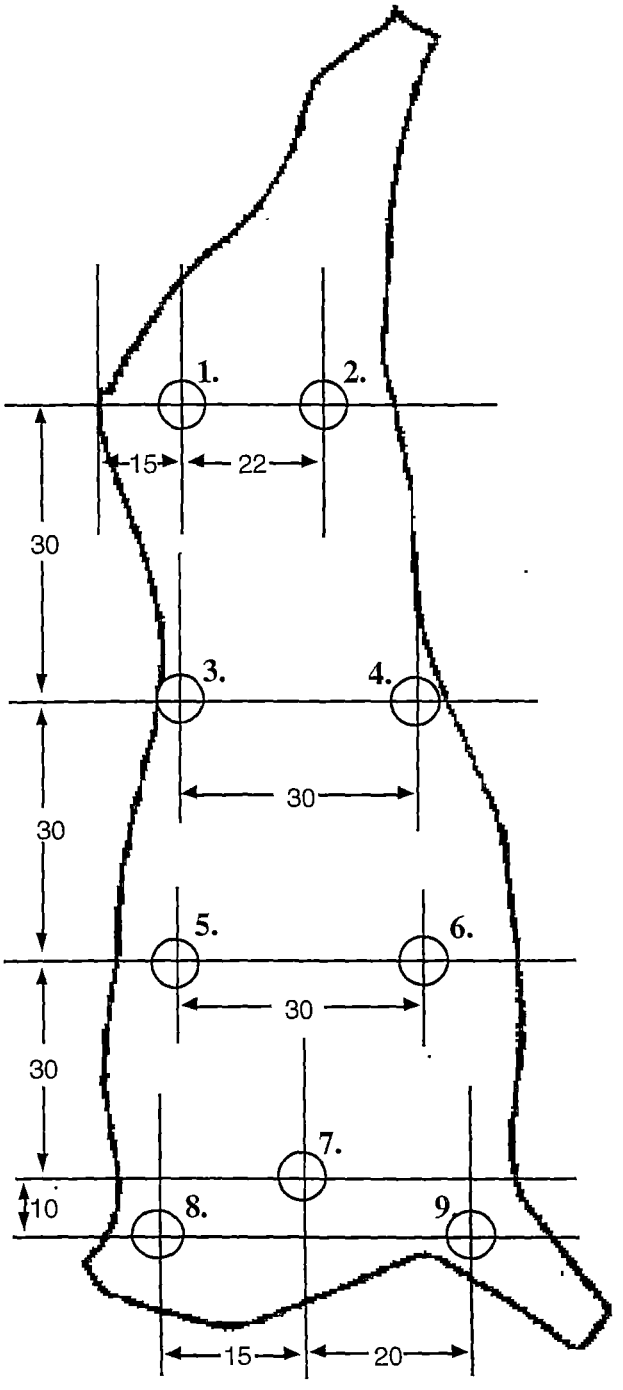
In a separate investigation, water activity measurement was made at sites 1, 2, 8

Table 4.1 Chilling pattern for a weekend chilling regime at Plant B.

Duration of treatment (h)*	Temperature set point (°C)	Fan speed (%)
8	8.5	110
12	9.5	60
Remainder	9.5	40

*Beginning immediately after chiller loading.

Figure 4.1 Approximate location of sampling sites for water activity measurement on the outside surface of a beef carcass side (not to scale). All values are cm.



and 9 (Figure 4.1) from a single carcass side located on an outside rail in a chiller. Carcasses were chilled in an overnight chilling regime. Two water activity sampling

sites were chosen at the end nearest the chiller ceiling (posterior end; sites 1 and 2) and two sites at the end nearest the chiller floor (anterior end; sites 8 and 9) of a beef carcass side. Sites 1 and 2 represented the carcass sampling sites closest to the source of chilling while sites 8 and 9 represented the carcass sites most distant from the source of chilling. Samples were taken immediately after chiller loading and another four times over the following 3h.

Plant B (Queensland)

Water activity sampling was done from six beef carcass sides over a weekend chilling regime. Sides were designated numbers 1717L, 1724L, 1768L, 1730R, 1751L and 1756L. The chiller contained four parallel rails. Sides 1717L, 1724L and 1768L were on outside rails while sides 1730R, 1751L and 1756L were on inside rails. The chilling pattern is given in Table 4.1. Chilled air was blown from a series of overhead fans. Samples 1.0mm in thickness were taken using an SGS at approximately 0, 3, 6, 9, 12, 15, 20, 40 and 56h after chiller loading from sites 5 and 6 (Figure 4.1), making sure that samples taken from the same region were taken from fresh sites each time. Plant B nominated sites 5 and 6 for sampling as these sites had extensive surface fat and could be trimmed after the experimental period without affecting carcass quality. Temperature was monitored at 15min intervals on all six sides at a number of locations on the carcass using multi-channel temperature loggers (16 Channel Grant Squirrel Model 1205 with type T thermocouples, Grant Instruments, UK). Temperature measurements were made at the butt, flank and brisket surface on all sides and at the deep butt on most sides (see Figure 1.2 for location of carcass regions). Measurement of air temperature around sides was made near the butt, neck and shoulder. Temperature measurement was made at a maximum of two sites for each side. Temperature measurement sites at the flank and brisket surface were very close to the water activity sampling sites (sites 5 and 6, respectively).

Bacteria were enumerated on beef carcass sides at two sites directly adjacent to water activity sampling sites by viable counts at approximately 0, 3, 6, 12, 20, 40 and

56h after chiller loading. Pseudomonads, coliforms and total viable count (TVC) were enumerated on surface tissue samples. A 30mm×30mm tissue sample was excised from the surface of carcasses adjacent to water activity sampling sites (within 1-2cm) using a sterile scalpel. Samples were placed in sterile stomacher bags and the mass recorded. 25ml of 0.1% peptone (Appendix 4.1) was added to stomacher bags and stomached for 1min. 1ml and 0.1ml of the stomached solution was pipetted onto each of two Pseudomonas C-F-C plates (Appendix 4.1), two PCA plates (Appendix 2.2) and two Petrifilm plates for coliforms and *E. coli* (Appendix 4.1) and incubated for 24-72h at 25°C.

Bacterial count per cm^2 of surface tissue was calculated using the following equation:

$$\text{count} = \frac{\text{average count} \times \text{dilution}}{(1/25\text{ml} + \text{sample mass}) \times \text{sample area}}$$

4.3.2.3 Comparison of observed and predicted bacterial numbers

Observed change in numbers of coliforms and pseudomonads on beef carcass sides at Plant B were compared with predicted change in numbers. Comparisons between predicted and observed change in bacterial numbers were only possible when there were initial and final bacterial counts obtained from specific carcass sites. At several carcass sites, bacterial density was enumerated at the beginning and end of chilling but not in between. These cases were not considered in the comparison with predicted bacterial numbers because there was considered to be an excessively long period of time (56h) between successive observed counts. Predictions of change in pseudomonad numbers were made for sides 1724L (site 6, 20-56h), 1768L (site 6, 0-3h) and 1756L (site 5, 40-56h; site 6, 20-56h). Predictions of change in coliform numbers were made for sides 1724L (site 5, 0-12h) and 1756L (site 6, 12-56h). From temperature history data at the flank and brisket (see Section 4.3.2.1), the surface temperature was recorded close to (within 15min) the times at which water activity measurements were made. Integration of growth rates between successive water

activity measurements was achieved using computer software similar to that used in 'Pseudomonas Predictor' (McMeekin and Ross, 1996). Using Excel software (Microsoft Excel Version 4.0 © 1985 - 1992. Microsoft Corporation, 1 Microsoft Way, Redmond, WA, USA) growth rate of coliforms and pseudomonads was calculated over each time interval between water activity measurements. Gill *et al.* (1996a) reported that there was no significant difference between numbers of *E. coli* and numbers of coliforms on beef carcasses. Therefore, observed numbers of coliforms in the present study were compared directly to numbers of *E. coli* predicted by a kinetic temperature/water activity model (Eqn. 17, Chapter 2). Predicted growth rates of *E. coli* were calculated only for temperature/water activity combinations at which growth was possible, determined using a growth/no growth model (p = 0.5) described in Chapter 3 (Eqn. 18). Predicted cell density of pseudomonads at the end of chilling was calculated using a temperature/water activity model for pseudomonads (T. Ross, pers. comm.). The pseudomonad model is given by:

$$\sqrt{k} = \frac{0.0251(T + 7.26)(1 - \exp(0.182(T - 41.4)))}{\sqrt{(a_w - 0.947)}} \qquad (21)$$

As no model is available for the growth/no growth boundary of pseudomonads, growth was assumed to occur on carcasses for all temperature/water activity combinations except when water activity was lower than 0.947.

Once *E. coli* and pseudomonad growth rate was calculated at each time interval between water activity sampling, growth rate was expressed as a function of the time between water activity determinations by dividing the time interval by the corresponding generation time (designated GT_{aw}). Average temperature over the time interval was calculated by averaging the initial and final temperature. Average water activity over the time interval was calculated in the same way. GT_{aw} was converted to an equivalent log increase at each time interval by using the following calculation:

$$\text{log increase} = \log.2^{GT_{aw}}$$

The log increase at each time interval between water activity sampling was summed to give the total predicted log increase over the period of time at which bacterial numbers were determined.

4.3.3 Starch as a water activity-lowering compound

A cut of beef with the carcass outer surface intact and minced beef were obtained from a local butcher. Cut beef tissue, carcass outer surface adipose tissue and minced beef samples were rolled in soluble and hydrolysed starch (Appendix 4.1) using forceps and water activity measured subsequently using a water activity meter (Aqualab model CX2, Decagon Devices, USA). The water activity of uncoated cut beef, adipose tissue and minced beef samples was also measured. Beef and adipose samples (approximately 40mm in diameter and 3-4mm in thickness) were cut to fit in water activity meter sample cups. Minced beef 3-4mm in depth was moulded into the base of a sample cup.

The effect of lowering the water activity of cut beef tissue, carcass outer surface adipose tissue and minced beef by the addition of hydrolysed starch was assessed in terms of the predicted growth rate of *E. coli*. Predicted growth rate response of *E. coli* with respect to temperature and water activity was calculated using a model for *E. coli* NT (R31) (Eqn. 17, Chapter 2). Growth rate was calculated at the water activities of beef samples before and after coating with hydrolysed starch and at temperatures simulating those of the beef carcass surface during the cooling of beef in a commercial chiller (20, 15, 12, 10 and 8°C). Growth rate response of *E. coli* was calculated only if growth was shown to occur by a model for the growth/no growth boundary for *E. coli* NT (R31) (Eqn. 18, Chapter 3).

4.4 Results

4.4.1 Conductivity method

Results of conductivity measurements of agar containing various levels of NaCl are presented in Figure 4.2. There was little change in conductivity over the water activity range 0.77-0.87. As water activity increased from 0.90-1.00, there was a decrease in conductivity until a value of 0.998 (NaCl concentration of 0.5% w/w) was reached, at which point there was a high degree of scatter in the data. Changing the separation distance of electrodes did not appear to have any effect on the conductivity.

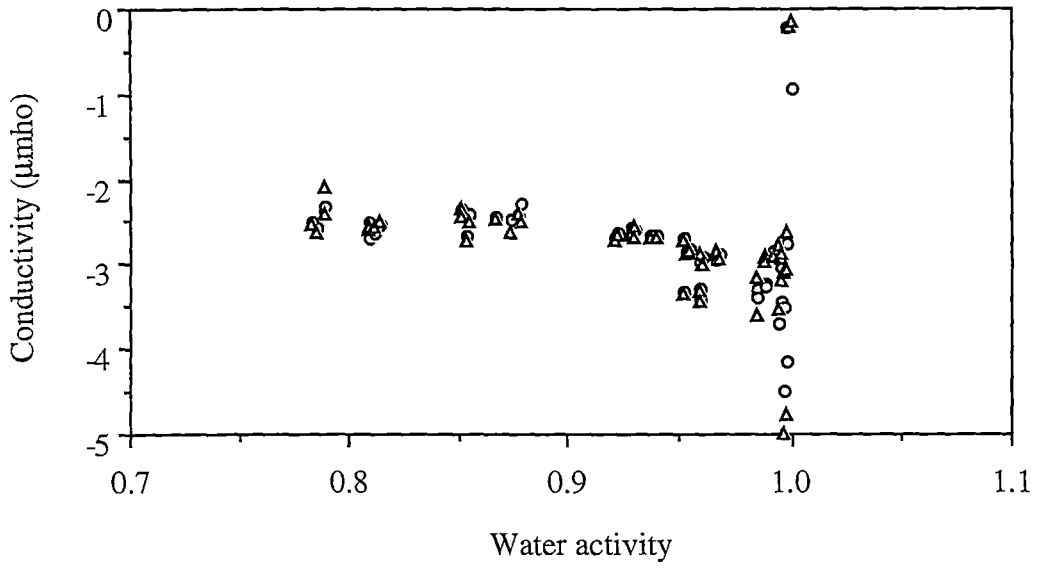


Figure 4.2 Conductivity of agar containing NaCl as a function of water activity. O, electrode separation distance of 20mm; Δ, electrode separation distance of 50mm.

4.4.2 Tissue excision method

4.4.2.1 Preliminary investigations

The measured water activity of PCA containing varying NaCl concentrations is presented in Figures 4.3a and 4.3b. For samples containing identical NaCl concentrations, initial water activity was very similar regardless of sample thickness. The sample showing the greatest reduction in water activity over 24h at all NaCl concentrations was the thinnest sample (1.0mm). The 1.5mm-thick samples showed the second highest reduction in water activity in most cases, while the 2.0mm-thick samples showed the slowest reduction in water activity over 24h. For 1.0mm-thick samples, the extent of water activity reduction was greatest at low NaCl concentration. At 0% NaCl, water activity of the 1.0mm sample decreased from 0.996 to 0.526 over 24h. At 10.5% NaCl the reduction was from 0.932 to 0.717 while at 25% NaCl reduction was from 0.817 to 0.640. For 1.5mm and 2.0mm samples, there was very little water activity reduction over 24h at 0 and 5.5% NaCl. Water activity reduction for 1.5mm samples was greatest at an NaCl concentration of 10.5% (from 0.935 to 0.821) while for 2.0 mm samples the greatest water activity reduction occurred at an NaCl concentration of 20.5% (from 0.859 to 0.763).

The measured water activity of beef carcass surface tissue of varying thickness is shown in Table 4.2. Thin tissue samples (1.0mm) were, on average, 0.025 water activity units lower than thick samples (3.0mm).

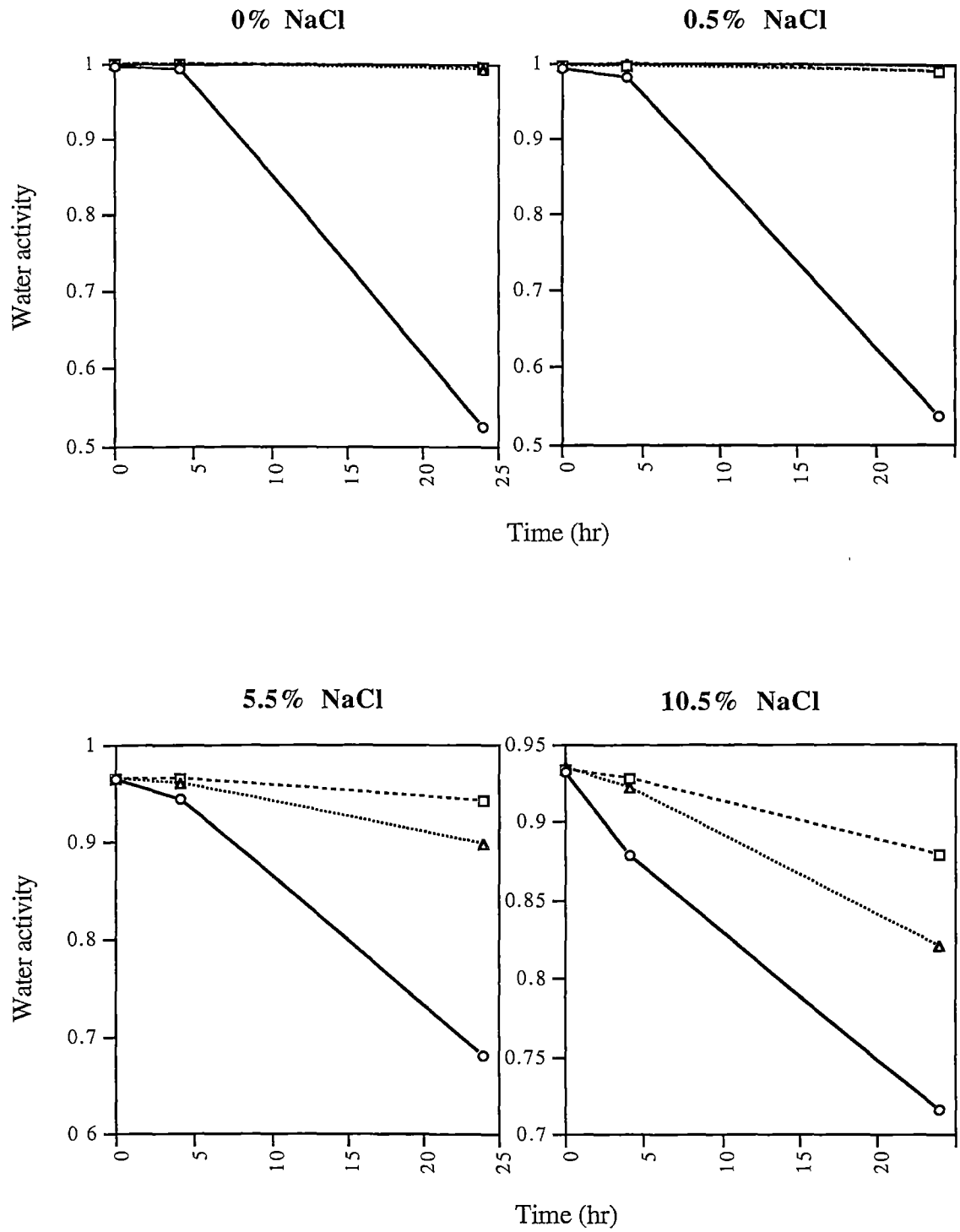


Figure 4.3a Change in water activity of PCA over time. O, agar thickness = 1.0mm; Δ, agar thickness = 1.5mm; T, agar thickness = 2.0mm.

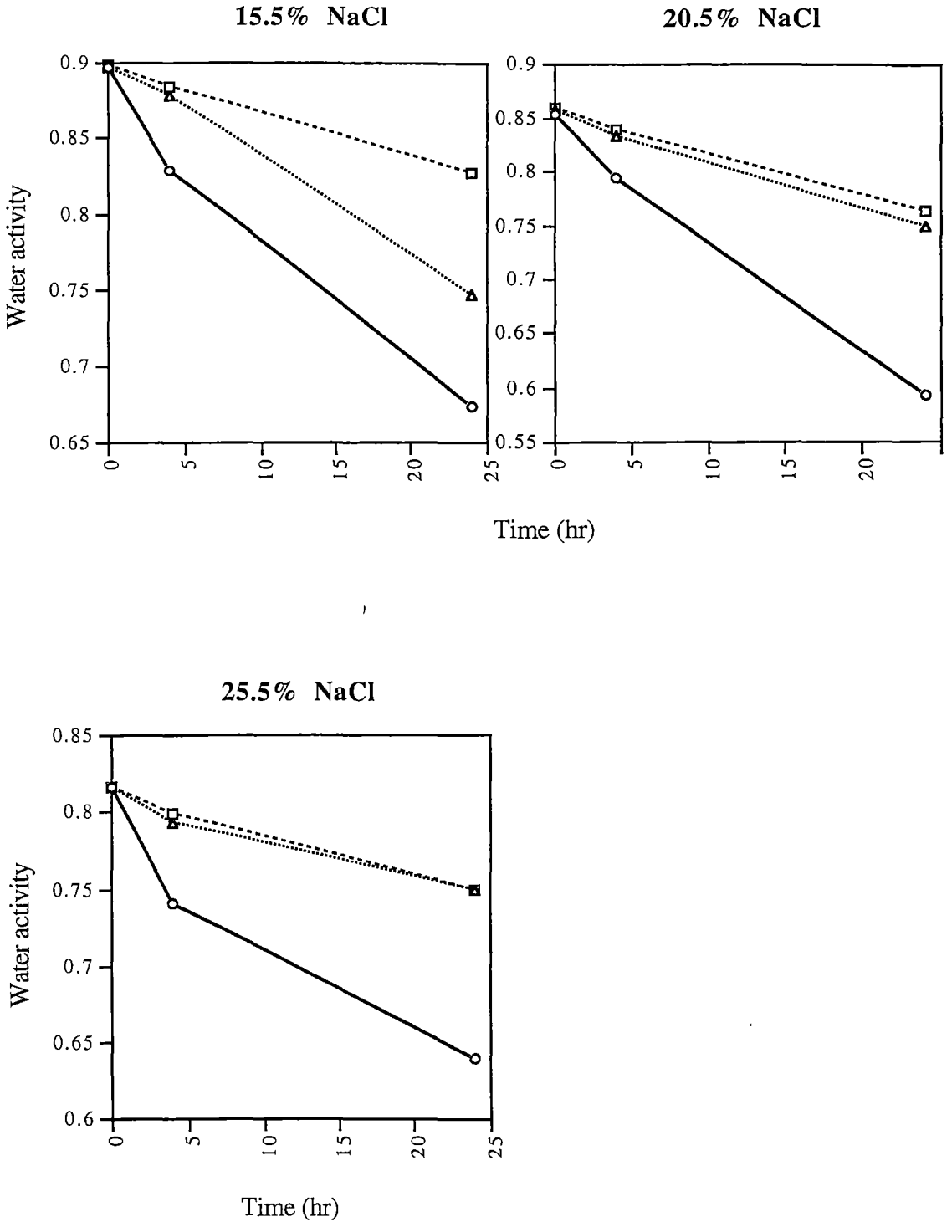


Figure 4.3b Change in water activity of PCA over time. O, agar thickness = 1.0mm; Δ, agar thickness = 1.5mm; T, agar thickness = 2.0mm.

Table 4.2 Variation of measured water activity of beef carcass surface tissue of varying thickness.

Sample thickness	Sample	a_w	Average a_w
	1	0.870	
1.0mm	2	0.884	
	3	0.928	0.894
3.0mm	1	0.912	
	2	0.936	
	3	0.910	0.919

4.4.2.2 Water activity mapping of beef carcasses

Plant A (Tasmania)

The measured water activity of nine sites on a single beef carcass side at Plant A is presented in Figure 4.4. Sampling was possible at all sites except for site 9, which was a particularly slippery, curved surface not amenable to sample excision using the SGS. Most sites were characterised by surface adipose tissue, but sites 4 and 6 were fat-free muscle tissue. Each sample, 1.0mm in thickness, was collected and read within approximately 15min, allowing eight sites on the side to be measured in approximately 100min. This was the fastest measurement time possible, with most of the time being for equilibration of the water activity meter.

There was no obvious trend in the water activity change between the initial sampling (within an hour of chiller loading) and approximately 6h later. All readings were above a water activity of 0.95. Sites 1, 5, 7 and 8 showed a slight decrease in water activity over 6h (average of 0.011 water activity units), while sites 2, 3, 4 and 6 showed a slight increase in water activity (average of 0.015 water activity units).

Figure 4.5 shows results for the water activity change of another beef carcass side (sites 1, 2, 8 and 9) over the first three hours of chilling. Tissue was excised successfully from all sites, including site 9, where tissue was amenable to sampling. There was a net decrease in water activity at all sites over 3h (average decrease of 0.017

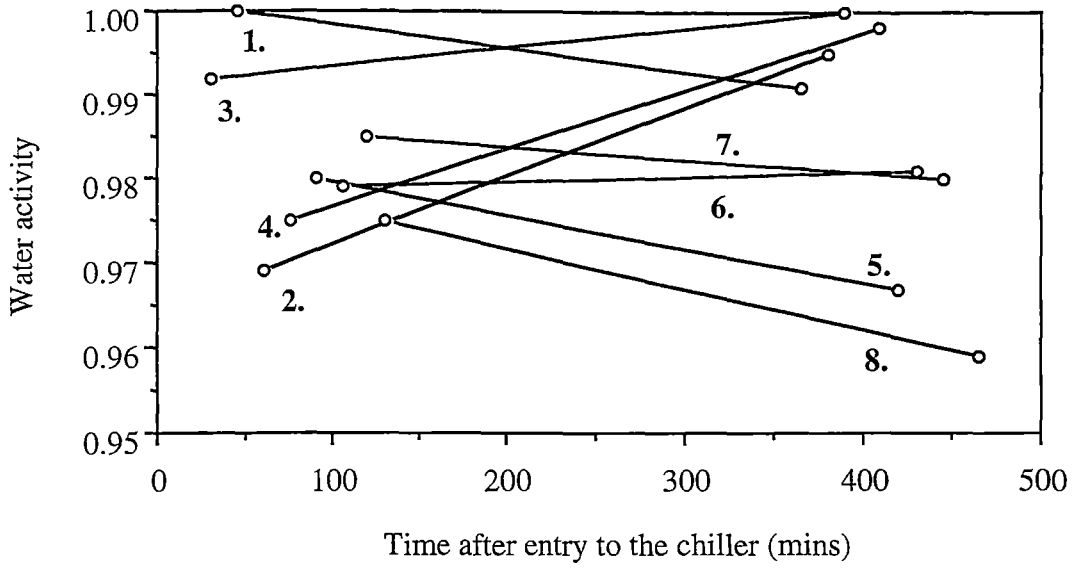


Figure 4.4 Change in water activity of a beef carcass side during the first 7h of chilling at Plant A. Values indicate carcass site (Figure 4.1).

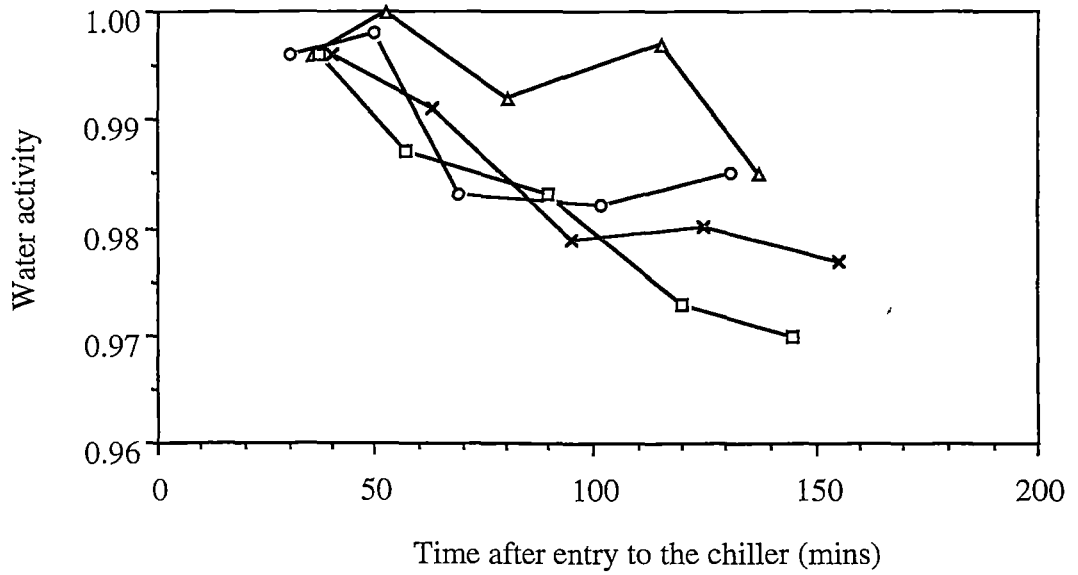


Figure 4.5 Change in water activity of a beef carcass side over the first 3h of chilling at Plant A. O, site 1; Δ, site 2; T, site 8; ×, site 9 (Figure 4.1).

water activity units), although there was an initial increase in water activity at sites 1 and 2. Site 8 showed the greatest net decrease in water activity (from 0.996 to 0.970), while sites 1 and 2 showed the smallest net decrease (from 0.996 to 0.985).

Plant B (Queensland)

Change in water activity at sites 5 and 6 on six beef carcass sides at Plant B is presented in Figures 4.6a-f. Initially, water activity was above 0.980 for eight of the 12 sites sampled. Of the sites with water activity below 0.980, two were above 0.970. Water activity at site 6 was almost always higher than at site 5. Water activity at site 5 fell to as low as 0.929 (side no. 1717L, approximately 20h after chiller loading) while at site 6 it fell to as low as 0.942 (side no. 1768R, approximately 30min after chiller loading). Both sides 1717L and 1768R were on outside rails. The highest variation in water activity occurred within the first 15-20h of chilling for all sides and at both sites. For all sides, the lowest water activity occurred within 20h of chiller loading. From 20h onwards, water activity of all sides was relatively high. At 56h, water activity was above 0.975 at site 5 for all sides except one (1717L) and above 0.980 at site 6 for all sides except one (1768R). There did not appear to be any difference in water activity profile between sides on outside rails and sides on inside rails. The lowest water activity values attained by each of the sides on outside rails were 0.929, 0.955 and 0.930 (average = 0.938) for side numbers 1717L, 1724L and 1768L, respectively. The lowest values for sides on inside rails were 0.937, 0.951 and 0.931 (average = 0.940) for 1730R, 1751L and 1756L, respectively.

Temperature profiles for the duration of weekend chilling at Plant B are given in Figures 4.7a and 4.7b. Profiles for all sides were very similar and so only one profile, for side number 1717L, is presented as an example (Figure 4.7a). The time course of chilling is given in Table 4.1. For the first 8h of chilling fan speed was at '110%' with a temperature set point of 8.5°C. Air temperature at the neck and butt of side 1717L fell very rapidly to approximately 7°C, 6h after chiller loading. Surface temperature at the butt, flank and brisket also fell rapidly to within the range of 9-11.5°C after 8h. For the

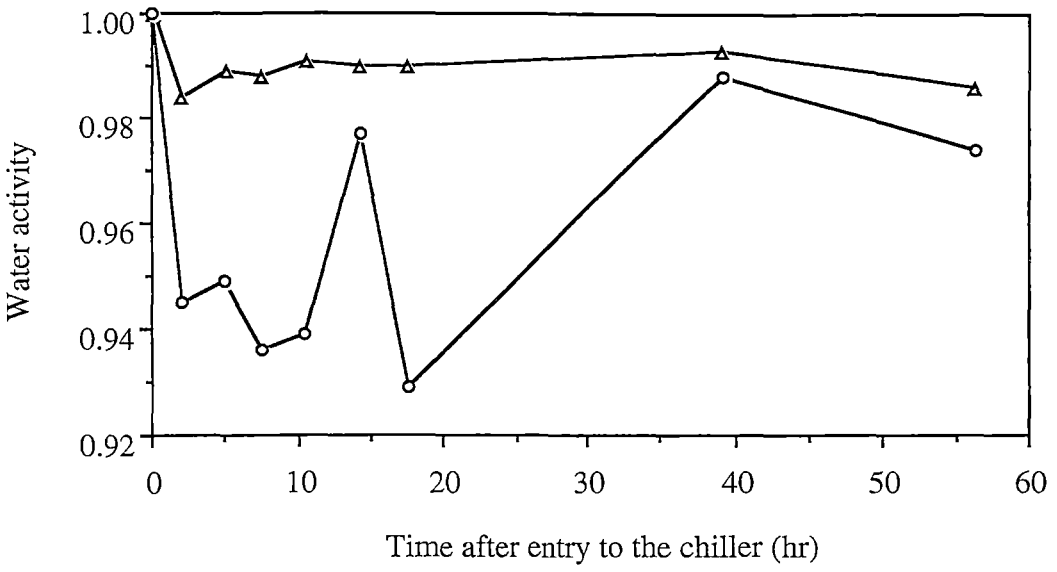


Figure 4.6a Change in water activity of a beef carcass side (no. 1717L, outside rail) over a weekend chilling regime at Plant B. O, site 5; Δ, site 6 (Figure 4.1).

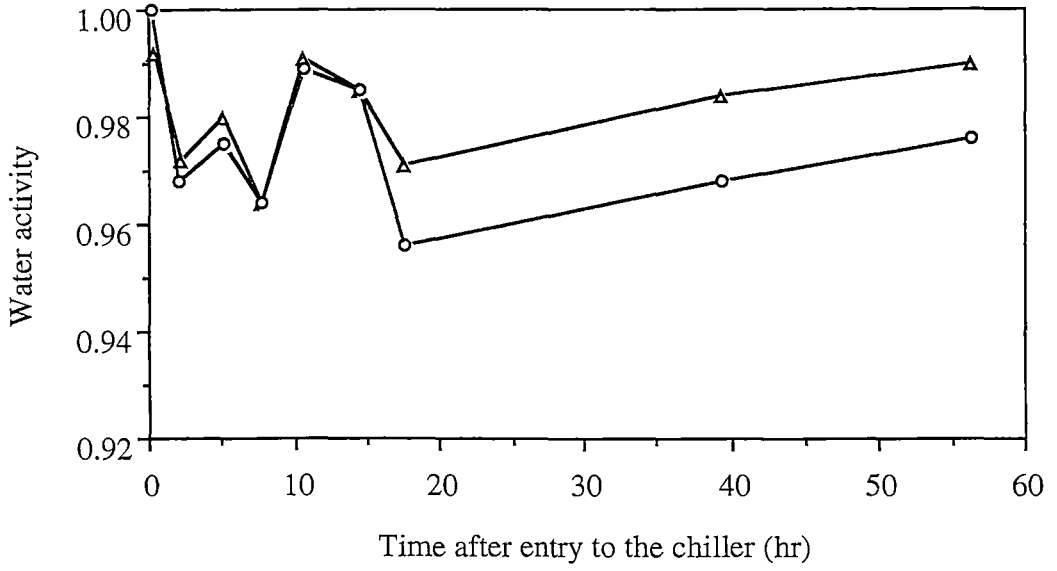


Figure 4.6b Change in water activity of a beef carcass side (no. 1724L, outside rail) over a weekend chilling regime at Plant B. O, site 5; Δ, site 6 (Figure 4.1).

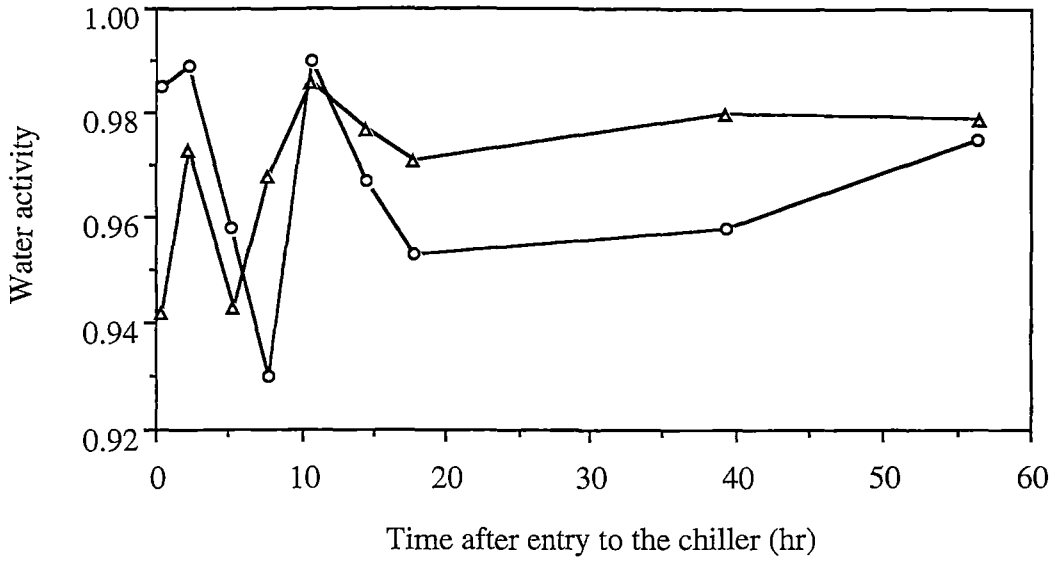


Figure 4.6c Change in water activity of a beef carcass side (no. 1768L, outside rail) over a weekend chilling regime at Plant B. O, site 5; Δ, site 6 (Figure 4.1).

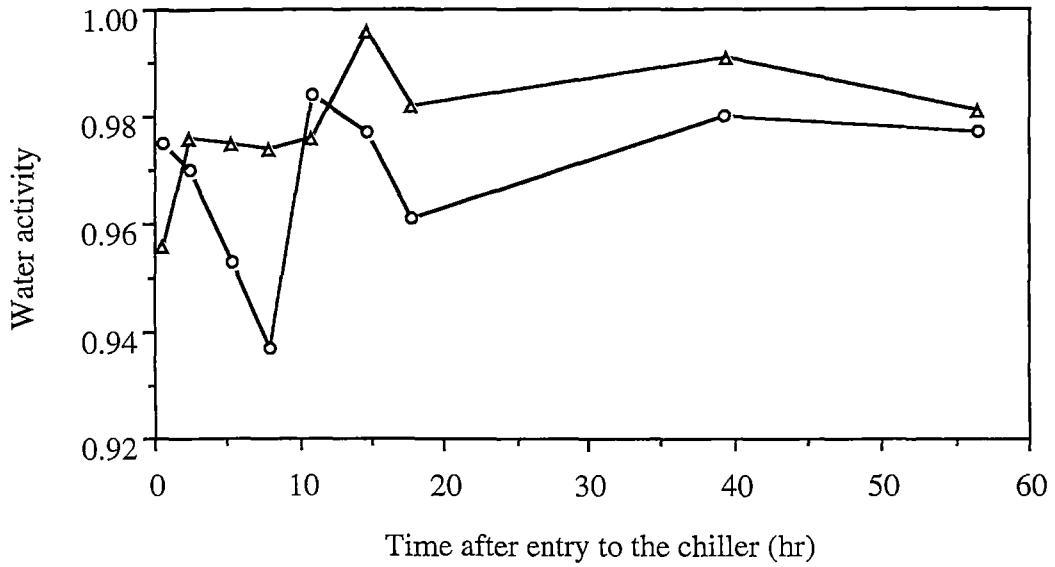


Figure 4.6d Change in water activity of a beef carcass side (no. 1730R, inside rail) over a weekend chilling regime at Plant B. O, site 5; Δ, site 6 (Figure 4.1).

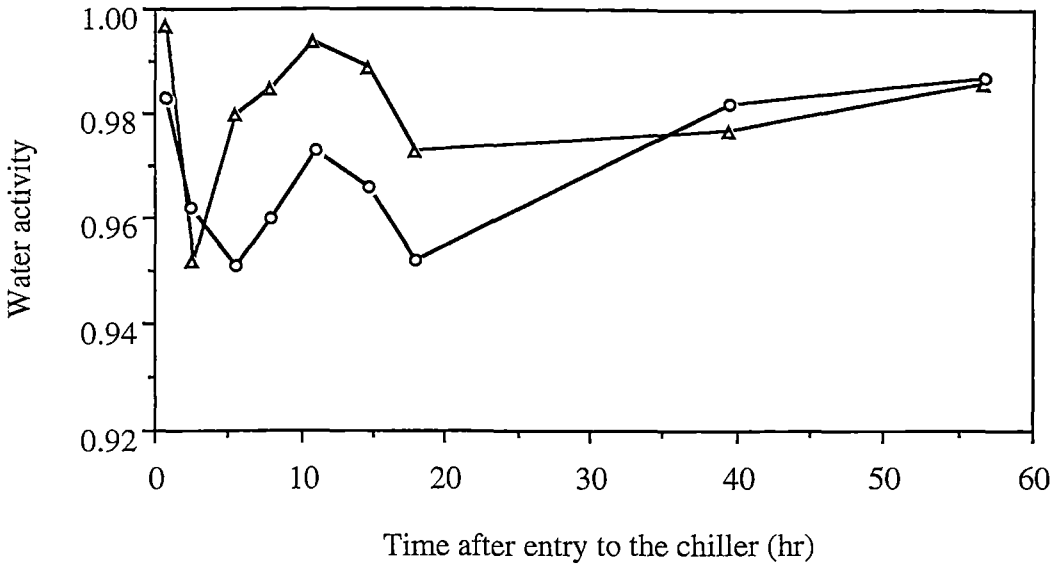


Figure 4.6e Change in water activity of a beef carcass side (no. 1751L, inside rail) over a weekend chilling regime at Plant B. O, site 5; Δ, site 6 (Figure 4.1).

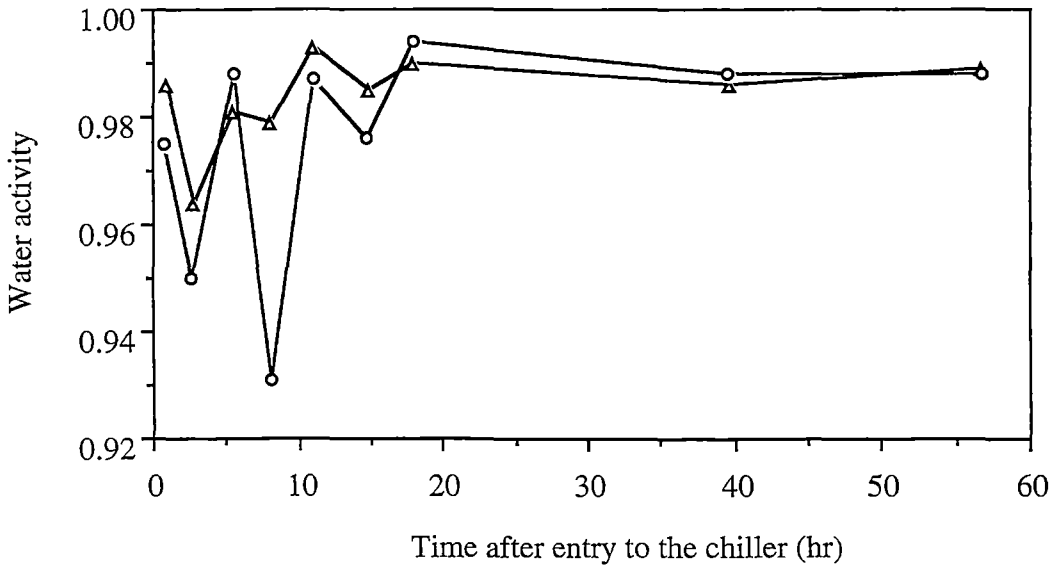


Figure 4.6f Change in water activity of a beef carcass side (no. 1756L, inside rail) over a weekend chilling regime at Plant B. O, site 5; Δ, site 6 (Figure 4.1).

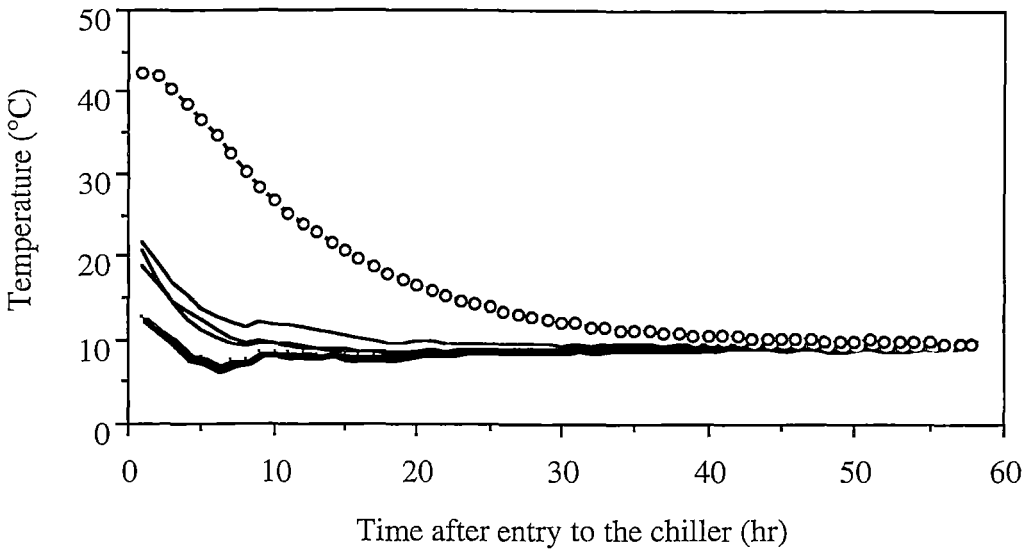


Figure 4.7a Change in temperature of a beef carcass side (no. 1717L, outside rail) over a weekend chilling regime at Plant B. O, deep-butt temperature; thin lines, surface temperature at butt, flank and brisket; thick lines, air temperature at neck and butt (see Figure 1.2 for carcass site locations).

next 12h, fan speed was reduced to 60% with a temperature set point of 9.5°C. Air and surface temperatures for side 1717L increased slightly, with air temperatures rising to approximately 9.5°C and surface temperatures rising by approximately 1°C. For the remainder of the chilling period, fan speed was further reduced to 40% with a temperature set point of 9.5°C. Over this period, all air and surface temperatures levelled off to approximately 9.5°C. Deep butt temperature declined at an ever-decreasing rate from an initial value of 42.4°C to 10°C, 48h after chiller loading and 9.5°C by the end of the chilling period.

There was very little difference between the temperature history of 1717L and the average temperature history of all six sides shown in Figure 4.7b. There was also little difference between the average of flank surface temperature profiles of sides on outside rails compared to those on inside rails.

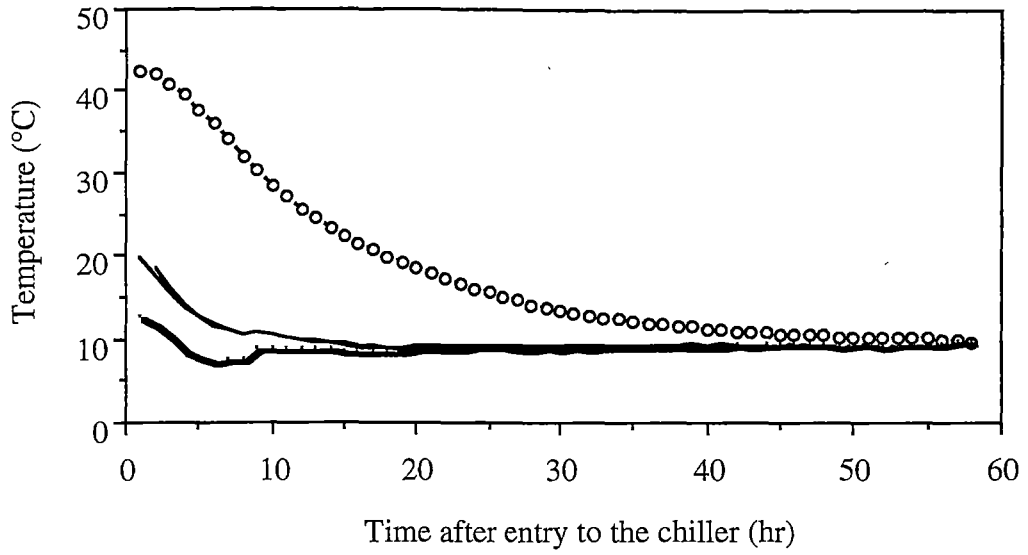


Figure 4.7b Average temperature history for beef carcass sides over a weekend chilling regime at Plant B. O, average deep butt temperature for six sides; thin lines, average flank surface temperatures for three sides on outside rails and three sides on inside rails; thick line, average of all air temperatures.

Data for the microbiological assessment of carcass sides is shown in Table 4.3. An assessment of the change in bacterial numbers over time was only possible in six cases (for pseudomonads: 1724L, site 6; 1768L, site 6; 1756L, sites 5 and 6; for coliforms: 1724L, site 5; 1756L, site 6). Numbers of pseudomonads on all sides were relatively low even after 56h, with the highest count of log 2.43 CFU/cm^2 occurring on side 1756L at site 6. Pseudomonads were only detected on one side (1756L) at site 5 and on four sides (1724L, 1768L, 1751L and 1756L) at site 6. In one case, pseudomonads were detected at early stages in the chilling process but not at the end (side 1768L, site 6, 0 and 3h after chiller loading).

Coliforms were detected at slightly higher levels than pseudomonads, but at approximately the same frequency. None of the coliforms detected were *E. coli* . Three sides (1717L, 1724L and 1756L) were found to be contaminated with coliforms at

Table 4.3 Density of pseudomonads, coliforms and total viable count on six beef carcass sides over a weekend chilling regime at Plant B.

		Carcass site number					
		5.*			6.*		
Side no.	Time after entry to chiller (hr)	Pseudo. (log CFU/cm^2)	Coliforms (log CFU/cm^2)	TVC (log CFU/cm^2)	Pseudo. (log CFU/cm^2)	Coliforms (log CFU/cm^2)	TVC (log CFU/cm^2)
	0		0.447			0.491	
	3						
	6						
1717L	12						
	20						
	40						
	56			1.51		1.51	3.76
	0		0.491			1.16	
	3						
	6						
1724L	12		0.477				
	20				0.699		
	40				0.699		
	56			2.67	2.24	2.84	4.02
	0				0.903		
	3				0.477		
	6						
1768L	12						
	20						
	40						
	56			3.12			2.56
	0						
	3						
	6						
1730R	12						
	20						
	40						
	56			3.25			1.85
	0						
	3						
	6						
	12						
1751L	20						
	40						
	56			2.14	0.301		3.28
	0						
	3						
	6						
1756L	12					0.949	
	20				0.602		
	40	1.81					
	56	1.84	1 52	3.99	2.43	1.83	4.01

*See Figure 4.1 for carcass site number locations; Pseudo. = pseudomonads; TVC = total viable count; no data indicates <1 CFU/cm^2 detected.

both sites 5 and 6 while the remaining sides were free of coliforms. The highest coliform count of log 2.84 CFU/cm^2 occurred on side 1724L at site 6. In general, coliforms were detected only at the end of chilling.

TVC at site 5 ranged from log 1.51 CFU/cm^2 (1717L) to log 3.99 CFU/cm^2 (1756L). The range at site 6 was log 1.85 CFU/cm^2 to 4.02 CFU/cm^2 (1724L). Counts were only detected at 56h for all sides.

4.4.2.3 Comparison of observed and predicted bacterial numbers

Predicted population density of pseudomonads and coliforms on beef carcasses (Plant B) at sites with initial and final observed bacterial numbers are presented in Table 4.4. In the six cases that comparisons were possible between observed and predicted change in bacterial numbers, bacterial growth rate was under-predicted in one case and over-predicted in five cases. For the comparison between observed and predicted change in pseudomonad numbers, predictions of a model for pseudomonads (Eqn. 21) over-predicted the growth rate in all four cases. Observed population densities of pseudomonads on side 1724L (site 6; 20-40-56h) remained at log 0.699 CFU/cm^2 at 20 and 40h and then increased to log 2.24 CFU/cm^2 at 56h. Pseudomonad population densities on the same side were predicted to increase from log 0.699 CFU/cm^2 (20h) to log 2.78 CFU/cm^2 (40h) and then to log 4.90 CFU/cm^2 (56h). Observed pseudomonad population density on side 1768L (site 6) decreased from log 0.903 CFU/cm^2 (0h) to log 0.477 CFU/cm^2 (3h) but was predicted to increase from log 0.903 CFU/cm^2 (0h) to log 1.14 CFU/cm^2 (3h). Observed pseudomonad population densities on side 1756L increased from log 1.81 CFU/cm^2 (40h) to log 1.84 CFU/cm^2 (56h) (predicted to increase from log 1.81 CFU/cm^2 (40h) to log 3.99 CFU/cm^2 (56h)) at site 5 and increased from log 0.602 CFU/cm^2 (40h) to log 2.43 CFU/cm^2 (56h) (predicted to increase from log 0.602 CFU/cm^2 (40h) to log 5.28 CFU/cm^2 (56h)) at site 6.

For the comparison between observed and predicted change in coliform numbers, predictions of a model for *E. coli* NT (R31) (Eqn. 17) over-predicted the growth rate of coliforms in one case and under-predicted the growth rate of coliforms in

Table 4.4 Predicted population density (log CFU/cm^2) of pseudomonads and coliforms (*E. coli*) on six beef carcass sides at Plant B.

		Carcass site number									
		5.*					6.*				
Side no.	Time after entry to chiller (hr)	Surface temp. (°C)	Water activity	Pseu	Gr. col ?	Col	Surface temp. (°C)	Water activity	Pseu	Gr. col ?	Col
	2.00	17.0	0.945		-		16.7	0.984		+	
	5.00	11.2	0.949		-		12.3	0.989		+	
	7.50	9.3	0.936		-		9.8	0.988		+	
1717L	10.50	9.3	0.939		-		9.5	0.991		+	
	14.25	8.9	0.977		-		9.0	0.99		+	
	17.50	8.5	0.929		-		8.7	0.99		+	
	39.25	9.3	0.988		+		9.3	0.993		+	
	56.25	9.4	0.974		-		9.6	0.986		+	
	1.75	17 9	0.968		+	0 491	18.5	0.972		+	
	4.75	13.7	0.975		+		12.8	0.98		+	
	7.25	11.9	0.964		+		10.7	0.964		-	
1724L	10.25	10.9	0.989		+		10.4	0.991		+	
	14.25	9.9	0.985		+	0.596	9.6	0.985		+	
	17.50	9.3	0.956		-		9.3	0.971	0.699	-	
	39.00	9.5	0.968		-		9.4	0.984	2.78	+	
	56.00	9.4	0.976		+		9.3	0.99	4.90	+	
	2.25	17.7	0.989		+		21.9	0.973	0.903	+	
	5.25	12.9	0.958		-		14.7	0.943	1.14	-	
	7.75	11.5	0.93		-		12.1	0.968		+	
1768L	10 75	11.0	0.99		+		11.4	0.986		+	
	14.5	10.0	0.967		-		10.6	0.977		+	
	17.75	9.6	0.953		-		10.0	0.971		+	
	39.25	9.7	0.958		-		9.7	0.98		+	
	56.5	9.6	0.975		+		9.4	0.979		+	
	2.25	19.3	0.97		+		17.3	0.976		+	
	5.25	12.9	0.953		-		12.5	0.975		+	
	7.75	11.0	0.937		-		10.2	0.974		+	
1730R	10.75	10.3	0.984		+		9.6	0.976		+	
	14.5	9.5	0.977		+		8.9	0.996		+	
	17.75	8.6	0.961		-		8.3	0.982		-	
	39.5	9.3	0.98		+		9.3	0.991		+	
	56.5	9.6	0.977		+		9.7	0.981		+	
	2.5	16.1	0.962		+		17.3	0.952		+	
	5.5	12.2	0.951		-		11.6	0.98		+	
	8	11.0	0.96		-		12.2	0.985		+	
	11	11.2	0.973		+		10.1	0.994		+	
1751L	14.5	10.3	0.966		-		9.5	0.989		+	
	18	9.3	0.952		-		8.8	0.973		-	
	39 5	9 4	0.982		+		9.4	0.977		+	
	56.5	9.4	0.987		+		9.4	0.986		+	
	2.5	17.1	0.95		-		17.6	0.964		+	
	5.5	11.8	0.988		+		10.5	0.981		+	
	8	9.8	0.931		-		8.4	0.979		-	
1756L	11	9.3	0.987		+		8.9	0.993		+	
	14.75	8.9	0.976		-		8.7	0.985		+	0.949
	18	8.5	0.994		+		8.4	0.99	0 602	+	
	39.5	9.0	0.988	1.81	+		9.0	0.986		+	
	56.75	9.5	0 988	3.99	+		9.1	0.989	5.28	+	1.37

*See Figure 4.1 for carcass site number locations; Pseu = pseudomonads (log CFU/cm^2); Gr. col? = growth of coliforms as predicted by a growth/no growth model for *E coli* (Eqn. 18) (+ = growth; - = no growth); col = coliforms (log CFU/cm^2).

another case. Observed coliform population density on side 1724L (site 5) decreased from log 0.491 CFU/cm^2 (0h) to log 0.477 CFU/cm^2 (12h) but was predicted to increase from log 0.491 CFU/cm^2 (0h) to log 0.596 CFU/cm^2 (12h). Observed coliform population density on side 1756L (site 6) increased from log 0.949 CFU/cm^2 (12h) to log 1.83 CFU/cm^2 (56h) but was predicted to increase from log 0.949 CFU/cm^2 (12h) to log 1.37 CFU/cm^2 (56h).

Predictions of a model for the growth/no growth boundary of *E. coli* (Eqn. 18) indicated that growth of coliforms was possible for most of the chilling period at site 6 on carcass sides. On average, growth was not possible at only one measured temperature/water activity combination for each carcass side during chilling. At site 5, 'no growth' temperature/water activity conditions occurred more frequently at an average of four occasions for each side during chilling. For comparisons between observed and predicted changes in coliform population density, temperature and water activity combinations encountered were always predicted to allow growth.

4.4.3 Starch as a water activity-lowering compound

The effect of starch used as a coating on the water activity of beef samples is shown in Table 4.5. Hydrolysed starch was found to give rise to a relatively large water activity reduction when coated on cut beef, adipose tissue and mince. Water activity was reduced by an average of 0.019 units compared to uncoated samples. Coating samples in soluble starch gave rise to small water activity reductions, with an average reduction of 0.003 units.

The effect of lowering the water activity of beef samples using hydrolysed starch is shown in terms of the predicted growth rate response of *E. coli* in Table 4.6. Hydrolysed starch gave rise the to greatest reduction in water activity on adipose tissue

Table 4.5 Water activity measurement of beef samples with and without added starch.

Sample	Water activity
cut beef tissue	0.993
cut beef tissue + soluble starch	0.989
cut beef tissue + hydrolysed starch	0.972
adipose tissue	0.991
adipose tissue + soluble starch	0.988
adipose tissue + hydrolysed starch	0.967
minced beef	0.996
minced beef + soluble starch	0.995
minced beef + hydrolysed starch	0.983

Table 4.6 Predicted generation times (h) of *E. coli* (Eqn. 18) on cut beef, adipose tissue and minced beef with and without a surface coating of hydrolysed starch.

		Temperature (°C)					
Sample	Water activity	20	15	12	10	8	GT_{coated}: $GT_{uncoated}$*
cut beef tissue	0.993	1.40	3.00	5.75	10.4	24.2	2.8
cut beef tissue + hydrolysed starch	0.972	3 87	8.29	15.9	28.7	NG‡	
adipose tissue	0.991	1.46	3.12	5.98	10.8	25.2	5.9
adipose tissue + hydrolysed starch	0.967	8.55	18.3	35.1	NG‡	NG‡	
minced beef	0.996	1.34	2.87	5.51	9.97	23.2	1.4
minced beef + hydrolysed starch	0.983	1.87	4.00	7.66	13.9	NG‡	

*GT_{coated} $GT_{uncoated}$ = the ratio of the predicted generation time (h) of *E. coli* on samples coated with hydrolysed starch to the predicted generation time of *E. coli* on samples with no added hydrolysed starch;

‡NG = no growth predicted by a model for the growth/no growth boundary for *E. coli* (Eqn. 18).

(from 0.991 to 0.967), which translated as a 5.9-fold reduction in the predicted growth rate of *E. coli* at 20, 15, 12 and 10°C. No growth was predicted at 8°C by a model for the growth/no growth boundary of *E. coli* NT (R31) (Eqn. 18, Chapter 3). Coating cut beef and minced beef with hydrolysed starch gave rise to smaller reductions in water activity than were observed at the surface of adipose tissue. Predicted reductions in the growth rate of *E. coli* on cut beef and minced beef were 2.8- and 1.4-fold, respectively.

4.5 Discussion

Preliminary Investigations

As mentioned in Section 1.3.4, after temperature, water activity is perhaps the most important environmental constraint affecting bacterial growth rate. Despite this, it is not routinely monitored during many food processing operations, including the fabrication of red meat carcasses. This may be due to the fact that only recently has water activity been able to be measured quickly (*ie.* under five minutes) and with a high degree of accuracy and precision.

Water activity is of particular relevance to processes such as beef production where the main cooling stage, chilling, does not serve to inhibit completely the growth of spoilage and pathogenic bacteria by temperature reduction alone. If beef sides are cooled to minimum temperatures in the range 9-10°C, all psychrotrophic and most mesophilic bacteria are still able to grow unless an additional hurdle such as water activity is also limiting to growth. If in addition to temperature the water activity on beef carcasses during chilling is known then the true efficacy of existing chilling regimes can be established. If water activity is sufficiently high during chilling such as to pose no hurdle to bacterial growth, then an ultimate chilling temperature of 9°C is insufficient to inhibit completely the growth of most spoilage and pathogenic bacteria. However, if water activity does provide a hurdle to growth then chilling temperature can be relaxed, thus saving energy.

As mentioned previously, there exist a number of methods for measuring water activity. These have been described by various workers (*eg.* Labuza *et al.*, 1976; Troller, 1983; Stamp *et al.*, 1984) and have varying accuracy and measurement time. Methods suitable for monitoring water activity of red meat carcasses during chilling (*ie.* those which are fast and accurate) have only been developed relatively recently. However, there exists no reported means of measuring water activity non-invasively on carcasses. One way in which this was attempted was to exploit the conductivity of the carcass surface.

Conductivity has been used extensively in microbiology for a number of applications, although it is usually described in terms of impedance, a function to which it is inversely related. Impedance is defined by Silley and Forsythe (1996) as "the resistance to flow of an alternating current as it passes through a conducting material". Measurement of impedance has been used for applications such as the enumeration of bacteria (*eg.* screening of animal feeds for *Salmonella* sp. (Gibson *et al.*, 1992)), assessment of degree of antibiotic resistance and testing for biocide efficacy (Silley and Forsythe, 1996). Impedance was also used as the basis for the development of an electronic fish freshness meter, where the aim was to develop a fast and non-destructive means for determining the quality of fish (Jason and Richards, 1975). The use of conductivity in the current study was investigated for similar reasons: fast and non-invasive measurement of water activity on the surface of beef carcasses. It was hypothesised that the conductivity between two metal electrodes placed on the carcass surface would be related to the ionic strength, or water activity, of the medium between them. An initial investigation using agar as the conductive medium showed that there was little change in conductivity over a large portion of the water activity range tested. When there was a change in conductivity with changing water activity, there was a high degree of scatter in the data, especially in agar containing no added NaCl. Silley and Forsythe (1996) noted that conductivity is temperature dependent and that a temperature increase of 1°C results in an average increase in conductivity of 1.8%. All experiments in the present study were carried out at room temperature with a likely temperature

fluctuation of only several degrees Celsius, which cannot explain the large scatter in some of the data. Scatter did not appear to be caused by electrode separation distance as there was no discernible difference in conductivity when electrodes were set at 20mm or 50mm apart. Jason and Richards (1975) in describing impedance explained that choice of electrodes was very important when measuring the electrical properties of biological surfaces. They stated that there can be a large error in impedance measurements caused by polarisation of electrical current between the electrode and surface material. Polarisation could be minimised by using a material such as graphite for the electrodes. Development of their electronic fish freshness meter eventually involved the use of four electrodes. It is evident that measurement of the electrical properties of surfaces is more complex than first thought. It was felt that finding a relationship between conductivity and water activity would clearly involve a more complex system than two stainless-steel electrodes of arbitrary size and so a more direct and simple method of water activity measurement was sought.

The simplest and most direct method of water activity measurement would be to use current meter technology in conjunction with a probe able to be placed directly onto the carcass surface. Initial discussions with the manufacturers of the Aqualab meter used in this laboratory indicated that modifications could be made to the existing meter to enable direct carcass surface measurement (Ross, pers. comm.). However, the working principle of the meter (see Section 1.3.4.1) requires that the sample temperature must come into equilibrium with the external temperature. This cannot be achieved inside a chiller where carcass and air temperatures are often dissimilar. Therefore, a method was required whereby carcass tissue was removed from the carcass without leading to quality losses through aesthetic or other damage. Removal of tissue only semi-invasively was thought to be best achieved using a surgical skin-grafting scalpel, which can be adjusted to remove layers of tissue as thin as 1mm.

Initial investigations were conducted to determine the sample thickness which best reflected the water activity at the *surface* of the carcass, as it is this measure which describes the conditions that surface contaminating bacteria experience. Results showed

that samples containing the same NaCl concentration had the same initial water activity, but that the thinnest sample (1.0mm) showed the fastest rate of drying. Thicker samples (1.5 and 2.0mm) showed slower rates of drying, thought to be due to moister underlying regions of agar replenishing the surface with water through diffusion. This process was evident in 1.0mm samples as well, but not for as long as for thicker samples. Delayed surface drying in 1.0mm samples was apparent at 0, 0.5 and 5.5% NaCl , but not at higher NaCl concentrations where water activity below the surface was insufficient to keep the surface moist through diffusion. Jason and Kent (1979) also noticed two phases of drying in a substrate containing NaCl. In cod muscle of varying salt content, there was an initial fast rate of drying attributed to the evaporation of free water, with a second slow rate of drying caused by the release of water molecules previously bound to sodium ions. As NaCl content increased, the initial phase was shortened due to a lower proportion of free water and a higher proportion of water bound to sodium ions. These results indicated that when attempting to measure the water activity at a surface, it is best to use the thinnest sample possible so as to avoid diffusion effects from underlying regions which may vary in water activity. However, measurements must be made quickly because thin samples lose water quickly by evaporation.

Water activity measurement of beef carcass surface tissue from a cut of beef obtained from a local butcher appeared to reinforce the observations made with agar samples. Three different 1.0mm samples had water activity values, on average, 0.025 units lower than three 3.0mm samples, suggesting that the surface water activity of thick samples was being bolstered by moister underlying tissue. These results are in concordance with the water activity meter operator's manual (Decagon Devices, Inc. USA) which suggests that material be homogenised before a reading is made. This includes material which may have a drier outer coating. As surface water activity is required to be measured in this case, the effects of underlying tissue must be eliminated as well as possible. As well as ensuring the thinnest sample possible, samples were cut

to fit meter sample cups as closely as possible so as not to expose any underlying tissue to the sample chamber headspace of the meter.

Abattoir Trials

The method developed from preliminary investigations was used to map the water activity of the beef carcass outside surface during chilling. This was first achieved at Plant A (Tasmania) by sampling from a large number of sites distributed symmetrically over the carcass surface during the first few hours of chilling. Water activity measurement of samples twice during the first 7h of chilling showed no obvious trend, with half of the sites showing a decrease in water activity between sampling times and half showing an increase. An increase or decrease did not appear to be associated with a single carcass region. It was not possible to draw any conclusions from such a small amount of data.

While there are no published findings relating to the measurement of water activity on red meat carcasses, there is a small amount of data for the water activity of meat samples. Lovett (1978) measured the surface water activity of beef lean and subcutaneous adipose tissue (SAT) by measuring weight loss of tissue held in a wind tunnel. Conditions inside the wind tunnel approximated those inside a chiller, with an initial sample temperature of 30°C, air velocity of 0.56m.s^{-1} and air temperature of 2°C. The air velocity is similar to that used in abattoir chillers (McPhail, pers. comm.). Their results suggested that the water activity of both lean and SAT samples decreased relatively quickly over the first hour of 'chilling' with a steady increase in water activity over the following 5h. The water activity of the lean sample fell to a minimum value of ~0.75, while the SAT sample fell to a value of ~0.52. A second trial at Plant A aimed to test the results of Lovett (1978), with water activity measurements being made on four sites on a carcass side over the first 3h of chilling. The reduction in water activity noted at all sites in this study did not match the extent seen by Lovett (1978). The lowest water activity attained was 0.970 (site 8). If water activity levels on the carcass surface fell to as low as 0.75 or even 0.5 for an hour during chilling, this would severely limit

the growth and even survival of all non-halophilic bacteria present (Troller and Christian, 1978). As mentioned in Section 1.3.4, death of *E. coli* occurs at a water activity of 0.53 regardless of the temperature (Hahn-Hägerdal, 1986). However, from the present study it appears extremely unlikely that water activity levels would ever fall this low. The dramatic fall in water activity observed by Lovett (1978) was possibly a function of the sample size (5g) which would be expected to have drying characteristics different to those of a carcass side.

A water activity/temperature measurement and bacterial enumeration trial over a weekend chilling regime at Plant B provided information about the whole chilling process. Water activity profiles on six carcasses appeared to follow a similar pattern, with the water activity at site 6 almost always being higher than that at site 5 (sites 5 and 6 correspond approximately to rib and brisket sites, respectively (Figure 1.2)). There was a large fluctuation in water activity over the first 20h of chilling, with values falling to as low as 0.929 and 0.942 for sites 5 and 6, respectively. Thereafter, water activity remained relatively high. This pattern is possibly a function of the chilling regime, where fan speeds and air temperature are alleviated 8h after chiller loading and again at 12h. Nottingham (1982) also pointed out that once carcasses have cooled to the temperature of the air, surface water activity is likely to increase as water diffuses from deeper tissues. This agrees with the average temperature profile found in this study, which shows that surface and air temperatures did not come into equilibrium until approximately 20h after chiller loading.

Bacterial load was unexpectedly low, with countable bacterial numbers mainly detected only by the end of chilling despite the bacterial enumeration protocol used in this study being sensitive to a minimum of 2.78 CFU/cm^2 (25ml dilution divided by 9cm^2 tissue sample area). Gill *et al.* (1996a) observed total viable counts of the order of log 2-3 CFU/cm^2 at sites approximating sites 5 and 6 before carcasses entered the chiller. Coliforms were also detected and were of the order of log 0.65-0.90 CFU/cm^2. In this study there was very little data collected for growth of coliforms on carcasses, but most counts and the highest counts were obtained from site 6. Pseudomonads were also

observed at a higher frequency at site 6 than at site 5. The lack of detection of pseudomonads at site 5 does not permit a comparison between the two sites in terms of pseudomonad counts.

There were few cases in which observed changes in bacterial numbers on carcasses could be compared with predicted changes in bacterial numbers because on most carcass sides, a countable population of bacteria was only enumerated once during chilling. When a comparison between observed and predicted change in bacterial numbers was possible, a model for the growth rate response of pseudomonads over-predicted growth rate in four out of four cases and a model for the growth rate response of *E. coli* both over- and under-predicted coliform growth rate. The inconsistent performance of both models (the pseudomonad model 'failed-safe' by a relatively large margin in all cases while the *E. coli* model 'failed-safe' once and 'failed-dangerous' once) may have been due to the fact that the predicted final bacterial population densities were calculated by summing population densities between successive water activity measurements. The time between water activity measurements was as much as 20h. Any variation in water activity and temperature during this time was not considered and so the discrepancies between observed and predicted change in bacterial numbers can be partly explained by this approximation. Temperature was logged every 15min but more accurate predictions would require that water activity measurements be made at approximately the same frequency.

Discrepancies between observed and predicted change in pseudomonad numbers could also be attributed to the fact that a growth/no growth model was not available for use in conjunction with the pseudomonad kinetic model. It is likely that there were temperature/water activity combinations used to predict growth rate by kinetic modelling but which did not actually permit growth. Therefore, predicted population densities would be expected to be lower than observed. Discrepancies between observed and predicted bacterial population densities may also be partly explained by the fact that water activity of the carcass surface was not measured exactly at the point of microbiological sampling but 1-2cm adjacent to it. On the same theme, in the present

study water activity was measured on carcasses at sites relatively distant from one another (see Figure 4.1) and using relatively large tissue sample size. The findings from an initial trial at Plant A showed that there was no obvious trend in the change of water activity at nine sites scattered symmetrically over a carcass side. This may be due to the fact that the water activity of carcasses is more heterogenous than could be measured in the present trials at Plants A and B. As the beef carcass is extremely heterogenous in its physical nature, with large differences in fat thickness on single carcasses and between carcasses, it is likely that water activity over the carcass surface changes on a very small scale and was only approximated in the present study when sampling was done using tissue 25mm×25mm in size. Ede and Partridge (1943) found that for mince samples of varying fat content subjected to drying in a wind tunnel, water loss occurred more rapidly in samples with low fat content compared with samples of higher fat content. A mechanism for this observation was not proposed. This may suggest that water loss at the beef carcass surface is dependent on the thickness of fat present. More accurate predictions of the change in bacterial growth on carcasses may not only require more frequent water activity sampling, but knowledge of the water activity on a much smaller scale.

The results of Gill *et al.* (1996a) agree with other sources (*eg.* Eustace, 1981) that carcasses are contaminated from a number of sources during slaughter and dressing and despite undergoing washing procedures before chilling, have measurable bacterial loads by the time they enter the chiller. Results of the present study confirm this to a small degree as there was slight contamination with pseudomonads and coliforms detected immediately after chiller loading on three carcass sides, although surprisingly no detection on TVC plates. Lack of growth on TVC plates at the early stages of chilling can possibly be explained by the fact contamination of carcasses is non-uniform and that only two carcass sites were screened for growth. Lack of uniformity of contamination is reported in the literature (*eg.* Dickson and Anderson, 1992; Jericho *et al.*, 1994). Relatively low water activities encountered on carcasses in the first 20h of chilling could explain inhibition of growth of contaminating bacteria, but cannot explain the complete

lack of detection of contaminants in many cases. The sudden increase in TVC at the end of chilling suggested that neither water activity nor temperature hurdles were sufficiently large to completely inhibit bacterial growth.

The measurement method developed in the present study offers meat processors an added tool for the design and operation of chillers. With temperature *and* water activity measurements available to microbiologists, kinetic modelling can be used in conjunction with probability modelling to make predictions of the growth rate of target microorganisms on beef carcasses and can assess changes in beef processing with respect to bacterial growth rate. Previously, the effects of altering chilling patterns could only be assessed with reference to air and carcass temperature. With the addition of water activity monitoring, the effects of fan speed and temperature set point can be observed not only by reference to temperature but also to surface drying-effects on the carcass. Investigations into altering chilling patterns are underway in Brisbane, Australia (McPhail, pers. comm.), and have already found that a reheating phase (temperature set point of 20°C) for 3h at the end of chilling leads to a slight decrease in water activity at both sites 5 and 6 (McPhail, pers. comm). Further studies will determine whether the favourable reduction in water activity more than compensates for the unfavourable increase in carcass surface temperature. One advantage of an elevated surface temperature at the end of chilling is that surface fat is softened giving rise to easier breaking of carcasses. Similar experiments were performed by Cutter *et al.* (1997) where rapid drying of carcasses was attempted at one or two points in the slaughter process using a forced-air heater. Air temperatures of about 400°C were administered for 15s. It was found that desiccation was more effective than water washing for removing bacterial contamination. However, growth inhibition may have been due to high temperature treatment rather than surface drying effects.

Suggested Protocol for Future Abattoir Trials

The trial described in the previous section is the first detailed investigation of red meat carcass surface water activity described in the literature. Water activity at the

carcass surface was mapped successfully during chilling, but predictions of the change in population densities of pseudomonads and coliforms either 'failed-safe' very conservatively or 'failed-dangerous'. In order to make more accurate predictions of the growth rates of surface contaminating bacteria, a number of changes would have to be made to the existing protocol. As the time between water activity measurements was of the order of 3-20h, more accurate bacterial growth rate predictions would require more frequent water activity measurements. Ideally, water activity measurements should be made as near as possible to the frequency of temperature measurements (15min in this study), especially when the change in surface water activity with time is most variable (the first 20h of chilling in the present study). To account for differences in surface water activity over the carcass, the sample used should be a small as possible and microbiological sampling should be done from the same sample used for water activity measurement. In order to gauge the effectiveness of this method for the prediction of the surface contaminant population change, higher initial bacterial numbers are required than were observed in the present study. Therefore, bacteria may have to be inoculated onto carcasses at the beginning of chilling at a measurable density. Initial investigations are underway in this laboratory to assess the use of *Klebsiella oxytoca* for such a purpose. This non-pathogenic bacterium has been approved for use by the Australian Quarantine and Inspection Service for use in an Australian abattoir but its growth rate characteristics on beef carcass compared to indicator organisms such as *E. coli* are, as yet, unknown.

Water Activity-Lowering Compounds

Besides chilling, carcass surface drying can be achieved by addition of water activity-lowering compounds. Traditionally, the humectant of choice for meat products has been NaCl, however an undesirable consequence of its use is its excessive saltiness and exacerbation of hypertension (Vallejo-Cordoba *et al.*, 1986). Starch was assessed for its water activity-lowering effect in the present study. Cut beef tissue, adipose tissue and minced beef were coated in soluble and hydrolysed starch and measured for water activity. Hydrolysed starch gave the greatest water activity reduction compared to

uncoated controls. The reduction on cut beef, adipose tissue and minced beef was equivalent to the addition of 3.5, 4.0 and 2.5% (w/w) NaCl and the reduction in the predicted growth rate of *E. coli* of 2.8-, 5.9- and 1.4-fold, respectively. Vallejo-Cordoba *et al.* (1986) assessed the use of soy protein hydrolysates for the reduction of water activity in ground beef and at the concentrations used, observed reductions equivalent to the addition of 1% NaCl. Therefore, reductions observed with the addition of hydrolysed starch are relatively significant and show promise for use in the meat industry where the use of an edible coating is acceptable. The water activity reduction on beef samples gave rise to a relatively large decrease in the predicted growth rate of *E. coli* on adipose tissue compared to the predicted decrease of *E. coli* growth rate on cut beef and minced beef. That the surfaces of beef carcass sides are largely covered in fatty tissue means that a starch coating would severely lower the growth rates of contaminating bacteria.

Appendix 4.1 Growth Media

Bacteriological Peptone (Oxoid, L37) (0.1%) was prepared by adding 1g peptone to 1 litre of distilled water. Sterilisation was achieved by autoclaving for 15min at 121°C.

Petrifilm Plates: Coliform and *E. coli* Count Plates, 3M, USA. Blue colonies with gas production were identified as *E. coli* and red colonies with gas production were identified as non-*E. coli* coliforms.

Pseudomonas C-F-C Agar was prepared according to the manufacturer's instructions using Pseudomonas Agar Base (Oxoid, CM 559) with the addition of Pseudomonas C-F-C Supplement (Oxoid, SR 103) and sterilised. Colonies with blue-green pigmentation and fluorescence under UV light were identified presumptively as *Pseudomonas aeruginosa.*

Starch - hydrolysed: Connaught Medical Research Laboratories.

Starch - soluble: Sigma, S-2630.

Appendix 4.2 Experimental Data

Table A4.1 Conductivity of PCA of varying water activity (Fig. 4.2).

Water activity	Conductivity (μmho) - 20mm*	Conductivity (μmho) - 50mm*	Water activity	Conductivity (μmho) - 20mm*	Conductivity (μmho) - 50mm*
0.998	-2.78	-3.05	0.996	-3.06	-2.86
0.998	-4.15	-2.60	0.992	-2.86	-2.91
0.997	-3.52	-3.10	0.994	-3 70	-3.53
0.996	-3.45	-3.19	0.989	-3.24	-2.94
0.967	-2.96	-2.83	0.989	-3.27	-2.91
0.968	-2.90	-2.92	0.985	-3.39	-3.58
0.929	-2.56	-2.65	0.985	-3.29	-3.15
0.930	-2.60	-2.53	0.854	-2.67	-2.71
0.867	-2.44	-2.45	0.855	-2.43	-2.49
0.878	-2.43	-2.38	0.812	-2.64	-2.54
0.855		-2.50	0.814	-2.55	-2.50
1.000	-0.93	-0.11	0.809	-2.51	-2.53
0.999		-0.20	0.809	-2.71	-2.56
0.998	-0.21	-4.76	0.785	-2.57	-2.61
0.997	-4.48	-4.98	0.783	-2.51	-2.53
0.961	-2.92	-2.99	0.959	-3.31	-3.31
0.959	-2.98	-2.87	0.959	-3.41	-3.41
0.929	-2.65	-2.61	0.952	-3.32	-3.32
0.930	-2.65	-2.67	0.954	-2.83	-2.83
0.879	-2.29	-2.47	0.953	-2.86	-2.86
0.875	-2.49	-2.63	0 952	-2.70	-2.70
0.851	-2.40	-2.42	0.938	-2.69	-2.69
0.851	-2.37	-2.33	0.940	-2.66	-2.66
0.788	-2.39	-2.08	0.922	-2.72	-2.72
0.788	-2.34	-2.38	0.923	-2.64	-2.64
0.994	-2.96	-2.76			

*Distance between electrodes.

Table A4.2 Change in water activity over time of PCA of varying water activity (Figs. 4.3a-b).

[NaCl] (%w/w)	Agar thickness (mm)	Time (h)	Water activity	[NaCl] (%w/w)	Agar thickness (mm)	Time (h)	Water activity
0	1	0	0.996		2	0	0.934
		4	0.994			4	0.928
		24	0.526			24	0.878
	1.5	0	0.999	15.5	1	0	0.897
		4	1.000			4	0.829
		24	0.994			24	0.674
	2	0	1.000		1.5	0	0.898
		4	1.000			4	0.879
		24	0.995			24	0.747
0.5	1	0	0.992		2	0	0.899
		4	0.981			4	0.885
		24	0.537			24	0.827
	1.5	0	0.995	20.5	1	0	0.854
		4	0.996			4	0.795
		24	0.991			24	0.593
	2	0	0.997		1.5	0	0.857
		4	0.999			4	0.832
		24	0.990			24	0.749
5.5	1	0	0.964		2	0	0.859
		4	0.943			4	0.840
		24	0.681			24	0.763
	1.5	0	0.965	25.5	1	0	0.817
		4	0.959			4	0.741
		24	0.896			24	0.640
	2	0	0.965		1.5	0	0.817
		4	0.964			4	0.793
		24	0.940			24	0.750
10.5	1	0	0.932		2	0	0.817
		4	0.879			4	0.799
		24	0.717			24	0.750
	1.5	0	0.935				
		4	0.922				
		24	0.821				

Table A4.3 Change in water activity of a single carcass side at Plant A over the first 7h of chilling (Fig. 4.4).

Carcass site no.	Time after chiller loading (min)	Water activity
1	45	1.000
	365	0.991
2	60	0.969
	380	0.995
3	30	0.992
	390	1.000
4	75	0.975
	410	0.998
5	90	0.980
	420	0.967
6	105	0.979
	430	0.981
7	120	0.985
	445	0.980
8	130	0.975
	465	0.959

Table A4.4 Change in water activity of a single carcass side at Plant A over the first 3h of chilling (Fig. 4.5).

Carcass site no.	Time after chiller loading (min)	Water activity
1	30	0.996
	50	0.998
	69	0.983
	102	0.982
	131	0.985
2	35	0.996
	52	1.000
	80	0.992
	115	0.997
	137	0.985
8	37	0.996
	57	0.987
	90	0.983
	120	0.973
	145	0.970
9	40	0.996
	63	0.991
	95	0.979
	125	0.980
	155	0.977

Table A4.5 Change in water activity of six carcass sides at Plant B over a weekend chilling regime (Figs. 4.6a-f).

Side/site no.	Time (h)	Water activity	Side/site no.	Time (h)	Water activity	Side/site no.	Time (h)	Water activity
1717L	0.07	1.000	1768L	0.33	0.985	1751L	0.67	0.983
Site 5	1.95	0.945	Site 5	2.17	0.989	Site 5	2.40	0.962
	5.00	0.949		5.20	0.958		5.40	0.951
	7.50	0.936		7.70	0.930		7.90	0.960
	10.50	0.939		10.70	0.990		10.90	0.973
	14.33	0.977		14.50	0.967		14.58	0.966
	17.50	0.929		17.72	0.953		17.90	0.952
	39.17	0.988		39.33	0.958		39.50	0.982
	56.25	0.974		56.42	0.975		56.62	0.987
1717L	0.12	1.000	1768L	0.42	0.942	1751L	0.75	0.997
Site 6	2.05	0.984	Site 6	2.23	0.973	Site 6	2.50	0.952
	5.05	0.989		5.25	0.943		5.45	0.980
	7.55	0.988		7.75	0.968		7.95	0.985
	10.55	0.991		10.65	0.986		10.85	0.994
	14.33	0.990		14.50	0.977		14.67	0.989
	17.55	0.990		17.72	0.971		17.90	0.973
	39.22	0.993		39.37	0.980		39.53	0.977
	56.28	0.986		56.47	0.979		56.65	0.986
1724L	0.22	1.000	1730R	0.50	0.975	1756L	0.78	0.975
Site 5	2.08	0.968	Site 5	2.30	0.970	Site 5	2.55	0.950
	5.10	0.975		5.30	0.953		5.50	0.988
	7.60	0.964		7.80	0.937		8.00	0.931
	10.60	0.989		10.80	0.984		11.00	0.987
	14.42	0.985		14.58	0.977		14.67	0.976
	17.63	0.956		17.82	0.961		18.00	0.994
	39.27	0.968		39.42	0.980		39.58	0.988
	56.33	0.976		56.52	0.977		56.70	0.988
1724L	0.28	0.992	1730R	0.58	0.956	1756L	0.83	0.986
Site 6	2.13	0.972	Site 6	2.37	0.976	Site 6	2.67	0.964
	5.15	0.980		5.35	0.975		5.55	0.981
	7.65	0.964		7.85	0.974		8.05	0.979
	10.65	0.991		10.75	0.976		10.95	0.993
	14.42	0.985		14.58	0.996		14.75	0.985
	17.63	0.971		17.80	0.982		18.00	0.990
	39.30	0.984		39.45	0.991		39.62	0.986
	56.38	0.990		56.57	0.981		56.73	0.989

Chapter 5. Conclusions and Projections

The present study offers microbiologists a number of tools for monitoring the current effectiveness of beef processing operations and assessing the effectiveness of modifications made to current operations. It was found in Chapter 2 that the growth rates of pathogenic strains of *E. coli* with respect to temperature were similar to predictions of a model for a non-pathogenic *E. coli* strain. Therefore, models can be generated safely in the laboratory using non-pathogenic strains of *E. coli.* These models can then be used to predict the growth rates of a wide array of pathogenic *E. coli* strains. That the generic *E. coli* model performed as well as published models for predicting the growth rate of *E. coli* in growth media and on a range of foods, including red meat carcasses, illustrated its accuracy as well as its versatility. Because *E. coli* is used as an indicator organism, the generic *E. coli* model would be suitable for assessing the degree of spoilage of beef carcasses at any stage throughout the slaughtering and dressing process and also for assessing the expected proliferation of pathogenic strains of *E. coli* if initially present.

The findings of Chapter 3 extended the capabilities of kinetic modelling. Although there were some problems finding a suitable model to describe the boundary between growth and no growth for a strain of pathogenic *E. coli* with respect to temperature and water activity, all models developed during the model refinement process modelled well the growth/no growth boundary. Knowledge of combinations of temperature and water activity which will not permit the growth of *E. coli* means that the kinetic modelling process need only be applied when conditions fall on the growth-side of the growth/no growth boundary. Predictions of the extent of bacterial growth will be inflated if those conditions not permitting growth are used in the modelling process. Also, food manufacturers can ensure that target organisms do not grow at all if conditions are never allowed to fall on the growth-side of the growth/no growth boundary specific for that organism.

The results of Chapter 2 and 3 could only be applied to the fabrication of beef carcasses if temperature *and* water activity history could be recorded. Temperature history is easily recorded using temperature loggers, but the measurement of beef carcass water activity has never been previously reported. Measurement of carcass surface water activity in the present study was achieved by excising 1.0mm-thick samples using a skin-grafting scalpel and measuring sample water activity in a dew-point water activity meter. Using temperature and water activity history during chilling, the predicted population densities of coliforms and pseudomonads on carcasses was calculated and compared to observed numbers. Predicted coliform population density was achieved using a combination of probability *and* kinetic modelling and agreed reasonably well with observed coliform numbers. Therefore, the present study offers a means to assess the beef production process in terms of both temperature and water activity at the carcass surface and using both kinetic and probability modelling.

The protocol described here could be extended to assess the growth of any target organism and any stage during fabrication. Also, the effects of process modification can be easily assessed in terms of bacterial growth. Investigations are currently being conducted at Plant B to monitor the effects of altered chilling patterns. The outcomes can be monitored in terms of bacterial growth and as a result of this study, water activity. Water activity (along with temperature) can not only be used to predict the growth rate of surface contaminants but is an indicator of fat hardness, so that changes in chilling patterns can now be shown to improve or worsen the ability of carcasses to be broken after chilling.

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Appendix 1. Common Materials

A2.1 Materials

Balances

Mettler PJ3600 DeltaRange®, Mettler Instrumente AG, CH8608 Greifensee-Zürich, Switzerland.

Sartorius Analytical AC 210 P, Sartorius Ag Göttingen, Germany.

pH meter

Corning M120, Corning Medical and Scientific, Scientific Instruments, Halstead, Essex, England, CO9 2DX with Orion 91-06 Ag/AgCl probe.

Pipettors

Fixopet: 100µL (fixed), 1000µL (fixed); Pluripet: 200-1000µL, Kartell Spa Via, Delle Industrie, 1 20082 Noviglio, Milan, Italy.

Pipetman®: 20-200µL, 200-1000µL, Gilson Medical Electronics (France) S.A., B.P. 45-95400, Villiers-le-Bel, France.

Oxford Macro-set: 5-10ml, Oxford Labware, Division of Sherwood Medical, St Louis, MO 63103 USA.

Spectrophotometry

Spectronic 20 (analogue) or 20D (digital) spectrophotometers (Milton Roy Co., USA) were used unless otherwise specified.

Temperature gradient incubator

Model TN3, Toyo Kagaku Sangyo Ltd., Japan.

Thermometry

A Fluke 51 K/J thermometer (John Fluke MFI Co. Inc., USA) was used with an Iron-Constantan thermocouple bead probe.

Timers

Electronic Clock-Timer, Model 870A, China.

Water activity meter

Aqualab model CX2, Decagon Devices, Inc., PO Box 835, Pullman, Washington 99163, USA.

Water baths

A range of shaking and static water baths were used including:

Lauda RC 20, RM 20, M 20, RM 6 (Lauda DR. R. Wobser GMBH & Co. K.G., Lauda-Könighofen, West Germany).

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